



HAL
open science

La double émulsion - évaporation solvant à usage thérapeutique : étude systématique et encapsulation de biomolécules, protéines et acides nucléiques

Dimah Ibraheem

► To cite this version:

Dimah Ibraheem. La double émulsion - évaporation solvant à usage thérapeutique : étude systématique et encapsulation de biomolécules, protéines et acides nucléiques. Génie des procédés. Université Claude Bernard - Lyon I, 2014. Français. NNT : 2014LYO10069 . tel-02401479

HAL Id: tel-02401479

<https://theses.hal.science/tel-02401479>

Submitted on 10 Dec 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

THESE

Présentée devant

L'UNIVERSITE CLAUDE BERNARD – Lyon 1

Pour l'obtention du Diplôme de DOCTORAT

Spécialité pharmacie industrielle et bonne fabrication

Par

Dimah IBHAHEEM

Titre:

« La double émulsion – évaporation solvant à usage thérapeutique: étude systématique et encapsulation de biomolécules, protéines et acides nucléiques »

Soutenue le

7 Mai 2014

Jury :

Salima Saidi

Serge Stoll

Pierre Lanteri

Ali Laayoun

Abdelhamid Elaissari

Hatem Fessi

Professeur Rapporteur (université d'Oran)

Professeur Rapporteur (université de Geneve)

Professeur Université Lyon-1

Docteur Société BioMérieux

Directeur de Recherche Directeur de thèse (CNRS)

Professeur Co-Directeur de thèse (Université Lyon-1)

Remerciements

A l'issu de ce travail, je souhaite présenter mes remerciements et toute ma gratitude aux gens qui m'ont soutenu et aidé pour en arriver là.

Tout d'abord, je veux remercier le Pr. Hatem FESSI, directeur du LAGEP, pour m'avoir accepté dans son laboratoire, d'avoir été à mon écoute et m'orienter particulièrement pendant les premiers jours en France et pour votre co-encadrement actif. En effet, malgré toutes ses fonctions et responsabilités, la porte de son bureau était toujours ouverte pour m'écouter, discuter des divers problèmes rencontrés lors de mon travail et trouver des solutions appropriées.

Mes remerciements à Madame le Professeur Salima SAIDI BESBES, Monsieur le Professeur Serge STOLL, Monsieur le Professeur Pierre LANTERI et Docteur Ali LAAYOUN qui ont accepté d'évaluer et de juger ce travail.

Je tiens à remercier profondément Dr. Abdelhamid ELAISSARI, l'encadrant de ma thèse. Cette thèse a été réalisée grâce à son suivi au quotidien. Ses conseils, ses recommandations et sa disponibilité ont grandement rendu facile l'achèvement de ce travail. Il a su m'offrir un cadre dynamique et plein d'enthousiasme, et me faire bénéficier de son expérience précieuse.

Monsieur ELAISSARI je vous remercie pour toutes les choses importantes que j'ai apprises de vous.

Merci infiniment à la superbe équipe du LAGEP et particulièrement à Nadia, Géraldine, Olivier, Jean Pierre, et Sébastien, merci pour votre disponibilité et votre coopération. Je remercie aussi Dr. Mohammad EISSA pour son aide et ses explications. Je remercie de tout mon cœur mes chers collègues du LAGEP pour votre aide et votre soutien. Vous avez toujours été à mes côtés. J'ai partagé avec vous des moments inoubliables et vous avez été comme une deuxième famille pour moi.

Un grand merci à ma merveilleuse amie Florence et à sa famille amicale pour m'avoir aidé à apprendre la langue française et surtout pour les moments inoubliables qu'on a passé ensemble.

Pour finir, je tiens à remercier tous ceux qui m'ont aidé de près ou de loin à compléter ce travail.

Sommaire

Sommaire	2
1. Introduction générale.....	5
2. Etude bibliographique	10
2.1. Les procédés d'encapsulations.....	10
2.2. La thérapie Génique et les techniques d'encapsulations	44
2.3. Protéines et peptides pour applications in vivo	59
3. Etude expérimentale	89
3.1. Etude systématique de l'émulsion double	89
3.2. Encapsulation des acides nucléiques	104
3.3. Encapsulations des protéines	112
4. Conclusion générale	128

Résumé de la thèse

Des biomolécules telles que les protéines et les acides nucléiques sont d'un grand intérêt dans l'immunothérapie, la thérapie génique tels que des cancers, le traitement de SIDA, les troubles auto-immuns liés à la déficience immunitaire sévère et combinés à autres maladies. Afin de cibler ces objectifs, l'encapsulation et le ciblage sont incontestablement nécessaires pour protéger les biomolécules contre la dégradation rapide et aussi pour éviter les effets secondaires.

Ainsi, dans le cadre de cette thèse, l'encapsulation de protéines et de l'ADN en utilisant le procédé basé sur la double émulsion évaporation de solvant a été étudiée. Mais avant de conduire l'étude expérimentale, l'état de l'art des procédés d'encapsulation a été effectué et une attention particulière a été consacrée à l'encapsulation de l'ADN et les protéines et aux procédés d'encapsulations basés sur la formulation des polymères biodégradables.

En ce qui concerne l'étude expérimentale, une étude systématique des paramètres pertinents sur les propriétés physico-chimique et colloïdales des particules a été examinée. Cette étude a permis de mettre en évidence les facteurs clés capables d'affecter non seulement le procédé mais aussi les propriétés colloïdales des particules élaborées (taille, distribution en taille, propriétés électrocinétiques et stabilité colloïdale...).

En second lieu, la formulation optimisée permettant l'obtention de tailles submicroniques a été utilisée pour l'encapsulation de l'ADN et les protéines. L'encapsulation de l'ADN a été étudiée en fonction de la concentration de l'ADN (sperme de saumon) et une encapsulation totale d'ADN a été observée sans modification ou altération des propriétés colloïdales des particules finales. L'encapsulation de protéines a été également examinée en utilisant HAS et une autre protéine fluorescente comme modèles. L'efficacité de l'encapsulation a été déterminée et trouvée de l'ordre de 100%. L'utilisation de la protéine fluorescente indique l'encapsulation totale avec une bonne répartition dans les cavités de la matrice polymère. En conclusion, le procédé de la double émulsion – évaporation de solvant est efficace pour l'encapsulation de l'ADN et des protéines sans modification des propriétés colloïdales des particules finales qui restent bien submicroniques.

Summary of the thesis

Biomolecules such as proteins and nucleic acids are of great interest in immunotherapy and gene therapy such as cancers, AIDS, autoimmune disorders, X-linked severe combined immune deficiency and many other diseases. In order to target such objectives, the encapsulation and the targeting are incontestably necessary principally to protect biomolecules against rapid degradation and also to avoid side effects.

Then, in this thesis, the encapsulation of proteins and DNA using double emulsion solvent evaporation technique was performed. But before the encapsulation, the state of the art of the encapsulation processes was performed and special attention has been dedicated to DNA and proteins encapsulation. Firstly, systematic study of double emulsion solvent evaporation process was investigated in order to point out the key parameters controlling the particles size and the colloidal stability. Secondly, the optimized formulation conditions leading to submicron colloidal was used for DNA and Proteins encapsulation. The DNA encapsulation was investigated as a function of DNA concentration and it was found total encapsulation of DNA without any modification of the colloidal properties. The protein encapsulation was examined using HAS and fluorescent proteins as model. The encapsulation efficiency was found to be high and reaches 100%. The use of fluorescent protein shows the total encapsulation and good distribution of proteins in polymer matrix. As a general conclusion, the high encapsulation efficiency of DNA and protein, revealed the compatibility of the used process to encapsulated hydrophilic biomolecules even in submicron size biodegradable colloidal submicron particles.

1. Introduction générale

Les biomolécules sont des macromolécules très importantes car elles participent à toutes les réactions physicochimiques qui se déroulent lors d'un cycle de vie d'un être vivant et qui assurent son entretien. Comme exemples de ces biomolécules on peut citer : les glucides, les lipides, les protéines et les acides nucléiques.

Après avoir compris l'importance et le rôle des biomolécules dans le corps humain, elles ont été mises à l'oeuvre pour le traitement d'un grand nombre de maladies graves et incurables telles que le cancer, le sida, et les anomalies génétiques. Dans cette direction, le développement du génie génétique basé sur l'utilisation de l'ADN ou d'une partie de l'ADN a largement stimulé l'idée de l'utilisation de biomolécules comme médicament thérapeutique. Il est à noter que toutes les biomolécules ont des caractéristiques physicochimiques qui limitent la possibilité de les utiliser directement *in vivo*. Ces biomolécules sont généralement macromoléculaires, polyanioniques et hydrophiles, ce qui limite leur pénétration cellulaire. En plus ces biomolécules sont fragiles et sensibles à la dégradation par des enzymes. Pour ces raisons il est nécessaire de masquer la charge négative portée par ces biomolécules et également de les protéger pour passer des barrières biologiques nombreuses lorsqu'elles sont utilisées ou associées à des vecteurs thérapeutiques. Pour ces raisons également les études de recherches se sont concentrées sur l'élaboration de vecteurs polymériques contenant ces biomolécules assurant ainsi la protection, le transport et également le ciblage. Les biomolécules souffrent d'un inconvénient majeur à savoir leur production à grande échelle et pour palier à ce problème, les chercheurs ont développé des techniques multiples permettant ainsi leur production industrielle et leur utilisation comme médicament thérapeutique.

Dans ce domaine, plusieurs vecteurs ont été utilisés. Les virus, comme les adénovirus, les virus adenoassociés et les virus herpétiques, viennent en tête des précoces vecteurs étudiés et utilisés pour transporter le gène jusqu'au noyau des cellules. Le virus peut accéder au noyau des cellules d'hôte et utiliser la machinerie cellulaire pour se répliquer et se disséminer dans le corps d'hôte. Profitant de sa vie dans le cycle biologique, cette approche 'virus' est largement utilisée comme vecteur de transport de gène sous le nom vecteur viral.

Malgré l'efficacité relativement élevée qui est atteinte par l'utilisation des vecteurs viraux, un grand nombre de problèmes demeurent tels que la difficulté de la production de ces vecteurs, l'immunogénicité, les risques biologiques et le coût de développement. Ce qui a incité à s'orienter vers d'autres alternatives basées sur la synthèse et la formulation capables d'assurer la même efficacité que les virus.

Les vecteurs non viraux sont apparus sous le nom de vecteurs synthétiques dans le but de répondre à ces demandes et à des risques biologiques liés aux vecteurs viraux. Au cours de ces dernières années, des vecteurs nombreux lipidiques ou polymériques cationiques ont été élaborés et évalués *in vitro* et *in vivo*. Puisque ces vecteurs synthétiques sont positivement chargés, ils peuvent complexer l'ADN via des interactions électrostatiques attractives, pour former des particules appelées lipoplexes (quand la complexation a lieu avec un lipide) ou polyplexes (quand la complexation s'effectue avec un polymère cationique). Malheureusement, ces systèmes présentent de nombreuses limitations en termes d'efficacité, de spécificité, de cytotoxicité, de biodisponibilité car l'ADN dans ces systèmes se trouve souvent à la surface des vecteurs particuliers et non encapsulé dans la matrice ou dans le cœur des vecteurs. Ainsi suite à cette fixation à la surface des vecteurs cationiques, la charge nette portée par les particules est négative.

En ce qui concerne les vecteurs de protéines, différentes méthodes ont été explorées pour l'encapsulation. Le procédé le plus étudié est la polymérisation interfaciale en utilisant comme semence des émulsions directes ou inverses. Ce procédé de polymérisation interfaciale conduit à des particules de taille submicronique. Mais, malgré des efforts permettant de réduire les quantités de solvants organiques et d'agents stabilisants utilisés, la présence dans le milieu réactionnel de traces de monomères et oligomères potentiellement toxiques reste un inconvénient majeur de ce procédé basé sur la chimie des polymères. D'autre part, le contrôle de la polymérisation (ou de degré de polymérisation) conduisant à la formation d'un polymère de masse molaire définie est parfois difficile à contrôler. En plus, les risques de dégradation du principe actif (ou de biomolécules encapsulées) dans le milieu réactionnel sont des limitations à prendre en considérations.

L'encapsulation est appliquée à grande échelle dans différents domaines, pharmaceutique, cosmétique et alimentaire, grâce aux avantages présentés par cette technique et à la diversité des procédés. Dans le domaine pharmaceutique, l'encapsulation de principe actif dans des particules assure sa protection contre les enzymes, le pH de milieu et l'effet mécanique et permet

également d'assurer le ciblage et le contrôle de la libération après fonctionnalisation de la surface des particules. Ceci augmente l'efficacité de médicament et réduit les effets secondaires. Il est à noter que dans ce domaine, différentes techniques d'encapsulations ont été étudiées. Malheureusement, l'encapsulation de biomolécules n'est pas très étudiée comme pour les molécules actives classiques. Les propriétés physico-chimiques des biomolécules telle que la sensibilité à des températures élevées, la nature des solvants, l'effet mécanique dénaturant lors de la formulation limitent l'utilisation d'un grand nombre de techniques conventionnelles et par conséquent, la production à grande échelle. Le polymère utilisé pour l'encapsulation doit être biodégradable et biocompatible, ce qui donne aux particules préparées la possibilité d'être utiliser in vivo et facilement éliminables par l'organisme.

Pour l'encapsulation de biomolécules, il faut un procédé utilisant des solvants non dénaturants, permettant l'encapsulation de molécules hydrophiles, utilisable à l'échelle industrielle, permettant également de répondre à un cahier des charges assez drastique imposé par l'application finale. C'est pour ces raisons que notre choix est porté sur l'utilisation de la double émulsion-évaporation de solvant comme procédé universel pour l'encapsulation de l'ADN et d'entités protéiques. En effet, cette technique de la double émulsion et évaporation de solvant est une méthode largement utilisée pour l'encapsulation de molécules actives hydrophiles. Ce procédé a été utilisé pour préparer des particules de taille largement supérieure à 5 micromètres.

Ainsi, l'objectif ultime de notre étude est d'examiner la possibilité d'encapsuler l'ADN et les protéines en utilisant le procédé de la double émulsion-évaporation de solvant. Pour se faire, nous avons structuré notre étude suivant un découpage présenté ci-dessous après une introduction situant le sujet dans son contexte scientifique.

Dans une première partie, nous avons réalisé une étude bibliographique la plus large possible et scindée en trois sous parties : (i) Une présentation des procédés d'encapsulation sans chimie et en se basant principalement sur la formulation des polymères biodégradables. L'un des objectifs de cette partie est de décrire de façon simple et accessible les procédés généralement utilisés pour cibler des applications in vivo. (ii) L'état de l'art sur l'encapsulation de l'ADN a été présenté et son utilisation dans la thérapie génique a été analysée. Dans cette revue, une attention particulière est dédiée à une présentation générale des techniques d'encapsulation de l'ADN uniquement. Enfin (iii) une analyse bibliographique de l'encapsulation des protéines et

l'immunothérapie a été présentée. Dans cette partie, nous avons cherché à décrire les procédés d'encapsulation adaptés aux protéines et les applications qui en découlent.

Une deuxième grande partie relative à l'étude expérimentale est scindée en trois sous parties : (i) une première sous partie dédiée à une étude systématique de la double émulsion-évaporation de solvant en utilisant le polycaprolactone comme polymère principal et le dichlorométhane comme solvant de ce dernier. Dans le cadre de cette étude fondamentale, une attention particulière est centrée sur l'étude de l'effet des paramètres opératoires et physico-chimiques sur les propriétés colloïdales des dispersions. (ii) L'étude de l'encapsulation de l'ADN en se basant sur l'étude systématique et en examinant l'effet de la concentration en ADN sur l'efficacité de l'encapsulation d'un côté et sur les propriétés colloïdales des particules finales de l'autre. (iii) Enfin, l'encapsulation des protéines en utilisant l'albumine de sérum humain (HSA) comme modèle et une protéine fluorescente a été étudiée en mettant l'accent sur l'efficacité de l'encapsulation et sur la distribution des protéines dans la matrice polymère. L'effet de la concentration en protéines sur le taux d'encapsulation et les propriétés colloïdales des particules finale a été examiné.

Ce manuscrit se termine par une conclusion générale récapitulant l'ensemble des résultats et en particulier l'intérêt de la double émulsion dans l'encapsulation de biomolécules.

Etude bibliographique

2. Etude bibliographique

2.1. Les procédés d'encapsulations

Différentes techniques sont utilisées pour préparer les formes particulières (micro et nano particules ou capsules). Ces particules permettent un meilleur transport de principe actif jusqu'à son site d'action.

L'encapsulation offre un grand nombre d'applications dans des domaines variées et en particulier dans le domaine pharmaceutique. Ainsi, le principe actif sensible peut être isolé et protégé des facteurs environnementaux nuisibles (humidité, chaleur, oxygène, lumière...). En plus, l'encapsulation assure une libération contrôlée de l'actif encapsulé (vitesse de relargage et site de libération). Par ailleurs, l'encapsulation permet de faciliter la manipulation et le stockage des médicaments en modifiant leurs caractéristiques physiques (la forme, le volume, la densité).

Les procédés utilisés pour la préparation des particules (des capsules et des sphères) sont très nombreux. Le choix du polymère et du procédé de fabrication dépend essentiellement des propriétés de la substance à encapsuler, de l'application ciblée et de l'effet recherché. Généralement, les procédés utilisant les biopolymères attire une grande d'attention, car ils sont biocompatibles et biodégradables et par conséquence, ils sont appropriés pour des applications in vivo.

L'encapsulation a une importance exceptionnelle quand les biomolécules (acides nucléiques, protéine, enzymes...) sont utilisées comme principes actifs. Les biomolécules jouent un rôle thérapeutique qui est de plus en plus crucial. L'utilisation de biomolécules pour le traitement des maladies graves, a ouvert de nouveaux horizons pour traiter des maladies résistantes aux traitements traditionnels. Malheureusement, les molécules hydrophiles et fragiles comme les protéines et les acides nucléiques, doivent être protégés contre l'effet des enzymes en milieu biologique. Leur libération doit être aussi contrôlée ce qui représente une des solutions à cette problématique.

Dans la revue ci-dessous, nous décrivons les techniques courantes utilisées pour l'encapsulation de molécules thérapeutiques. Lors de cette revue une attention particulière est dédiée aux techniques à base de polymères préformés:

- Nanoprécipitation : la phase organique miscible à l'eau (composé d'un solvant polaire, un ou plusieurs principes actifs et un polymère) est ajoutée à une phase aqueuse (contient l'émulsifiant), sous agitation mécanique. Ceci conduit à l'obtention de nanoparticules ou de nanosphères. Généralement, cette technique est utilisée pour l'encapsulation de substances hydrophobes.
- Emulsion et diffusion de solvant: pour préparer des particules en utilisant cette méthode, on a besoin de trois phases. Une phase organique (contient le polymère et le principe actif hydrophobe), une phase aqueuse (contient un agent stabilisant), et un diluant (normalement une large quantité de l'eau). Après préparation de l'émulsion huile dans eau, une grande quantité d'eau est ajoutée, ce qui entraîne la diffusion de solvant organique dans la phase aqueuse. Le polymère précipite rapidement et donne lieu à la formation des particules. Cette technique est principalement utilisée pour l'encapsulation de substances hydrophobes.
- Emulsion simple et évaporation de solvant: une émulsion simple type huile dans eau est préparée, puis la phase organique contenant le polymère et le principe actif est évaporée. Cette évaporation de la phase organique induit la précipitation de polymère permettant alors l'obtention des particules dans la phase aqueuse.
- Double émulsion évaporation de solvant (émulsion multiple): L'émulsion multiple peut être préparée par plusieurs techniques. Cette méthode, comme son nom l'indique est réalisée en deux étapes. Tout d'abord, la première émulsion est préparée en utilisant un tensioactif approprié afin d'assurer la stabilité de cette émulsion. Celle-ci jouera le rôle de la phase intérieure. La deuxième émulsion est préparée en utilisant généralement un autre type de tensioactif. L'énergie nécessaire pour préparer les deux émulsions est fournie soit par ultrasonication ou homogénéisation à forte énergie.
- Le spray dring : C'est une technique très simple basée sur l'utilisation d'une solution de médicament (contenant un polymère) atomisée et pulvérisée dans une chambre de séchage où les gouttelettes sont rapidement séchées à l'air chaud pour donner naissance à des particules.
- Techniques de fluides supercritiques: Ces procédés mettent en œuvre les fluides supercritiques comme le CO₂ supercritique, état dans lequel ces composés présentent des propriétés particulières en fonction de la température et/ou de la pression. Le polymère et la substance active sont dissous dans le CO₂ supercritique puis lorsque cet ensemble

passer par une buse, une diminution importante de la pression entraîne la précipitation de polymère sous forme de particules.

- Gélification ionique : Cette méthode est principalement utilisée avec des polymères hydrophiles naturels. Ces polymères comprennent la gélatine, l'alginate, le chitosane et l'agarose. Cette technique est basée sur la transition du polymère de l'état liquide à l'état gélifique. Les particules sont obtenues après refroidissement des gouttelettes d'émulsion de gélatine à température inférieure au point de gélification dans un bain de glace.
- Couche par couche: Ce procédé est basé sur l'adsorption d'un polymère sur une semence de particules. Cette adsorption est de type électrostatique généralement réalisée en contrôlant la salinité et le pH du milieu d'adsorption. Afin d'avoir une écorce d'épaisseur importante, l'adsorption d'un deuxième polymère est réalisée sur le premier. L'actif peut se trouver dans la semence formant le cœur des particules finales. Les particules obtenues après avoir éliminé le noyau solide, sont vésiculaires et sont appelées capsules polyélectrolytiques.

Cet article fournit aussi des informations sur les polymères les plus utilisés et qui sont classés en deux classes :

- Les polymères naturels comme le chitosane, la dextrine, la gélatine etc.
- Les polymères synthétiques :
 - polyesters biodégradables comme PLGA, PLA, PCLetc.
 - polymères acryliques comme l'Eudragit.
 - polymères pegylés comme chitosane – PEG, MPEG-PCL, PLA-PEG, MPEG-PLA.

Dans cette revue, il est également rapporté les principales applications des particules développées au cours des dernières années et leurs principales propriétés.

Review article:

**PARTICLES FROM PREFORMED POLYMERS AS CARRIERS
FOR DRUG DELIVERY**

K. Miladi^{a,b}, D. Ibraheem^a, M. Iqbal^a, S. Sfar^b, H. Fessi^a, A. Elaissari^{a*}

^a University of Lyon, F- 69622, Lyon, France; Lyon 1 University, Villeurbanne, CNRS, UMR 5007, Laboratoire d'Automatique et de Génie des Procédés, LAGEP-CPE-308G, 43 bd. du 11 Nov.1918, F-69622, Villeurbanne, France

^b University of Monastir, Laboratoire de pharmacie galénique, Rue Avicenne, 5000 Monastir, Tunisia

* Corresponding author: Phone: +33-472431841, Fax: +33-472431682
E-mail: elaissari@lagep.univ-lyon1.fr

ABSTRACT

Biodegradable and biocompatible polymers are widely used for the encapsulation of drug molecules. Various particulate carriers with different sizes and characteristics have been prepared by miscellaneous techniques. In this review, we reported the commonly used preformed polymer based techniques for the preparation of micro and nano-structured materials intended for drug encapsulation. A description of polymer-solvent interaction was provided. The most widely used polymers were reported and described and their related research studies were mentioned. Moreover, principles of each technique and its crucial operating conditions were described and discussed. Recent applications of all the reported techniques in drug delivery were also reviewed.

Keywords: Drug delivery, particles, polymer, encapsulation, carriers, operating conditions

INTRODUCTION

Particulate carriers have gained tremendous interest during the last decades which permitted to deliver many hydrophilic and hydrophobic molecules. Obtained particles present small size which facilitates their absorption. These drug delivery systems protect active pharmaceutical ingredients from degradation, enhance biopharmaceutical properties and could provide passive or active targeting or sustained delivery. Bio-medical applications of the developed carriers are continuously growing (Ahmad, 2013; Soares, 2013; Miladi et al., 2013). Although, they present different physico-chemical properties, the used polymers are

mainly biocompatible and biodegradable. A multitude of techniques are used to obtain these particles. These methods differ by their principles and the nature of drug molecules that could be encapsulated. Some successfully marketed products led to an enlargement of the applications and the interest given by researchers to these drug delivery systems. Choice of the technique and operating conditions is crucial to obtain formulations bearing good properties for *in vitro* and *in vivo* applications. In this review, we will focus on polymeric particles and give a scope about the most used polymers. We will also describe the common preformed polymer based techniques used

for the encapsulation of drug molecules. We will also review the major applications of the developed particles during the last years and their main properties.

1. POLYMER-SOLVENT INTERACTIONS

Many techniques that rely on preformed polymers have been used for the preparation of particulate carriers. Although these methods are quite different, they generally share a unique principle which is polymer precipitation. Precipitation of the polymer occurs either when a non solvent is added or after subsequent decrease of its solubility in a solvent. Many parameters could influence polymer solubility such as, solvent nature, pH, salinity and temperature of the dispersion medium. Solubility of polyelectrolytes in water, for example, is highly pH and salinity dependent (Genes, 1979), while that of poly(alkyl acrylamide) and poly(alkyl methacrylamide), is mainly temperature dependent (Elaissari, 2002). In fact, nanoprecipitation and emulsion based techniques are based on the addition of a non solvent to the polymer which causes its precipitation. However, ionic gelation technique, for instance, in which generally a polyelectrolyte is used as polymer, is based on the addition of a salt or an oppositely charged polymer. This results in a change in the salinity of the medium and the appearance of electrostatic interactions and thus, leads to polymer precipitation. The thermodynamic behavior of the polymer in a given solution is highly dependent on the Flory χ -parameter. This parameter is defined as the free energy change per solvent molecule (in $k_B T$ units) when a solvent-solvent contact is shifted to a solvent-polymer contact. It is expressed by the following mathematical equations:

$$\chi = \frac{\Delta G}{k_B T} = \frac{\Delta H - T\Delta S}{k_B T} = \frac{1}{2} - A \left(1 - \frac{\theta}{T}\right)$$

Equation (1)

where k_B and T are Boltzmann constant and temperature, respectively; A and θ parameters are defined as follows:

$$A = \frac{2\Delta S + k_B}{2k_B} \quad \text{Equation (2)}$$

$$\theta = \frac{2\Delta H}{2\Delta S + k_B} \quad \text{Equation (3)}$$

It can be seen that the A parameter is directly related to entropy changes, whereas θ temperature is a function of both entropic and enthalpic variations. When θ temperature = T , the corresponding Flory χ -parameter = $1/2$, at which the second Virial coefficient is equal zero (Elias, 2003). The latter can be easily determined from light scattering measurements of a diluted polymer solution. At θ temperature conditions, the binary interactions among constituents will be negligible and only the excluded volume effects will be predominant. Consequently, the solvent will be a good solvent for the polymer when $\chi < 1/2$ and a poor one when $\chi > 1/2$ (Minost et al., 2012).

2. COMMONLY USED POLYMERS FOR ENCAPSULATION

Several polymers have been used for drug encapsulation but only biodegradable and biocompatible ones are suitable for biomedical applications. The biodegradability of a polymer is acquired by the presence of a labile function such as ester, orthoester, anhydride, carbonate, amide, urea or urethane in their backbone. These polymers could be of natural (polysaccharides and protein based polymers) or synthetic (polyesters) nature (Pillai and Panchagnula, 2001). The most commonly used polymers for drug encapsulation are polyesters (lactide and glycolide copolymers, poly- ϵ -caprolactone), acrylic polymers (polymethacrylates) and polyamides (gelatin and albumin). The selection of the right polymer is a crucial step to obtain particles that are suitable for a well-defined application. In fact, polymers' structures are highly differ-

ent and their surface and bulk properties are highly relevant for the obtaining of the desirable biological application. Copolymers could be also used to monitor the hydrophobicity of the materials. Some polymers are poly(ethyleneglycol) (PEG) copolymerized in order to decrease nanoparticle recognition by the reticular endothelial sys-

tem. Table 1 contains examples of the most used biocompatible and biodegradable polymers in encapsulation. Some polymers, especially those having mucoadhesive properties, could also be used for coating the nanocarriers (Mazzafarro et al., 2012; Zandanel and Vauthier, 2012).

Table 1: Commonly used polymers

Materials	References
Polymers	
<i>Natural polymers</i>	
Chitosan	Elmizadeh et al., 2013; Fàbregas et al., 2013; Khalil et al., 2012; Konecni et al., 2012; Du et al., 2009; Bernkop-Schnürch et al., 2006; Gan et al., 2005; Asada et al., 2004
Dextran	Liang et al., 2013; Dai et al., 2012; Sajadi Tabassi et al., 2008; Koten et al., 2003
Dextran derivatives	Kanthamneni et al., 2012; Kauffman et al., 2012; Aumelas et al., 2007; Miyazaki et al., 2006
Cyclodextrins	Çirpanli et al., 2009; Memişoğlu et al., 2003; Pariot et al., 2002; Lemos-Senna et al., 1998
Gelatin	Nahar et al., 2008; Balthasar et al., 2005; Vandervoort and Ludwig, 2004; Bruschi et al., 2003
<i>Synthetic polymers</i>	
<i>Biodegradable polyesters</i>	
PLGA	Gyulai et al., 2013; Beck-Broichsitter et al., 2012; Morales-Cruz et al., 2012; Beck-Broichsitter et al., 2011; Nehilla et al., 2008; Song et al., 2008; Budhian et al., 2007; Bozkir and Saka, 2005; Fonseca et al., 2002; Yang et al., 1999; Govender et al., 1999
PLA	Bazylińska et al., 2013; Fredriksen and Grip 2012; Kadam et al., 2012; Kumari et al., 2011; Ataman-Onal et al., 2006; Lamalle-Bernard et al., 2006; Hyvönen et al., 2005; Katare et al., 2005; Chorny et al., 2002; Leo et al., 2000
PCL	Behera and Swain, 2012; Guerreiro et al., 2012; Hernán Pérez de la Ossa et al., 2012; Khayata et al., 2012; Arias et al., 2010; Wang et al., 2008; Limayem Blouza et al., 2006; Tewa-Tagne et al., 2006; Yang et al., 2006; Le Ray et al., 2003; Chawla and Amiji 2002; Raval et al., 2011; Hombreiro Pérez et al., 2000; Benoit et al., 1999; Masson et al., 1997
Poly(lactide-co-glycolide-co-caprolactone)	Zhang et al., 2006
<i>Acrylic polymers</i>	
Eudragit	Hao et al., 2013; Das et al., 2010; Eidi et al., 2010; Trapani et al., 2007; Galindo-Rodríguez et al., 2005; Haznedar and Dortuñ 2004; Pignatello et al., 2002
<i>Others</i>	
Polyvinylbenzoate	Labruère et al., 2010
<i>Pegylated polymers</i>	
Chitosan-PEG	Seo et al., 2009
MPEG-PCL	Falamarzian and Lavasanifar, 2010; Xin et al., 2010
PCL-PEG-PCL	Suksiriworapong et al., 2012; Huang et al., 2010; Gou et al., 2009
Poly(caprolactone)-poly(ethylene oxide)-poly(lactide)	Hu et al., 2003
PLA-PEG	Sacchetin et al., 2013; Essa et al., 2010; Ishihara et al., 2010; Vila et al., 2005; Vila et al., 2004; Govender et al., 2000; Huang et al., 1997
PLA-PEG-PLA	Chen et al., 2011; Ruan and Feng 2003
MPEG-PLA	Zheng et al., 2010; Dong and Feng, 2007; Dong and Feng, 2004

2.1 Natural polymers

2.1.1 Chitosan

Chitosan is obtained by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and cell walls of fungi. It is a cationic and biodegradable polysaccharide consisting of repeating D-glucosamine and N-acetyl-D-glucosamine units, linked via (1-4) glycosidic bonds. Chitosan is non toxic and can be digested in the physiological environment, either by lysozymes or by chitinases, which are present in the human intestine and in the blood. These properties led to increased interest for this polymer in pharmaceutical research and industry as a carrier for drug delivery (Mao et al., 2010). In addition, chitosan has mucoadhesive properties owing to its positive charge that allows interaction with the negatively-charged mucosal surface. Consequently, the use of chitosan as a matrix (Patil and Sawant, 2011) or as a coating material (Mazzarino et al., 2012) in drug encapsulation had become a promising strategy to prolong the residence time, to increase the absorption of active molecules through the mucosa (Mao et al., 2010; Alpar et al., 2005) and also for targeted delivery (Park et al., 2010).

2.1.2 Dextran and its derivatives

Dextran polymers are produced by bacteria from sucrose. Chemical synthesis is also possible. These glucose polymers consist predominantly of linear α -1,6-glucosidic linkage with some degree of branching via 1,3-linkage. Dextran-based microspheres have got much attention because of their low toxicity, good biocompatibility and biodegradability, which are of interest for application in biomedical and pharmaceutical fields (Mehvar, 2000). Many dextran polymers such as Sephadex® (cross-linked dextran microspheres) as well as Spherex® (cross-linked starch microspheres) were used as carriers for drug delivery. Other derivatives of dextran and

starch including diethyl aminoethyl dextran and polyacryl starch have also been used for mucosal drug delivery. Illum et al. (2001) proposed some mechanisms to explain absorption enhancement effects of cross-linked starch and dextran microspheres intended to nasal delivery which are: (1) Deposition of the microspheres in the less or non ciliated anterior part of the nasal cavity and slower nasal clearance; (2) Retention of the formulation in the nasal cavity for an extended time period because of the bioadhesive properties of the microspheres and (3) The local high drug concentration provided by the gelled system in close contact with the epithelial absorptive surface (Illum et al., 2001).

2.1.3 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides that contain at least six D-(+) glucopyranose units which are attached by α -(1,4) glycosidic bonds. They have been widely used for the formulation of drugs with bioavailability concerns resulting from poor solubility, poor stability and severe side effects. There are 3 natural CDs which are α -, β -, and γ -CDs (with 6, 7, or 8 glucose units respectively) (Challa et al., 2005). In addition, amphiphilic cyclodextrins are synthetic derivatives of natural cyclodextrins. Such derivatives are able to self-organize in water to form micelles and nano-aggregates, which is interesting for pharmaceutical applications, mainly, encapsulation (Gèze et al., 2002). In fact, amphiphilic cyclodextrins have recently been used to prepare nanoparticles and nanocapsules without surfactants and have shown high drug-loading capacity with favorable release properties (Lemos-Senna et al., 1998; Çirpanli et al., 2009; Duchêne, 1999). They have also been used for targeting and for increasing drug loading (Duchêne et al., 1999).

2.1.4 Gelatin

Gelatin is a natural polymer that is derived from collagen. It is commonly used for pharmaceutical and medical applications because of its biodegradability and biocompatibility in physiological environments. Gelatin is attractive for use in controlled release due to its nontoxic, bioactive properties and inexpensive price. It is also a polyampholyte having both cationic and anionic groups along with hydrophilic groups. Mechanical properties, swelling behavior and thermal properties of gelatin depend significantly on its crosslinking degree (Young et al., 2005).

2.2 Biodegradable polyesters

Polyester-based polymers are among of the most widely investigated materials for drug delivery. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers poly(lactic acid-co-glycolic acid) (PLGA) along with poly- ϵ -caprolactone are some of the well-defined biomaterials with regard to design and performance for drug-delivery applications.

2.2.1 PLGA

PLGA, a copolymer of lactic acid and glycolic acid, has generated tremendous interest due to its excellent biocompatibility, biodegradability, and mechanical strength. PLGA is approved by the US FDA and European Medicine Agency (EMA) in various drug delivery systems in humans. In order to improve the formulation of controlled drug delivery systems, an understanding of the physical, chemical, and biological properties of polymers is helpful. In fact, the polymer is commercially available with different molecular weights and copolymer compositions. The degradation time can vary from several months to several years, depending on the molecular weight and copolymer ratio (Danhier et al., 2012). For example, lactic acid is more hydrophobic than glycolic acid and, therefore, lactide-rich PLGA copoly-

mers are less hydrophilic, absorb less water, and subsequently, degrade more slowly (Dinarvand et al., 2011). PLGA particles are widely used to encapsulate active molecules with a broad spectrum of pharmaceutical applications (Danhier et al., 2012; Menei et al., 2005; Singh et al., 2004).

2.2.2 PLA

PLA is a biocompatible and biodegradable synthetic polyester which undergoes scission in the body to monomeric units of lactic acid. The latter is a natural intermediate in carbohydrate metabolism. PLA possess good mechanical properties and it is largely used for the preparation of particles (Gupta and Kumar, 2007).

2.2.3 PCL

It was in 1930s that the ring-opening polymerization of PCL was studied. The biodegradable property of this synthetic polymer was first identified in 1973. PCL is suitable for controlled drug delivery due to its high permeability to many drugs and non-toxicity (Sinha et al., 2004). Molecular weight dependent surface hydrophobicity and crystallinity of PCL are the causes for its slower biodegradation in two distinct phases such as random non-enzymatic cleavage and enzymatic fragmentation. Lipophilic drugs are generally distributed uniformly in the matrix while hydrophilic drugs tend to move towards the interface and remain on the surface of PCL formulation in adsorbed state. Diffusion was described as the only possible mechanism by which the lipophilic drugs release from PCL particles as they were shown to be intact for a much longer duration *in vivo*. However, two phenomena could be implicated in hydrophilic drugs' release. Highly lipophilic drugs that resist complete diffusion are released upon surface erosion by enzymatic action while hydrophilic drugs that accumulate at the interface during the formulation processes are released by desorption at the initial period of release study

or dosage intake. This results in a biphasic drug release pattern for PCL particles with much higher burst release for hydrophilic drugs than lipophilic ones (Dash and Konkimalla, 2012).

2.3 Pegylated polymers

Many of the above cited polymers could be conjugated to PEG chains, which allows the enhancement of their hydrophilicity and permits the obtaining of a stealth surface that could protect the prepared carriers from degradation by the cells belonging to the reticuloendothelial system. Conjugation to PEG confers also bioadhesive properties for the carriers (Yoncheva et al. 2005).

3. Used methods for the encapsulation of active molecules

3.1 Nanoprecipitation

The nanoprecipitation technique was first developed by Fessi et al. in 1986 (Devissaguet et al., 1991). The technique allows the obtaining of either nanospheres or nanocapsules. The organic phase could be added to the aqueous phase under magnetic stirring. This one-step process allows the instantaneous and reproducible formation of monodisperse nanoparticles. Nanoprecipitation is simple, is by far the fastest, most reproducible, and industrially feasible preparation procedure of nanospheres (Vauthier and Bouchemal, 2009). Practically, two miscible phases are required: an or-

ganic solvent in which the polymer is dissolved and an aqueous phase (non-solvent of the polymer). The most common used organic solvents are ethanol and acetone. Such solvents are miscible in water and easy to remove by evaporation. Some oils could be added to these solvents to allow the dissolving of the active (Rosset et al., 2012). As Figure 1 shows, the method is based on the addition of one phase to the other under moderate magnetic stirring which causes the interfacial deposition of a polymer after displacement of the organic solvent from the organic solution. This leads to the formation of a suspension of nanoparticles. The organic phase could be a mixture of solvents such as, mixture of acetone with water or ethanol etc. Similarly, the aqueous phase could consist of a mixture of non-solvents and could contain surfactants. Commonly used polymers are biodegradable polyesters, especially PCL, PLA and PLGA (Rao and Geckeler, 2011). Particle formation process includes three basic steps which are, particle nucleation, molecular growth and aggregation. The rate of every step has a crucial impact on the particle size distribution. Supersaturation is the driving force that manages all of these steps, namely, particles nucleation rate. Supersaturation, itself, is influenced by fluid dynamics and mixing. In fact, low stirring rate results in low nucleation rates while higher mixing rates give high nucleation rates (Lince et al., 2008).

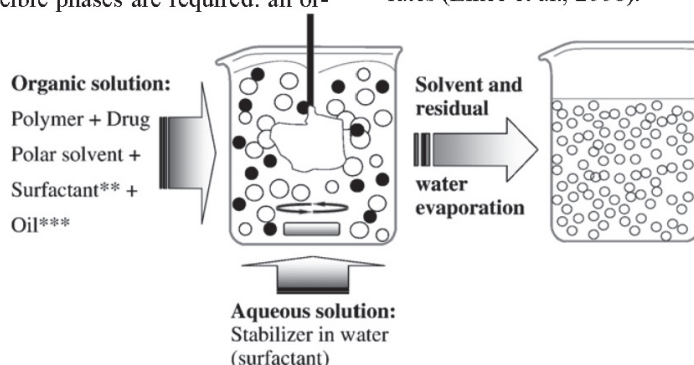


Figure 1: The nanoprecipitation technique (Pinto Reis et al., 2006)

Operational parameters that should be controlled include the organic phase to non organic phase ratio, the concentration of the polymer and the stabilizer and the amount of the drug. Every one of these parameters may exert an impact on the characteristics of the obtained nanoparticles (size, uniformity and charge). In fact, an increase of the polymer amount generally increases particles' size (Chorny et al., 2002; Simşek et al., 2013; Dong and Feng, 2004; Asadi et al., 2011). The same effect was obtained after increasing the polymer molecular weight (Limayem Blouza et al., 2006; Holgado et al., 2012). These findings were explained by an increase of the viscosity of the organic phase which rendered solvent diffusion more difficult and thus, led to larger nanoparticles' size. The effect of increasing organic phase volume seems conflicting: some studies showed that it causes a decrease of the particles size (Dong and Feng, 2004) while others showed the opposite phenomenon (Asadi et al., 2011). Increasing the water phase amount leads to a decrease of the particles size as a result of the increased diffusion of the water-miscible solvent in the aqueous phase and thus, the more rapid precipitation of the polymer and formation of nanoparticles (Budhian et al., 2007). An increase of the surfactant amount generally causes a decrease of the particles size and reduces size distribution (Contado et al., 2013; Siqueira-Moura et al., 2013). Some studies did not, however, found significant change following surfactant amount increase (Dong and Feng, 2004). The nature of the surfactant may also influence the particles' size (Limayem Blouza et al., 2006). Increasing mixing rate decreases the particles size as it causes faster diffusion rate (Asadi et al., 2011). Theoretical drug loading may also influence particles size and drug loading (Govender et al., 1999). Nanoprecipitation is generally designed for the encapsulation of hydrophobic drug molecules (Seju et al., 2011; Katara and Majumdar, 2013; Seremeta et al., 2013). Such actives may be

dissolved within the organic phase. Bilati et al. (2005) described a nanoprecipitation technique intended to the encapsulation of hydrophilic molecules but the size of the obtained particles was not sufficiently uniform (Bilati et al., 2005). To further improve the reproducibility of the nanoprecipitation technique and make it more convenient for industrial applications, membrane contactor and microfluidic technology were successfully used (Khayata et al., 2012; Xie and Smith, 2010). These techniques allow better size control within different batches of particles. Table 2 contains some examples of the applications of the nanoprecipitation technique in drug delivery during the last years. It can be concluded that polyesters are among the most used polymers for the preparation of the nanoparticles by this technique.

3.2 Emulsion diffusion (ESD)

ESD was first developed by Quintanar-Guerrero and Fessi (Quintanar-Guerrero et al., 1996) to prepare PLA based nanospheres. Three liquid phases are needed in this technique: an organic phase, an aqueous phase and a dilution phase. The organic phase generally contains the polymer and the hydrophobic drug. The aqueous phase is a solution of a stabilizing agent while the dilution phase usually consists of a large volume of water. Mutual saturation of the aqueous and organic phase allows further obtaining of a thermodynamically equilibrated emulsion upon high speed homogenization. Subsequent addition of an excess of water enables the diffusion of the organic solvent from the dispersed phase resulting in precipitation of the polymer and the formation of the particles (Figure 2). Commonly used polymers in this method include PCL, PLA and Eudragit® (Mora-Huertas et al., 2010). Table 3 shows that the technique is mainly used for the encapsulation of hydrophobic molecules. However, hydrophilic molecules may also be encapsulated by a modified solvent diffusion method using an aqueous inner phase (Ma

et al., 2001). Operating conditions affecting the size of the obtained particles include external/internal phase ratio, emulsification stirring rate, volume and temperature of water for dilution, polymer amount and concentration of the stabilizer (Quintanar-Guerrero et al., 1996; Mora-Huertas et al., 2010). Influence of high shear homogenization and sonication on the particles size was assessed and it was found that sonication was more efficient for particle size reduction. The nature of the surfactant influenced also the particles size. In fact, when Pluronic F68 (PF68), didodecyltrimethylammonium bromide (DMAB) and polyvinylalcohol (PVA) were compared, DMAB gave the smallest particles but with the lowest encapsulation efficiency (Jain et al., 2011). Particles size was also described to increase with an increase of initial drug amount (Youm et al., 2012), polymer

amount (Youm et al., 2012; Esmaeili et al., 2011) and the oil phase volume (Esmaeili et al., 2011; Poletto et al., 2008). An increase of the surfactant amount resulted in a decrease of the size but it seems that above some level further significant size reduction is no longer possible (Jain et al., 2011; Surassmo et al., 2010). An increase of the homogenization rate led to a decrease of the particles' size (Jain et al., 2011; Kwon et al., 2001; Galindo-Rodríguez et al., 2005). Likely, the same effect was obtained following an increase of the temperature and the volume of added water (Kwon et al., 2001; Song et al., 2006). The nature of the organic solvent also influenced particle size (Song et al., 2006). Table 3 shows some of the recent applications of the ESD technique.

Table 2: Applications of the nanoprecipitation technique

Encapsulated molecule	Polymer	Size (nm)	Zeta potential (mV)	Reference
Doxorubicin	Gelatin-co-PLA-DPPE	131.5-161.1	-	Han et al., 2013
Aceclofenac	Eudragit RL 100	75.5-184.4	22.5 - 32.6	Katara and Majumdar, 2013
Doxorubicin	Dextran-b-polycaprolactone	95-123.3	-	Li et al., 2013
Chloroaluminum phthalocyanine	PLGA	220.3-326.3	-17.7-(-40.9)	Siqueira-Moura et al., 2013
Efavirenz	PCL and Eudragit® RS 100	89.5 - 173.9	-17.9-53.8	Seremeta et al., 2013
Paclitaxel	PLGA	50 - 150	-15 - (-20)	Wang et al., 2013
Retinoic acid	PLA	153.6-229.8	-27.4-(-20.9)	Almouazen et al., 2012
Brimonidine Tartrate	Eudragit® RL 100	123.5 - 140.2	13.1- 20.8	Khan et al., 2012
Vitamin E	PCL	123-320	-24.5-(-1.46)	Khayata et al., 2012
Paclitaxel	Hydrophobized pullulan	127.6-253		Lee et al., 2012
Curcumin	PCL, chitosan	104-125	(-0.099)-79.8	Mazzarino et al., 2012
Diclofenac	PCL	152	-50	Mora-Huertas et al., 2012
Amphotericin B	PLGA	86-153	-31.4-(-9.1)	Van de Ven et al., 2012
Epirubicin	Poly(butyl cyanoacrylate)	217-235	-4.5-(-0.1)	Yordanov 2012
Camptothecin	Beta-cyclodextrin	281	-13	Cirpanli et al., 2011
	PLGA	187	-0.06	
	PCL	274	-19	
Naringenin	Eudragit® E	90	-	Krishnakumar et al., 2011
Olanzapine	PLGA	91.2	-23.7	Seju et al., 2011

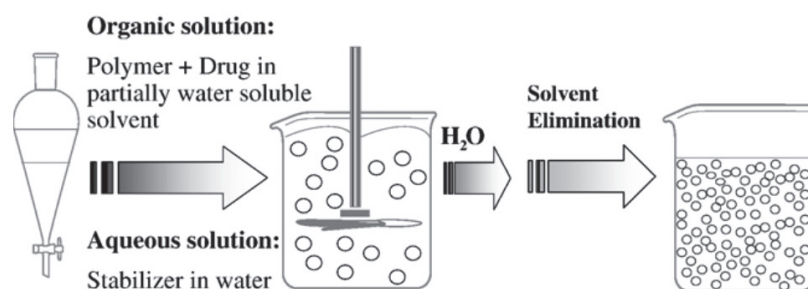


Figure 2: Emulsion diffusion technique (Pinto Reis et al., 2006)

Table 3: Applications of the emulsion diffusion method

Encapsulated molecule	Polymer	Size (μm)	Zeta potential (mV)	Reference
Articaine	PCL	-	-	Campos et al., 2013
Omeprazole	Eudragit L 100-55	0.256.3- 0.337	8.92 - 16.53	Hao et al., 2013
Curcumin	Polyurethane and polyurea	0.216- 4.901	-	Souguir et al., 2013
Matricaria recutita L. extract	PEG-PBA-PEG	0.186- 0.446	-	Esmaeili et al., 2011
Bovine serum albumin	Chitosan	81-98	-	Karnchanajindanun et al., 2011
Alendronate	PLGA	0.145	-4.7	Cohen-Sela et al., 2009
An oligonucleotide	PLA	0.390	-	Delie et al., 2001

3.3 Simple Emulsion evaporation (SEE)

The SEE technique is widely used in the field of particulate carriers' development. This method was first developed by (Vanderhoff et al., 1979). It consists on the formation of a simple emulsion followed by the evaporation of the organic solvent. Subsequent precipitation of the polymer allows the obtaining of the particles (Figure 3). Practically, for oil in water emulsion method, the polymer is dissolved in a volatile and non miscible organic solvent such as chloroform, ethylacetate or dichloromethane. This organic phase, in which the drug and the polymer are dissolved, is then dispersed by high speed homogenization or by sonication in an aqueous phase containing a surfactant. Once an oil-in-water (o/w)

emulsion is obtained, the evaporation of the organic solvent permits the precipitation of the polymer and thus, the formation of the particles. As it can be seen in Table 4, SEE is generally used for the encapsulation of hydrophobic drugs (O'Donnell and McGinity, 1997). The evaporation of the organic solvent is obtained by moderately stirring the emulsion at room temperature or under high temperature and low pressure conditions. The obtained particles can be then harvested by ultracentrifugation or filtration, then washed and lyophilized. Membrane technology was also used to prepare particles by the simple emulsion technique (Doan et al., 2011). Another alternative of the technique is the use of water in oil emulsion method that is suitable for the encapsulation of hydrophilic active molecules. Particulate carriers are obtained after evap-

oration of the water phase which causes the precipitation of the hydrophilic polymer (Banerjee et al., 2012). Parameters that have to be managed include organic phase to water phase ratio, nature of the surfactant and its concentration, stirring rate, polymer amount and evaporation rate. Decreasing the organic solvent volume resulted generally in a decrease of particle size (Budhian et al., 2007). Particle size could also be decreased by increasing surfactant amount (Valot et al., 2009; Manchanda et al., 2010; Khaled et al., 2010; Su et al., 2009), increa-

sing stirring rate (Su et al., 2009; Lee et al., 2012; Avachat et al., 2011; Yadav and Sawant, 2010) or increasing aqueous phase volume (Adibkia et al., 2011). However, an increase of polymer amount generally increases particles' size (Doan et al., 2011; D'Aurizio et al., 2011; Adibkia et al., 2011; Agnihotri and Vavia, 2009). Table 4 shows the applications of the SEE technique in drug delivery. Polyesters were widely used for the encapsulation of hydrophobic drugs.

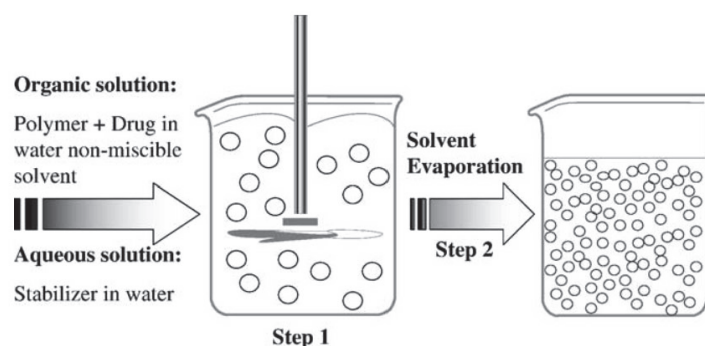


Figure 3: Simple emulsion solvent evaporation (Pinto Reis et al., 2006)

Table 4: Applications of simple emulsion solvent evaporation technique

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Curcumin	PLGA and PLGA-PEG	0.161-0.152	-	Khalil et al., 2013
Efavirenz	PCL and Eudragit® RS 100	0.083-0.219	53	Seremeta et al., 2013
Human amylin	PCL	0.202	-	Guerreiro et al., 2012
Azithromycin	PLGA	14.11-15.29	-	Li et al., 2012
Teniposide	PLGA	0.113-0.135	-36.6-(-23.1)	Mo et al., 2012
Camptothecin	PCL-PEG-PCL	4.2-5.4	-	Dai et al., 2011
Naproxen	PLGA	352-824	-	Javadzadeh et al., 2010
Doxorubicin	PLGA	0.137-0.164	-12.3-(-9.9)	Manchanda et al., 2010
Dexamethasone	PLGA	5.18-7	-	Rawat and Burgess, 2010
Ibuprofen	Eudragit RSPO	14-51.1	-	Valot et al., 2009

3.4 Double emulsion evaporation (DEE)

Double emulsion technique is suitable for the encapsulation of hydrophilic molecules (see Table 5 and Figure 4). Generally, the method consists on the dispersion of an aqueous phase in a non miscible organic solvent to form the first emulsion (W1/O). This dispersion is performed under high shear homogenization or low power sonication for a short time. This step is followed by the dispersion of the obtained emulsion in a second aqueous phase containing a hydrophilic emulsifier. Again, homogenization could be carried under high shear homogenization or with a sonication probe. When sonication is used, it must be performed at low power and within a short period of time to not break the first emulsion (Giri et al., 2013). After the formation of the multiple emulsion, evaporation of the volatile organic solvent under low pressure (by a rotary evaporator) or at ambient temperature allows the obtaining of the particulate carriers (Figure 4). There are other types of multiple emulsions like w/o/o or o/w/o (Giri et al., 2013). A lot of parameters may influence the properties of the obtained particles such as, relative phases' ratio (Khoee et al., 2012), amount of the polymer, its nature and molecular weight (Zambaux et al., 1998; Péan et al., 1998; Van de Ven et al., 2011), nature of the surfactants and their amounts (Zhao et al., 2007; Khoee and Yaghoobian, 2009; Dhanaraju et al., 2004), homogenization speed (Eley and Mathew, 2007; Basarkar et al., 2007), the composition of the external phase (Péan et al., 1998; Tse et al., 2009) and evaporation speed (Khoee et al., 2012). Operating conditions may also influence strongly encapsulation efficiency (Tse et al., 2009; Billon et al., 2005; Silva et al., 2013; Zhou et al., 2013; Karataş et al., 2009; Hachicha et al., 2006; Al-Kassas, 2004; Cun et al., 2011; Gaignaux et al., 2012; Cun et al., 2010). Membrane technique and microfluidic devices were also used to prepare particulate carriers by the

DES method (Vladisavljević and Williams, 2008; van der Graaf et al., 2005).

3.5 Spray drying

Spray drying is a simple process which gained too much interest due to its cost-effectiveness and scalability (Sou et al., 2011). Practically, a polymer containing drug solution is atomized and sprayed into a drying chamber where droplets are dried by heated air (See Figure 5). Reduction of droplets' size that follows atomization allows the obtaining of an enormous surface area between droplets and the drying gas. The subsequent precipitation of the polymer permits the encapsulation of the drug within the obtained particulate carriers. The evaporation of the solvent occurs within a very short period of time. Consequently, the materials never reach the inlet temperature of drying gas. This is very attractive for encapsulating heat-sensitive drug molecules like proteins (Cal and Sollohub, 2010; Sollohub and Cal, 2010; Prata et al., 2013). Many operating conditions could influence the properties of the obtained particles. Parameters to be controlled include the drying air temperature and humidity (Bruschi et al., 2003), the rate and fluid dynamics of the air flow, the atomization process (Droplet size, spray rate, spray mechanism) and the composition of ingredients and excipients in the feeding solution (Rattes and Oliveira, 2007). PLA (Baras et al., 2000; Gander et al., 1996; Sastre et al., 2007; Muttill et al., 2007), PLGA (Wang and Wang, 2002; Mu and Feng, 2001; Castelli et al., 1998; Bittner et al., 1999; Prior et al., 2000; Conti et al., 1997), PCL, methacrylate polymers (Esposito et al., 2002; Año et al., 2011; Cruz et al., 2010; Hegazy et al., 2002; Raffin et al., 2008) and chitosan (He et al., 1999; Giunchedi et al., 2002; Cevher et al., 2006) are among the most used polymers in spray-drying method. As Table 6 shows, the technique allowed the obtaining of mainly microparticles bearing better drug solubility and sustained release.

Table 5: Applications of the double emulsion technique

Encapsulated molecule	Polymer	Size (μm)	Zeta potential (mV)	Reference
Vancomycin	PLGA	0.450-0.466	-7.2(-3.5)	Zakeri-Milani et al., 2013
Prostaglandin E1	PLGA	7-22.5	-	Gupta and Ahsan, 2011
Deoxyribonuclease I	PLGA	0.190-0.349	-	Osman et al., 2011
S. equi antigens	PCL	0.242-0.450	-53.1-38.7	Florindo et al., 2009
Hepatitis B surface antigen	PLGA	1-5	0.51-14	Thomas et al., 2009
Plasmid DNA	PLGA	1.9-4.6	-24.6(-22.9)	Tse et al., 2009

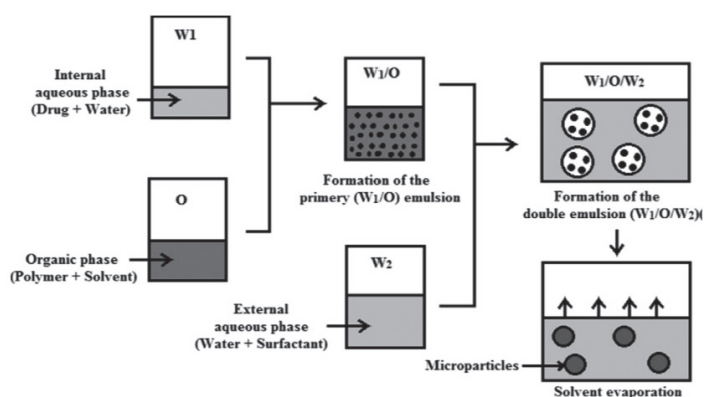


Figure 4: Double emulsion solvent evaporation technique (Giri et al., 2013)

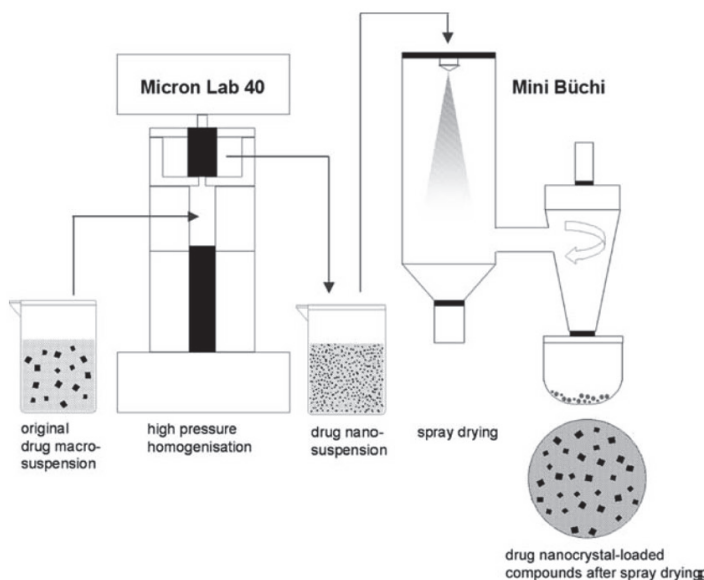


Figure 5: The spray drying method (Pinto Reis et al., 2006)

Table 6: Applications of the spray drying technique

Encapsulated molecule	Polymer	Size (μm)	Zeta potential (mV)	Reference
Nimodipine	PLGA	1.9-2.37	-	Bege et al., 2013
Theophylline	Eudragit RS30D	< 60	-	Garekani et al., 2013
Ofloxacin	PLA	2.6-4.9	-	Palazzo et al., 2013
Sodium diclofenac	PGA-co-PDL	2.3	-32.2	Tawfeek, 2013
	PEG-PGA-co-PDL	3.9	-29.9	
	and mPEG-co-(PGA-co-PDL)	2.5	-31.2	
Sodium fluoride	Chitosan	3.4-5.3	-	Keegan et al., 2012
Plasmid	Chitosan	2.5-11.7	-	Mohajel et al., 2012
Heparin	PLGA	2.5-3.8	-63.5 - (-28.2)	Yildiz et al., 2012
Alendronate	Eudragit® S100	13.8	-	Cruz et al., 2010
Zolmitriptan	Chitosan glutamate and Chitosan base	2.6-9.4	-	Alhalaweh et al., 2009
Triamcinolone	PLGA	0.5-1.5	-	da Silva et al., 2009
Acyclovir	Chitosan	18.7-34.9	-	Stulzer et al., 2009

3.6 Supercritical fluid technology (SFT)

In the recent years, novel particle formation techniques using supercritical fluids (SCF) have been developed in order to overcome some of the disadvantages of conventional techniques that are: (1) poor control of particle size and morphology; (2) degradation and loss of biological activity of thermo sensitive compounds; (3) low encapsulation efficiency and (4) low precipitation yield (Santos et al., 2013). Moreover, SFT presents the main advantage of not requiring the use of toxic solvents. In fact, SCF based technologies have attracted enormous interest for the production of microparticles and nanoparticles (Table 7), since their emergence in the early 1990s (Sanli et al., 2012).

The supercritical state is achieved when a substance is exposed to conditions above its critical pressure and temperature. In such conditions, the fluid will have liquid-like density and, thus, solvating properties that are similar to those of liquids and, at the same time, gas-like mass transfer properties. Carbon dioxide (CO_2) is the most commonly used critical fluid. In fact, CO_2 is nontoxic, nonflammable and easy recyclable. Moreover, CO_2 has moderate critical parameters of CO_2 (a critical pressure of 7.4

MPa and a critical temperature of 304.1 K) and low price and is highly available which makes it very attractive from an economical point of view and also for the processing of labile compounds (Elizondo et al., 2012). Supercritical fluid technology methods can be divided in four methods which are rapid expansion of supercritical solution (RESS), Particles from gas saturated solutions (PGSS), gas antisolvent (GAS) and supercritical antisolvent process (SAS). These methods depend on whether CO_2 was used as a solvent, a solute or an antisolvent. Figure 6 shows the experimental set up of the RESS technique. In the RESS technique, the drug and the polymer are first dissolved in supercritical CO_2 in high pressure chamber. The subsequent passing of the solution through a nozzle results in a rapid decrease of the pressure and thus, a precipitation of the drug particles embedded in the polymer matrix and their recovery in the extraction unit (Byrappa et al., 2008). Many parameters such as the density of the SCF (Pressure and temperature of supercritical fluid) (Kalani and Yunus, 2012), flow rate of drug-polymer solution and/or CO_2 and formulation variables (Martin et al., 2002) could influence the size of the obtained particles. Table 7 shows that SFT was used for

the processing of nanoparticles and micro-particles mainly based on polyesters.

3.7 Ionic gelation (IG)

IG method is used mainly with natural hydrophilic polymers to prepare particulate carriers. These polymers include gelatin, alginate, chitosan and agarose. IG has the advantage of not using organic solvents. The technique is based on the transition of the polymer from liquid state to a gel (Figure 7). For instance, gelatin based particles are obtained after the hardening of the droplets of emulsified gelatin solution. The particles are obtained after cooling gelatin emulsion droplets below the gelation point in an ice bath. For alginate, however, particles are produced by drop-by-drop extrusion of the sodium alginate solution into the

calcium chloride solution. Sodium alginate is, in fact, a water-soluble polymer that gels in the presence of multivalent cations such as calcium. Chitosan particles are prepared by spontaneous formation of complexes between the positively charged chitosan and polyanions (tripolyphosphate or gelatin) or by the gelation of a chitosan solution dispersed in an oil emulsion (Mahapatro and Singh, 2011). Figure 7 illustrates the gelation mechanism of polysaccharides. At high temperatures, a random coil conformation is assumed. With decreasing temperature, the aggregation of double helices structure forms the physical junctions of gels (Rees and Welsh, 1977). Table 8 displays some recent applications of IG. This technique has been mainly used to prepare chitosan nanoparticles.

Table 7: Applications of the SCF technology

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Hydrocortisone acetate	PLGA	1-5	-	Falco et al., 2013
17 α -methyltestosterone	PLA	5.4-20.5	13.9 - 67.7	Sacchetin et al., 2013
Paracetamol	PLA	0.301-1.461	-	Kalani and Yunus, 2012
5-fluorouracil	PLLA-PEG/PEG	0.175	-	Zhang et al., 2012
Human growth hormone	PLGA	93	-	Jordan et al., 2010
Azacytidine	PLA	2	-	Argemí et al., 2009
Bovine serum albumin	PLA	2.5	-	Kang et al., 2009
Retinyl palmitate	PLA	0.040-0.110	-	Sane and Limtrakul, 2009
Indomethacin	PLA	2.35	-	Kang et al., 2008

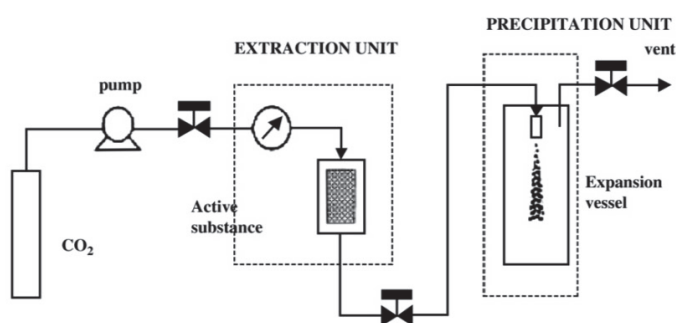


Figure 6: Schematic presentation of the experimental set up for the RESS process (Byrappa et al., 2008)

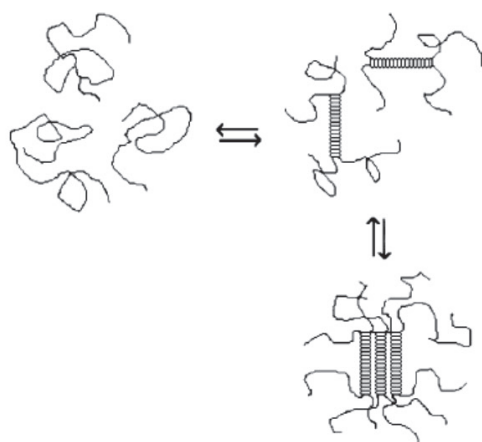


Figure 7: Gelation mechanism of polysaccharides in water (Guenet, 1992)

Table 8: Some applications of the ionic gelation technique

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Articaine hydrochloride	Alginate/chitosan	0.340-0.550	-22 - (-19)	de Melo et al., 2013
TNF-α siRNA	Trimethyl chitosan-cysteine	0.146	25.9	He et al., 2013
Paclitaxel	O-carboxymethyl chitosan	0.130-0.180	-30 - (-12)	Maya et al., 2013
pDNA	Chitosan	0.153-0.403	46.2-56.9	Cadete et al., 2012
Gemcitabine	Chitosan	0.095	-	Derakhshandeh and Fathi, 2012
Dexamthasone sodium phosphate	Chitosan	0.256-0.350	-	Doustgani et al., 2012
Itraconazole	Chitosan	0.190-0.240	11.5-18.9	Jafarinejad et al., 2012
5-fluorouracil and leucovorin	Chitosan	0.040-0.097	25.6-28.9	Li et al., 2011
Insulin	Chitosan and arabic gum	0.172-0.245	35.7-43.4	Avadi et al., 2010
CKS9 peptide sequence	Chitosan	0.226	-	Yoo et al., 2010

3.8 Layer by layer

Polyelectrolyte self assembly is also called layer-by-layer (LbL) assembly process. The earliest technology was based on the assembly of colloidal particles on a solid core (Iler, 1966). From the 1990s, applications were expanded. LbL allowed, in fact, the assembly of polyelectrolyte films using biopolymers, proteins, peptides, poly-

saccharides and DNA (Powell et al., 2011). This approach was first developed by Sukhorukov et al. (Sukhorukov et al., 1998). Polyelectrolytes are classified according to their origin. Standard synthetic polyelectrolytes include poly(styrene sulfonate) (PSS), poly(dimethyldiallylammonium chloride) (PDDA), poly(ethylenimine) (PEI), poly(N-isopropyl acrylamide) (PNIPAM), poly-

(acrylic acid) (PAA), poly (methacrylic acid) (PMA), poly(vinyl sulfate) (PVS) and poly(allylamine) (PAH). Natural polyelectrolytes include nucleic acids, proteins and polysaccharides such as, alginic acid, chondroitin sulfate, DNA, heparin, chitosan, cellulose sulfate, dextran sulfate and carboxymethylcellulose (de Villiers et al., 2011). The obtained particles are vesicular and are called polyelectrolyte capsules. Assembly process is based on irreversible electrostatic attraction that leads to polyelectrolyte adsorption at supersaturating polyelectrolyte concentrations. Other interactions such as, hydrogen bonds, hydrophobic interactions and Van der Waals forces were also described (de Villiers et al., 2011). A colloidal template that serves to the adsorption of the polyelectrolyte is also needed. The commonly used cores for the formulated particles are derived from stabilized colloidal dispersions of charged silica, charged poly(styrene) spheres, metal oxides, polyoxometalates and conducting liquid crystalline polymers. Carrier systems can be functionalized with stimuli-responsive components that respond to temperature, pH and

ionic strength. The polymers/colloids used in LbL technique can also be functionalized to alter their properties preceding layer by layer assembly. Experimental parameters that have to be managed include coating material concentration, ion concentration and the pH of the medium (Vergaro et al., 2011). Polymer assembly occurs after incubation of the template in the polymer solution or by decreasing polymer solubility by drop-wise addition of a miscible solvent (Radtchenko et al., 2002). This procedure could be repeated with a second polymer to allow sequential deposition of multiple polymer layers (Figure 8). LbL presents advantages over several conventional coating methods: (1) simplicity of the process and equipment; (2) its suitability for coating most surfaces; (3) availability and abundance of natural and synthetic colloids; (4) flexible application to objects with irregular shapes and sizes; (5) formation of stabilizing coats and (6) control over the required multilayer thickness (de Villiers et al., 2011). Table 9 contains some recent applications of LbL technique.

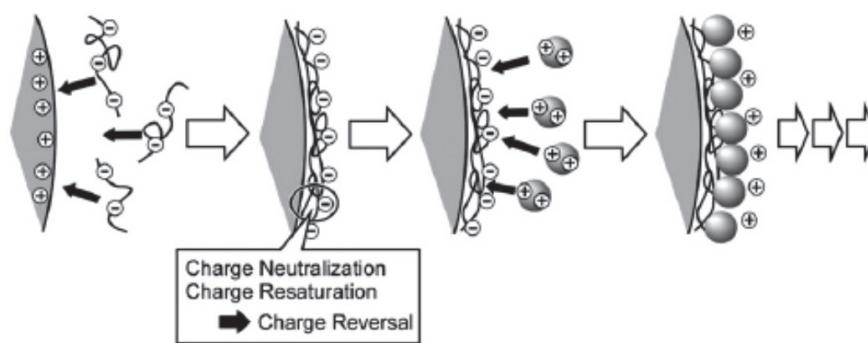


Figure 8: The layer by layer technique based on electrostatic interaction (Ariga et al., 2011)

Table 9: Applications of the layer-by-layer technique

Active	Polyelectrolytes	Size (µm)	Zeta potential (mV)	References
Kaempferol	Sodium Alginate and protamine sulfate	0.161	- 8.9	Kumar et al., 2012
Designed peptide DP-2015	Poly-L-glutamic acid and poly-L-lysine	-	-	Powell et al., 2011
5-fluorouracil	Poly(L-glutamic acid) and chitosan	1	25-40	Yan et al., 2011
Plasmid DNA	Plasmid DNA and reducible hyperbranched poly(amidoamine) or polyethylenimine	-	-	Blacklock et al., 2009
Artemisinin	Alginate, gelatin and chitosan	0.806	-33	Chen et al., 2009
Insulin	Glucose oxidase and catalase	6	-	Qi et al., 2009
Heparin	Poly(styrene sulfonate) and chitosan	1	-10.4	Shao et al., 2009
Acyclovir	Poly(vinyl galactose ester-co-methacryloxyethyl trimethylammonium chloride) and poly(sodium 4-styrenesulfonate)	-	-	Zhang et al., 2008a
Propranolol hydrochloride	Poly(vinyl galactose ester-co-methacryloxyethyl trimethylammonium chloride) and Poly(sodium 4-styrenesulfonate)	5-15.6	-	Zhang et al., 2008b

CONCLUSION

Encapsulation of active molecules is a crucial approach that has been widely used for many biomedical applications. It permits enhancement of bioavailability of molecules, sustained delivery, passive or active targeting and decrease of toxicity and side effects. These formulations can render some active molecules more suitable for a specific route such as the delivery of proteins by the oral route or the delivery of some drugs via the blood brain barrier. Thus, they enhance efficiency, patient compliance and allow successful management of diseases. Many biodegradable and biocompatible polymers were investigated. The choice of the technique and the suitable polymer is a crucial step. It depends on the physicochemical properties of the drug to be encapsulated. The management of operating conditions is also a hard task to monitor particles' properties and to enhance drug loading. Recent research works are focus-

ing on active targeting by the coating the carriers by biomolecules that specifically recognize a well-defined cell receptor. One can also notice a shift for more 'intelligent' drug delivery systems. Responsive materials, for example, react to a specific physiological stimulus such as a variation of pH to release the encapsulated drug. Other thermo-sensitive materials deliver drugs at a specific temperature. It can be noted also that more attention is paid to safer methods that avoid the use of organic solvents (RESS) or to techniques that provide better reproducibility and easy scalability (microfluidics and membrane emulsification technology), which could be attractive for industrial processing.

REFERENCES

- Adibkia K, Javadzadeh Y, Dastmalchi S, Mohammadi G, Niri FK, Alaei-Beirami M. Naproxen-eudragit rs100 nanoparticles: preparation and physicochemical characterization. *Colloids Surf B Biointerfaces* 2011;83:155–9.
- Agnihotri SM, Vavia PR. Drug loaded poly[lac(glc-leu)] microparticles: formulation and release characteristics. *Colloids Surf B Biointerfaces* 2009;74:336–9.
- Ahmad H. A special issue on polymer and hybrid particles for biomedical applications. *J Colloid Sci Biotechnol* 2013;2:153–4.
- Al-Kassab R. Design and in vitro evaluation of gentamicin-eudragit microspheres intended for intravitreal administration. *J Microencapsul* 2004;21:71–81.
- Alhalaweh A, Andersson S, Velaga SP. Preparation of zolmitriptan-chitosan microparticles by spray drying for nasal delivery. *Eur J Pharm Sci* 2009;38:206–14.
- Almouazen E, Bourgeois S, Boussaïd A, Valot P, Malleval C et al. Development of a nanoparticle-based system for the delivery of retinoic acid into macrophages. *Int J Pharm* 2012;430:207–15.
- Alpar HO, Somavarapu S, Atuah KN, Bramwell VW. Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and dna delivery. *Adv Drug Deliv Rev* 2005;57:411–30.
- Año G, Esquisabel A, Pastor M, Talavera A, Cedré B et al. A new oral vaccine candidate based on the microencapsulation by spray-drying of inactivated vibrio cholerae. *Vaccine* 2011;29:5758–64.
- Argemí A, Vega A, Subra-Paternault P, Saurina J. Characterization of azacytidine/poly(l-lactic) acid particles prepared by supercritical antisolvent precipitation. *J Pharm Biomed Anal* 2009;50:847–52.
- Arias JL, López-Viota M, Sáez-Fernández E, Ruiz MA. Formulation and physicochemical characterization of poly(ϵ -caprolactone) nanoparticles loaded with flutafur and diclofenac sodium. *Colloids Surf B Biointerfaces* 2010;75:204–8.
- Ariga K, Lvov YM, Kawakami K, Ji Q, Hill JP. Layer-by-layer self-assembled shells for drug delivery. *Adv Drug Deliv Rev* 2011;63:762–71.
- Asada M, Takahashi H, Okamoto H, Tanino H, Danjo K. Theophylline particle design using chitosan by the spray drying. *Int J Pharm* 2004;270:167–74.
- Asadi H, Rostamizadeh K, Salari D, Hamidi M. Preparation of biodegradable nanoparticles of triblock pla-peg-pla copolymer and determination of factors controlling the particle size using artificial neural network. *J Microencapsul* 2011;28:406–16.
- Ataman-Önal Y, Munier S, Ganée A, Terrat C, Durand P-Y et al. Surfactant-free anionic pla nanoparticles coated with hiv-1 p24 protein induced enhanced cellular and humoral immune responses in various animal models. *J Control Release* 2006;112:175–85.
- Aumelas A, Serrero A, Durand A, Dellacherie E, Leonard M. Nanoparticles of hydrophobically modified dextrans as potential drug carrier systems. *Colloids Surf B Biointerfaces* 2007;59:74–80.
- Avachat AM, Bornare PN, Dash RR. Sustained release microspheres of ropinirole hydrochloride: effect of process parameters. *Acta Pharm Zagreb Croat* 2011;61:363–76.
- Avadi MR, Sadeghi AMM, Mohammadpour N, Abedin S, Atyabi F et al. Preparation and characterization of insulin nanoparticles using chitosan and arabic gum with ionic gelation method. *Nanomed Nanotechnol Biol Med* 2010;6:58–63.
- Balthasar S, Michaelis K, Dinuer N, von Briesen H, Kreuter J, Langer K. Preparation and characterization of antibody modified gelatin nanoparticles as drug carrier system for uptake in lymphocytes. *Biomaterials* 2005;26:2723–32.
- Banerjee P, Deb J, Roy A, Ghosh A, Chakraborty P. Fabrication and development of pectin microsphere of metformin hydrochloride. *ISRN Pharm* 2012;230621. doi: 10.5402/2012/230621.
- Baras B, Benoit M-A, Gillard J. Parameters influencing the antigen release from spray-dried poly(dl-lactide) microparticles. *Int J Pharm* 2000;200:133–45.
- Basarkar A, Devineni D, Palaniappan R, Singh J. Preparation, characterization, cytotoxicity and transfection efficiency of poly(dl-lactide-co-glycolide) and poly(dl-lactic acid) cationic nanoparticles for controlled delivery of plasmid dna. *Int J Pharm* 2007;343:247–54.

- Bazylińska U, Lewińska A, Lamch L, Wilk KA. Polymeric nanocapsules and nanospheres for encapsulation and long sustained release of hydrophobic cyanine-type photosensitizer. *Colloids Surf Physicochem Eng Asp* 2013.
- Beck-Broichsitter M, Ruppert C, Schmehl T, Guenther A, Betz T et al. Biophysical investigation of pulmonary surfactant surface properties upon contact with polymeric nanoparticles in vitro. *Nanomed Nanotechnol Biol Med* 2011;7:341–50.
- Beck-Broichsitter M, Schweiger C, Schmehl T, Gessler T, Seeger W, Kissel T. Characterization of novel spray-dried polymeric particles for controlled pulmonary drug delivery. *J Control Release* 2012; 158:329–35.
- Bege N, Renette T, Endres T, Beck-Broichsitter M, Hänggi D, Kissel T. In situ forming nimodipine depot system based on microparticles for the treatment of posthemorrhagic cerebral vasospasm. *Eur J Pharm Biopharm* 2013;84:99–105.
- Behera T, Swain P. Antigen adsorbed surface modified poly-ε-caprolactone microspheres stimulates both adaptive and innate immune response in fish. *Vaccine* 2012;30:5278–84.
- Benoit MA, Baras B, Gillard J. Preparation and characterization of protein-loaded poly(ε-caprolactone) microparticles for oral vaccine delivery. *Int J Pharm* 1999;184:73–84.
- Bernkop-Schnürch A, Heinrich A, Greimel A. Development of a novel method for the preparation of submicron particles based on thiolated chitosan. *Eur J Pharm Biopharm* 2006;63:166–72.
- Bilati U, Allemann E, Doelker E. Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles. *Eur J Pharm Sci* 2005;24:67–75.
- Billon A, Chabaud L, Gouyette A, Bouler J-M, Merle C. Vancomycin biodegradable poly(lactide-co-glycolide) microparticles for bone implantation. influence of the formulation parameters on the size, morphology, drug loading and in vitro release. *J Microencapsul* 2005;22:841–52.
- Bittner B, Mäder K, Kroll C, Borchert H-H, Kissel T. Tetracycline-hcl-loaded poly(dl-lactide-co-glycolide) microspheres prepared by a spray drying technique: influence of γ-irradiation on radical formation and polymer degradation. *J Control Release* 1999; 59:23–32.
- Blacklock J, You Y-Z, Zhou Q-H, Mao G, Oupický D. Gene delivery in vitro and in vivo from bioreducible multilayered polyelectrolyte films of plasmid dna. *Biomaterials* 2009;30:939–50.
- Bozkir A, Saka OM. Formulation and investigation of 5-fu nanoparticles with factorial design-based studies. *Il Farm* 2005;60:840–6.
- Bruschi M, Cardoso ML, Lucchesi M, Gremião MP. Gelatin microparticles containing propolis obtained by spray-drying technique: preparation and characterization. *Int J Pharm* 2003;264:45–55.
- Budhian A, Siegel SJ, Winey KI. Haloperidol-loaded plga nanoparticles: systematic study of particle size and drug content. *Int J Pharm* 2007;336: 367–75.
- Byrappa K, Ohara S, Adschiri T. Nanoparticles synthesis using supercritical fluid technology - towards biomedical applications. *Adv Drug Deliv Rev* 2008; 60:299–327.
- Cadete A, Figueiredo L, Lopes R, Calado CCR, Almeida AJ, Gonçalves LMD. Development and characterization of a new plasmid delivery system based on chitosan-sodium deoxycholate nanoparticles. *Eur J Pharm Sci* 2012;45:451–8.
- Cal K, Sollohub K. Spray drying technique. i: hardware and process parameters. *J Pharm Sci* 2010;99: 575–86.
- Campos EVR, de Melo NFS, de Paula E, Rosa AH, Fraceto LF. Screening of conditions for the preparation of poly(ε-caprolactone) nanocapsules containing the local anesthetic articaine. *J Colloid Sci Biotechnol* 2013;2:106–11.
- Castelli F, Conti B, Maccarrone DE, Conte U, Puglisi G. Comparative study of 'in vitro' release of anti-inflammatory drugs from poly(lactide-co-glycolide) microspheres. *Int J Pharm* 1998;176:85–98.
- Cevher E, Orhan Z, Mülazimoğlu L, Şensoy D, Alper M et al. Characterization of biodegradable chitosan microspheres containing vancomycin and treatment of experimental osteomyelitis caused by methicillin-resistant staphylococcus aureus with prepared microspheres. *Int J Pharm* 2006;317:127–35.
- Challa R, Ahuja A, Ali J, Khar RK. Cyclodextrins in drug delivery: an updated review. *AAPS Pharm Sci Tech* 2005;6:E329–57.

- Chawla JS, Amiji MM. Biodegradable poly(ϵ -caprolactone) nanoparticles for tumor-targeted delivery of tamoxifen. *Int J Pharm* 2002;249:127–38.
- Chen A-Z, Zhao Z, Wang S-B, Li Y, Zhao C, Liu Y-G. A continuous reprecipitation process to prepare PLA-PEG-PLA microparticles. *J Supercrit Fluids* 2011;59:92–7.
- Chen Y, Lin X, Park H, Greever R. Study of artemisinin nanocapsules as anticancer drug delivery systems. *Nanomed Nanotechnol Biol Med* 2009;5:316–22.
- Chorny M, Fishbein I, Danenberg HD, Golomb G. Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. *J Control Release* 2002;83:389–400.
- Çirpanlı Y, Bilensoy E, Lale Doğan A, Çaliş S. Comparative evaluation of polymeric and amphiphilic cyclodextrin nanoparticles for effective camptothecin delivery. *Eur J Pharm Biopharm* 2009;73:82–9.
- Çirpanlı Y, Allard E, Passirani C, Bilensoy E, Lemaire L et al. Antitumoral activity of camptothecin-loaded nanoparticles in 9L rat glioma model. *Int J Pharm* 2011;403:201–6.
- Cohen-Sela E, Chorny M, Koroukhov N, Danenberg HD, Golomb G. A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in PLGA nanoparticles. *J Control Release* 2009;133:90–5.
- Contado C, Vighi E, Dalpiaz A, Leo E. Influence of secondary preparative parameters and aging effects on PLGA particle size distribution: a sedimentation field flow fractionation investigation. *Anal Bioanal Chem* 2013;405:703–11.
- Conti B, Bucolo C, Giannavola C, Puglisi G, Giunchedi P, Conte U. Biodegradable microspheres for the intravitreal administration of acyclovir: in vitro/in vivo evaluation. *Eur J Pharm Sci* 1997;5:287–93.
- Cruz L, Assumpção E, Andrade SF, Conrado DJ, Kulkamp IC et al. Gastroresistant microparticles containing sodium alendronate prevent the bone loss in ovariectomized rats. *Eur J Pharm Sci* 2010;40:441–7.
- Cun D, Foged C, Yang M, Frøkjær S, Nielsen HM. Preparation and characterization of poly(DL-lactide-co-glycolide) nanoparticles for siRNA delivery. *Int J Pharm* 2010;390:70–5.
- Cun D, Jensen DK, Maltesen MJ, Bunker M, Whiteside P et al. High loading efficiency and sustained release of siRNA encapsulated in PLGA nanoparticles: quality by design optimization and characterization. *Eur J Pharm Biopharm* 2011;77:26–35.
- D'Aurizio E, van Nostrum CF, van Steenberghe MJ, Sozio P, Siepmann F et al. Preparation and characterization of poly(lactide-co-glycolic acid) microspheres loaded with a labile antiparkinson prodrug. *Int J Pharm* 2011;409:289–96.
- Da Silva AA Jr, de Matos JR, Formariz TP, Rossanezi G, Scarpa MV et al. Thermal behavior and stability of biodegradable spray-dried microparticles containing triamcinolone. *Int J Pharm* 2009;368:45–55.
- Dai C, Wang Y, Hou X. Preparation and protein adsorption of porous dextran microspheres. *Carbohydr Polym* 2012;87:2338–43.
- Dai M, Xu X, Song J, Fu S, Gou M et al. Preparation of camptothecin-loaded PLGA microspheres for the treatment of colorectal peritoneal carcinomatosis and tumor growth in mice. *Cancer Lett* 2011;312:189–96.
- Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. PLGA-based nanoparticles: an overview of biomedical applications. *J Control Release* 2012;161:505–22.
- Das S, Suresh PK, Desmukh R. Design of eudragit RL 100 nanoparticles by nanoprecipitation method for ocular drug delivery. *Nanomed Nanotechnol Biol Med* 2010;6:318–23.
- Dash TK, Konkimalla VB. Poly- ϵ -caprolactone based formulations for drug delivery and tissue engineering: a review. *J Control Release* 2012;158:15–33.
- De Melo NFS, Campos EVR, de Paula E, Rosa AH, Fraceto LF. Factorial design and characterization studies for articaine hydrochloride loaded alginate/chitosan nanoparticles. *J Colloid Sci Biotechnol* 2013;2:146–52.
- De Villiers MM, Otto DP, Strydom SJ, Lvov YM. Introduction to nanocoatings produced by layer-by-layer (LbL) self-assembly. *Adv Drug Deliv Rev* 2011;63:701–15.
- Delie F, Berton M, Allémann E, Gumy R. Comparison of two methods of encapsulation of an oligonucleotide into poly(D,L-lactic acid) particles. *Int J Pharm* 2001;214:25–30.

- Derakhshandeh K, Fathi S. Role of chitosan nanoparticles in the oral absorption of gemcitabine. *Int J Pharm* 2012;437:172–7.
- Devissaguet J-P, Fessi H, Puisieux F. Process for the preparation of dispersible colloidal systems of a substance in the form of nanocapsules. US Patent 5049322, 1991.
- Dhanaraju MD, Jayakumar R, Vamsadhara C. Influence of manufacturing parameters on development of contraceptive steroid loaded injectable microspheres. *Chem Pharm Bull (Tokyo)* 2004;52:976–9.
- Dinarvand R, Sepehri N, Manoochehri S, Rouhani H, Atyabi F. Polylactide-co-glycolide nanoparticles for controlled delivery of anticancer agents. *Int J Nanomed* 2011;6:877–95.
- Doan TVP, Couet W, Olivier JC. Formulation and in vitro characterization of inhalable rifampicin-loaded plga microspheres for sustained lung delivery. *Int J Pharm* 2011;414:112–7.
- Dong Y, Feng SS. Methoxy poly(ethylene glycol)-poly(lactide) (mpeg-pla) nanoparticles for controlled delivery of anticancer drugs. *Biomaterials* 2004;25:2843–9.
- Dong Y, Feng SS. In vitro and in vivo evaluation of methoxy polyethylene glycol-poly(lactide) (mpeg-pla) nanoparticles for small-molecule drug chemotherapy. *Biomaterials* 2007;28:4154–60.
- Doustgani A, Farahani EV, Imani M, Doulabi AH. Dexamethasone sodium phosphate release from chitosan nanoparticles prepared by ionic gelation method. *J Colloid Sci Biotechnol* 2012;1:42–50.
- Du W-L, Niu S-S, Xu Y-L, Xu Z-R, Fan C-L. Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions. *Carbohydr Polym* 2009;75:385–9.
- Duchêne D. Cyclodextrins in targeting application to nanoparticles. *Adv Drug Deliv Rev* 1999;36:29–40.
- Duchêne D, Ponchel G, Wouessidjewe D. Cyclodextrins in targeting: application to nanoparticles. *Adv Drug Deliv Rev* 1999;36:29–40.
- Eidi H, Joubert O, Attik G, Duval RE, Bottin MC et al. Cytotoxicity assessment of heparin nanoparticles in nr8383 macrophages. *Int J Pharm* 2010;396:156–65.
- Elaissari A. Thermally sensitive latex particles. In: Birdi K (ed.): *Handbook of surface and colloid chemistry*, 2nd ed. Boca Raton, FL: CRC Press, 2002.
- Eley JG, Mathew P. Preparation and release characteristics of insulin and insulin-like growth factor-one from polymer nanoparticles. *J Microencapsul* 2007;24:225–34.
- Elias H-G. *An introduction to plastics*. Weinheim: Wiley-VCH, 2003.
- Elizondo E, Veciana J, Ventosa N. Nanostructuring molecular materials as particles and vesicles for drug delivery, using compressed and supercritical fluids. *Nanomedicine* 2012;7:1391–1408.
- Elmizadeh H, Khanmohammadi M, Ghasemi K, Hassanzadeh G, Nassiri-Asl M, Gamarudi AB. Preparation and optimization of chitosan nanoparticles and magnetic chitosan nanoparticles as delivery systems using box-behnken statistical design. *J Pharm Biomed Anal* 2013;80:141–6.
- Esmaili A, Saremnia B, Koohian A, Rezazadeh S. Mechanism of nanocapsules of matricaria recutita l. extract formation by the emulsion-diffusion process. *Superlattices Microstruct* 2011;50:340–9.
- Esposito E, Cervellati F, Menegatti E, Nastruzzi C, Cortesi R. Spray dried eudragit microparticles as encapsulation devices for vitamin c. *Int J Pharm* 2002;242:329–34.
- Essa S, Rabanel JM, Hildgen P. Effect of aqueous solubility of grafted moiety on the physicochemical properties of poly(D,L-lactide) (plla) based nanoparticles. *Int J Pharm* 2010;388:263–73.
- Fàbregas A, Miñarro M, García-Montoya E, Pérez-Lozano P, Carrillo C et al. Impact of physical parameters on particle size and reaction yield when using the ionic gelation method to obtain cationic polymeric chitosan-tripolyphosphate nanoparticles. *Int J Pharm* 2013;446:199–204.
- Falamarzian A, Lavasanifar A. Optimization of the hydrophobic domain in poly(ethylene oxide)-poly(ε-caprolactone) based nano-carriers for the solubilization and delivery of amphotericin b. *Colloids Surf B Biointerfaces* 2010;81:313–20.
- Falco N, Reverchon E, Della Porta G. Injectable plga/hydrocortisone formulation produced by continuous supercritical emulsion extraction. *Int J Pharm* 2013;441:589–97.

- Florindo HF, Pandit S, Lacerda L, Gonçalves LMD, Alpar HO, Almeida AJ. The enhancement of the immune response against *s. equi* antigens through the intranasal administration of poly- ϵ -caprolactone-based nanoparticles. *Biomaterials* 2009;30:879–91.
- Fonseca C, Simões S, Gaspar R. Paclitaxel-loaded plga nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity. *J Control Release* 2002;83:273–86.
- Fredriksen BN, Grip J. Plga/pla micro- and nanoparticle formulations serve as antigen depots and induce elevated humoral responses after immunization of atlantic salmon (*salmo salar* L.). *Vaccine* 2012;30:656–67.
- Gaignaux A, Réeff J, Siepmann F, Siepmann J, De Vriese C et al. Development and evaluation of sustained-release clonidine-loaded plga microparticles. *Int J Pharm* 2012;437:20–8.
- Galindo-Rodríguez SA, Puel F, Briçon S, Al-lémann E, Doelker E, Fessi H. Comparative scale-up of three methods for producing ibuprofen-loaded nanoparticles. *Eur J Pharm Sci* 2005;25:357–67.
- Gan Q, Wang T, Cochrane C, McCarron P. Modulation of surface charge, particle size and morphological properties of chitosan-*tpg* nanoparticles intended for gene delivery. *Colloids Surf B Biointerfaces* 2005;44:65–73.
- Gander B, Johansen P, Nam-Trân H, Merkle HP. Thermodynamic approach to protein microencapsulation into poly(d,l-lactide) by spray drying. *Int J Pharm* 1996;129:51–61.
- Garekani HA, Moghaddam ZF, Sadeghi F. Organic solution versus aqueous dispersion of eudragit rs in preparation of sustained release microparticles of theophylline using spray drying. *Colloids Surf B Biointerfaces* 2013;108:374–9.
- Gennes P-G. *Scaling concepts in polymer physics*. Ithaca, NY: Cornell University Press, 197.
- Gèze A, Aous S, Baussanne I, Putaux J, Defaye J, Wouessidjewe D. Influence of chemical structure of amphiphilic β -cyclodextrins on their ability to form stable nanoparticles. *Int J Pharm* 2002;242:301–5.
- Giri TK, Choudhary C, Ajazuddin, Alexander A, Badwaik H, Tripathi DK. Prospects of pharmaceuticals and biopharmaceuticals loaded microparticles prepared by double emulsion technique for controlled delivery. *Saudi Pharm J* 2013;21:125–41.
- Giunchedi P, Juliano C, Gavini E, Cossu M, Sorrenti M. Formulation and in vivo evaluation of chlorhexidine buccal tablets prepared using drug-loaded chitosan microspheres. *Eur J Pharm Biopharm* 2002;53:233–9.
- Gou M, Zheng L, Peng X, Men K, Zheng X et al. Poly(ϵ -caprolactone)-poly(ethylene glycol)-poly(ϵ -caprolactone) (pcl-peg-pcl) nanoparticles for honokiol delivery in vitro. *Int J Pharm* 2009;375:170–6.
- Govender T, Stolnik S, Garnett MC, Illum L, Davis SS. Plga nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. *J Control Release* 1999;57:171–85.
- Govender T, Riley T, Ehtezazi T, Garnett MC, Stolnik S et al. Defining the drug incorporation properties of pla-peg nanoparticles. *Int J Pharm* 2000;199:95–110.
- Guenet J-M. *Thermoreversible gelation of polymers and biopolymers*. New York: Academic Press, 1992.
- Guerreiro LH, Da Silva D, Ricci-Junior E, Girard-Dias W, Mascarenhas CM et al. Polymeric particles for the controlled release of human amylin. *Colloids Surf B Biointerfaces* 2012;94:101–6.
- Gupta AP, Kumar V. New emerging trends in synthetic biodegradable polymers – polylactide: a critique. *Eur Polym J* 2007;43:4053–74.
- Gupta V, Ahsan F. Influence of pei as a core modifying agent on plga microspheres of pge₁, a pulmonary selective vasodilator. *Int J Pharm* 2011;413:51–62.
- Gyulai G, Péntzes CB, Mohai M, Csempesz F, Kiss É. Influence of surface properties of polymeric nanoparticles on their membrane affinity. *Eur Polym J* 2013;49:2495–503.
- Hachicha W, Kodjikian L, Fessi H. Preparation of vancomycin microparticles: importance of preparation parameters. *Int J Pharm* 2006;324:176–84.
- Han S, Li M, Liu X, Gao H, Wu Y. Construction of amphiphilic copolymer nanoparticles based on gelatin as drug carriers for doxorubicin delivery. *Colloids Surf B Biointerfaces* 2013;102:833–41.
- Hao S, Wang B, Wang Y, Zhu L, Wang B, Guo T. Preparation of eudragit I 100-55 enteric nanoparticles by a novel emulsion diffusion method. *Colloids Surf B Biointerfaces* 2013;108:127–33.

- Haznedar S, Dortunç B. Preparation and in vitro evaluation of eudragit microspheres containing acetazolamide. *Int J Pharm* 2004;269:131–40.
- He P, Davis SS, Illum L. Chitosan microspheres prepared by spray drying. *Int J Pharm* 1999;187:53–65.
- He C, Yin L, Tang C, Yin C. Multifunctional polymeric nanoparticles for oral delivery of tnf- α sirna to macrophages. *Biomaterials* 2013;34:2843–54.
- Hegazy N, Demirel M, Yazan Y. Preparation and in vitro evaluation of pyridostigmine bromide micro-particles. *Int J Pharm* 2002;242:171–4.
- Hernán Pérez de la Ossa D, Ligresti A, Gil-Alegre ME, Aberturas MR, Molpeceres J et al. Poly- ϵ -caprolactone microspheres as a drug delivery system for cannabinoid administration: development, characterization and in vitro evaluation of their anti-tumoral efficacy. *J Control Release* 2012;161:927–32.
- Hombreiro Pérez M, Zinutti C, Lamprecht A, Ubrich N, Astier A et al. The preparation and evaluation of poly(ϵ -caprolactone) microparticles containing both a lipophilic and a hydrophilic drug. *J Control Release* 2000;65:429–38.
- Hu Y, Jiang X, Ding Y, Zhang L, Yang C et al. Preparation and drug release behaviors of nimodipine-loaded poly(caprolactone)-poly(ethylene oxide)-polylactide amphiphilic copolymer nanoparticles. *Biomaterials* 2003;24:2395–404.
- Huang Y-Y, Chung T-W, Tzeng T. Drug release from pla/peg microparticulates. *Int J Pharm* 1997;156:9–15.
- Huang Y, Gao H, Gou M, Ye H, Liu Y et al. Acute toxicity and genotoxicity studies on poly(ϵ -caprolactone)-poly(ethylene glycol)-poly(ϵ -caprolactone) nanomaterials. *Mutat Res Toxicol Environ Mutagen* 2010;696:101–6.
- Hyvönen S, Peltonen L, Karjalainen M, Hirvonen J. Effect of nanoprecipitation on the physicochemical properties of low molecular weight poly(l-lactic acid) nanoparticles loaded with salbutamol sulphate and beclomethasone dipropionate. *Int J Pharm* 2005;295:269–81.
- Iler RK. Multilayers of colloidal particles. *J Colloid Interface Sci* 1966;21:569–94.
- Illum L, Fisher AN, Jabbal-Gill I, Davis SS. Bioadhesive starch microspheres and absorption enhancing agents act synergistically to enhance the nasal absorption of polypeptides. *Int J Pharm* 2001;222:109–19.
- Ishihara T, Takahashi M, Higaki M, Mizushima Y, Mizushima T. Preparation and characterization of a nanoparticulate formulation composed of peg-pla and pla as anti-inflammatory agents. *Int J Pharm* 2010;385:170–5.
- Jafarinejad S, Gilani K, Moazeni E, Ghazi-Khansari M, Najafabadi AR, Mohajel N. Development of chitosan-based nanoparticles for pulmonary delivery of itraconazole as dry powder formulation. *Powder Technol* 2012;222:65–70.
- Jain AK, Swarnakar NK, Godugu C, Singh RP, Jain S. The effect of the oral administration of polymeric nanoparticles on the efficacy and toxicity of tamoxifen. *Biomaterials* 2011;32:503–15.
- Javadzadeh Y, Ahadi F, Davaran S, Mohammadi G, Sabzevari A, Adibkia K. Preparation and physico-chemical characterization of naproxen-plga nanoparticles. *Colloids Surf B Biointerfaces* 2010;81:498–502.
- Jordan F, Naylor A, Kelly CA, Howdle SM, Lewis A, Illum L. Sustained release high microsphere formulation produced by a novel supercritical fluid technology: in vivo studies. *J Control Release* 2010;141:153–60.
- Kadam RS, Tyagi P, Edelhauser HF, Kompella UB. Influence of choroidal neovascularization and biodegradable polymeric particle size on transscleral sustained delivery of triamcinolone acetonide. *Int J Pharm* 2012;434:140–7.
- Kalani M, Yunus R. Effect of supercritical fluid density on nanoencapsulated drug particle size using the supercritical antisolvent method. *Int J Nanomed* 2012;7:2165–72.
- Kang Y, Wu J, Yin G, Huang Z, Yao Y et al. Preparation, characterization and in vitro cytotoxicity of indomethacin-loaded plla/plga microparticles using supercritical co2 technique. *Eur J Pharm Biopharm* 2008;70:85–97.
- Kang Y, Yang C, Ouyang P, Yin G, Huang Z et al. The preparation of bsa-plla microparticles in a batch supercritical anti-solvent process. *Carbohydr Polym* 2009;77:244–9.

- Kanthamneni N, Sharma S, Meenach SA, Billet B, Zhao J-C et al. Enhanced stability of horseradish peroxidase encapsulated in acetalated dextran microparticles stored outside cold chain conditions. *Int J Pharm* 2012;431:101–10.
- Karataş A, Sonakin O, Kiliçarslan M, Baykara T. Poly (epsilon-caprolactone) microparticles containing levobunolol hcl prepared by a multiple emulsion (w/o/w) solvent evaporation technique: effects of some formulation parameters on microparticle characteristics. *J Microencapsul* 2009;26:63–74.
- Karnchanajindanun J, Srisa-ard M, Baimark Y. Genipin-cross-linked chitosan microspheres prepared by a water-in-oil emulsion solvent diffusion method for protein delivery. *Carbohydr Polym* 2011;85:674–80.
- Katara R, Majumdar DK. Eudragit rl 100-based nanoparticulate system of aceclofenac for ocular delivery. *Colloids Surf B Biointerfaces* 2013;103:455–62.
- Katare YK, Muthukumaran T, Panda AK. Influence of particle size, antigen load, dose and additional adjuvant on the immune response from antigen loaded pla microparticles. *Int J Pharm* 2005;301:149–60.
- Kauffman KJ, Kanthamneni N, Meenach SA, Pierson BC, Bachelder EM, Ainslie KM. Optimization of rapamycin-loaded acetalated dextran microparticles for immunosuppression. *Int J Pharm* 2012;422:356–63.
- Keegan GM, Smart JD, Ingram MJ, Barnes L-M, Burnett GR, Rees GD. Chitosan microparticles for the controlled delivery of fluoride. *J Dent* 2012;40:229–40.
- Khaled KA, Sarhan HA, Ibrahim MA, Ali AH, Naguib YW. Prednisolone-loaded plga microspheres. *in vitro* characterization and *in vivo* application in adjuvant-induced arthritis in mice. *AAPS Pharm Sci Tech* 2010;11:859–69.
- Khalil NM, do Nascimento TCF, Casa DM, Dalmolin LF, de Mattos AC et al. Pharmacokinetics of curcumin-loaded plga and plga-peg blend nanoparticles after oral administration in rats. *Colloids Surf B Biointerfaces* 2013;101:353–60.
- Khalil SKH, El-Feky GS, El-Banna ST, Khalil WA. Preparation and evaluation of warfarin-β-cyclodextrin loaded chitosan nanoparticles for transdermal delivery. *Carbohydr Polym* 2012;90:1244–53.
- Khan MS, Vishakante GD, Bathool A. Development and characterization of brimonidine tartrate loaded eudragit nanosuspensions for ocular drug delivery. *J Colloid Sci Biotechnol* 2012;1:122–8.
- Khayata N, Abdelwahed W, Chehna MF, Charcosset C, Fessi H. Preparation of vitamin e loaded nanocapsules by the nanoprecipitation method: from laboratory scale to large scale using a membrane contactor. *Int J Pharm* 2012;423:419–27.
- Khoe S, Yaghoobian M. An investigation into the role of surfactants in controlling particle size of polymeric nanocapsules containing penicillin-g in double emulsion. *Eur J Med Chem* 2009;44:2392–9.
- Khoe S, Sattari A, Atyabi F. Physico-chemical properties investigation of cisplatin loaded polybutyladipate (pba) nanoparticles prepared by w/o/w. *Mater Sci Eng C* 2012;32:1078–86.
- Konecsni K, Low NH, Nickerson MT. Chitosan-tripolyphosphate submicron particles as the carrier of entrapped rutin. *Food Chem* 2012;134:1775–9.
- Koten JW, Van Luyn MJA, Cadée JA, Brouwer L, Hennink WE et al. Il-2 loaded dextran microspheres with attractive histocompatibility properties for local il-2 cancer therapy. *Cytokine* 2003;24:57–66.
- Krishnakumar N, Sulfikkarali N, Rajendra Prasad N, Karthikeyan S. Enhanced anticancer activity of naringenin-loaded nanoparticles in human cervical (hela) cancer cells. *Biomed Prev Nutr* 2011;1:223–31.
- Kumar A, Gupta GK, Khedgikar V, Gautam J, Kushwaha P et al. *In vivo* efficacy studies of layer-by-layer nano-matrix bearing kaempferol for the conditions of osteoporosis: a study in ovariectomized rat model. *Eur J Pharm Biopharm* 2012;82:508–17.
- Kumari A, Yadav SK, Pakade YB, Kumar V, Singh B et al. Nanoencapsulation and characterization of albizia chinensis isolated antioxidant quercitrin on pla nanoparticles. *Colloids Surf B Biointerfaces* 2011;82:224–32.
- Kwon H-Y, Lee J-Y, Choi S-W, Jang Y, Kim J-H. Preparation of plga nanoparticles containing estrogen by emulsification-diffusion method. *Colloids Surf Physicochem Eng Asp* 2001;182:123–30.
- Labruère R, Sicard R, Cormier R, Tuross E, West L. Poly(vinyl benzoate) nanoparticles for molecular delivery: studies on their preparation and *in vitro* properties. *J Control Release* 2010;148:234–40.
- Lamalle-Bernard D, Munier S, Compagnon C, Charles M-H, Kalyanaraman VS et al. Coadsorption of hiv-1 p24 and gp120 proteins to surfactant-free anionic pla nanoparticles preserves antigenicity and immunogenicity. *J Control Release* 2006;115:57–67.

- Le Ray A-M, Chiffolleau S, Iooss P, Grimandi G, Gouyette A et al. Vancomycin encapsulation in biodegradable poly(ϵ -caprolactone) microparticles for bone implantation. influence of the formulation process on size, drug loading, in vitro release and cytocompatibility. *Biomaterials* 2003;24:443–9.
- Lee SJ, Hong G-Y, Jeong Y-I, Kang M-S, Oh J-S et al. Paclitaxel-incorporated nanoparticles of hydrophobized polysaccharide and their antitumor activity. *Int J Pharm* 2012;433:121–8.
- Lemos-Senna E, Wouessidjewe D, Duchêne D, Lesieur S. Amphiphilic cyclodextrin nanospheres: particle solubilization and reconstitution by the action of a non-ionic detergent. *Colloids Surf B Biointerfaces* 1998;10:291–301.
- Leo E, Forni F, Bernabei MT. Surface drug removal from ibuprofen-loaded pla microspheres. *Int J Pharm* 2000;196:1–9.
- Li B, Wang Q, Wang X, Wang C, Jiang X. Preparation, drug release and cellular uptake of doxorubicin-loaded dextran-b-poly(ϵ -caprolactone) nanoparticles. *Carbohydr Polym* 2013;93:430–7.
- Li P, Wang Y, Peng Z, She F, Kong L. Development of chitosan nanoparticles as drug delivery systems for 5-fluorouracil and leucovorin blends. *Carbohydr Polym* 2011;85:698–704.
- Li X, Chang S, Du G, Li Y, Gong J et al. Encapsulation of azithromycin into polymeric microspheres by reduced pressure-solvent evaporation method. *Int J Pharm* 2012;433:79–88.
- Liang D, Fu X, Liao M, Yuan W, Su J. Development of dextran microparticles loaded with il-1ra of high-encapsulation efficiency and high-bioactivity by a novel method without exposing il-1ra to water–oil interfaces. *Powder Technol* 2013;235:299–302.
- Limayem Blouza I, Charcosset C, Sfar S, Fessi H. Preparation and characterization of spironolactone-loaded nanocapsules for paediatric use. *Int J Pharm* 2006;325:124–31.
- Lince F, Marchisio DL, Barresi AA. Strategies to control the particle size distribution of poly- ϵ -caprolactone nanoparticles for pharmaceutical applications. *J Colloid Interface Sci* 2008;322:505–15.
- Ma J, Feng P, Ye C, Wang Y, Fan Y. An improved interfacial coacervation technique to fabricate biodegradable nanocapsules of an aqueous peptide solution from polylactide and its block copolymers with poly(ethylene glycol). *Colloid Polym Sci* 2001;279:387–92.
- Mahapatro A, Singh DK. Biodegradable nanoparticles are excellent vehicle for site directed in-vivo delivery of drugs and vaccines. *J Nanobiotechnol* 2011;9:55.
- Manchanda R, Fernandez-Fernandez A, Nagesetti A, McGoron AJ. Preparation and characterization of a polymeric (plga) nanoparticulate drug delivery system with simultaneous incorporation of chemotherapeutic and thermo-optical agents. *Colloids Surf B Biointerfaces* 2010;75:260–7.
- Mao S, Sun W, Kissel T. Chitosan-based formulations for delivery of dna and sima. *Adv Drug Deliv Rev* 2010;62:12–27.
- Martin TM, Bandi N, Shulz R, Roberts CB, Kompella UB. Preparation of budesonide and budesonide-pla microparticles using supercritical fluid precipitation technology. *AAPS Pharm Sci Tech* 2002;3:E18.
- Martín-Banderas L, Alvarez-Fuentes J, Durán-Lobato M, Prados J, Melguizo C, Fernández-Arévalo M et al. Cannabinoid derivate-loaded plga nanocarriers for oral administration: formulation, characterization, and cytotoxicity studies. *Int J Nanomed* 2012;7:5793-806.
- Masson V, Maurin F, Fessi H, Devissaguet JP. Influence of sterilization processes on poly(ϵ -caprolactone) nanospheres. *Biomaterials* 1997;18:327–35.
- Maya S, Kumar LG, Sarmiento B, Sanoj Rejinold N, Menon D et al. Cetuximab conjugated o-carboxymethyl chitosan nanoparticles for targeting egfr overexpressing cancer cells. *Carbohydr Polym* 2013;93:661–9.
- Mazzaferro S, Bouchemal K, Maksimenko A, Skanji R, Opolon P, Ponchel G. Reduced intestinal toxicity of docetaxel loaded into mucoadhesive nanoparticles, in mouse xenograft model. *J Colloid Sci Biotechnol* 2012;1:210–7.
- Mazzarino L, Travelet C, Ortega-Murillo S, Otsuka I, Pignot-Paintrand I et al. Elaboration of chitosan-coated nanoparticles loaded with curcumin for mucoadhesive applications. *J Colloid Interface Sci* 2012;370:58–66.
- Mehvar R. Dextrans for targeted and sustained delivery of therapeutic and imaging agents. *J Control Release* 2000;69:1–25.

- Memişoğlu E, Bochet A, Şen M, Duchêne D, Hincal AA. Non-surfactant nanospheres of progesterone inclusion complexes with amphiphilic β -cyclodextrins. *Int J Pharm* 2003;251:143–53.
- Menei P, Montero-Menei C, Venier M-C, Benoit J-P. Drug delivery into the brain using poly(lactide-co-glycolide) microspheres. *Expert Opin Drug Deliv* 2005;2:363–76.
- Miladi K, Sfar S, Fessi H, Elaissari A. Drug carriers in osteoporosis: preparation, drug encapsulation and applications. *Int J Pharm* 2013;445:181–95.
- Minost A, Delaveau J, Bolzinger M-A, Fessi H, Elaissari A. Nanoparticles via nanoprecipitation process. *Recent Pat Drug Deliv Formul* 2012;6:250–8.
- Miyazaki Y, Onuki Y, Yakou S, Takayama K. Effect of temperature-increase rate on drug release characteristics of dextran microspheres prepared by emulsion solvent evaporation process. *Int J Pharm* 2006;324:144–51.
- Mo L, Hou L, Guo D, Xiao X, Mao P, Yang X. Preparation and characterization of teniposide plga nanoparticles and their uptake in human glioblastoma u87mg cells. *Int J Pharm* 2012;436:815–24.
- Mohajel N, Najafabadi AR, Azadmanesh K, Vatnara A, Moazeni E et al. Optimization of a spray drying process to prepare dry powder microparticles containing plasmid nanocomplex. *Int J Pharm* 2012;423:577–85.
- Mora-Huertas CE, Fessi H, Elaissari A. Polymer-based nanocapsules for drug delivery. *Int J Pharm* 2010;385:113–42.
- Mora-Huertas CE, Garrigues O, Fessi H, Elaissari A. Nanocapsules prepared via nanoprecipitation and emulsification-diffusion methods: comparative study. *Eur J Pharm Biopharm* 2012;80:235–9.
- Morales-Cruz M, Flores-Fernández GM, Morales-Cruz M, Orellano EA, Rodríguez-Martínez JA et al. Two-step nanoprecipitation for the production of protein-loaded plga nanospheres. *Results Pharma Sci* 2012;2:79–85.
- Mu L, Feng S. Fabrication, characterization and in vitro release of paclitaxel (taxol®) loaded poly (lactic-co-glycolic acid) microspheres prepared by spray drying technique with lipid/cholesterol emulsifiers. *J Control Release* 2001;76:239–54.
- Muttill P, Kaur J, Kumar K, Yadav AB, Sharma R, Misra A. Inhalable microparticles containing large payload of anti-tuberculosis drugs. *Eur J Pharm Sci* 2007;32:140–50.
- Nahar M, Mishra D, Dubey V, Jain NK. Development, characterization, and toxicity evaluation of amphotericin b-loaded gelatin nanoparticles. *Nanomed Nanotechnol Biol Med* 2008;4:252–61.
- Nehilla B, Bergkvist M, Popat K, Desai T. Purified and surfactant-free coenzyme q10-loaded biodegradable nanoparticles. *Int J Pharm* 2008;348:107–14.
- O'Donnell PB, McGinity JW. Preparation of microspheres by the solvent evaporation technique. *Adv Drug Deliv Rev* 1997;28:25–42.
- Osman R, Kan PL, Awad G, Mortada N, El-Shamy A-E, Alpar O. Enhanced properties of discrete pulmonary deoxyribonuclease i (dnasei) loaded plga nanoparticles during encapsulation and activity determination. *Int J Pharm* 2011;408:257–65.
- Palazzo F, Giovagnoli S, Schoubben A, Blasi P, Rossi C, Ricci M. Development of a spray-drying method for the formulation of respirable microparticles containing ofloxacin-palladium complex. *Int J Pharm* 2013;440:273–82.
- Pariot N, Edwards-Lévy F, Andry M-C, Lévy M-C. Cross-linked β -cyclodextrin microcapsules. ii. retarding effect on drug release through semi-permeable membranes. *Int J Pharm* 2002;232:175–81.
- Park JH, Saravanakumar G, Kim K, Kwon IC. Targeted delivery of low molecular drugs using chitosan and its derivatives. *Adv Drug Deliv Rev* 2010;62:28–41.
- Patil SB, Sawant KK. Chitosan microspheres as a delivery system for nasal insufflation. *Colloids Surf B Biointerfaces* 2011;84:384–9.
- Péan JM, Venier-Julienne MC, Boury F, Menei P, Denizot B, Benoit JP. Ngf release from poly(d,l-lactide-co-glycolide) microspheres. effect of some formulation parameters on encapsulated ngf stability. *J Control Release* 1998;56:175–87.
- Pignatello R, Bucolo C, Ferrara P, Maltese A, Puleo A, Puglisi G. Eudragit rs100® nanosuspensions for the ophthalmic controlled delivery of ibuprofen. *Eur J Pharm Sci* 2002;16:53–61.
- Pillai O, Panchagnula R. Polymers in drug delivery. *Curr Opin Chem Biol* 2001;5:447–51.

- Pinto Reis C, Neufeld RJ, Ribeiro AJ, Veiga F. Nanoencapsulation i. methods for preparation of drug-loaded polymeric nanoparticles. *Nanomed Nanotechnol Biol Med* 2006;2:8–21.
- Poletto FS, Fiel LA, Donida B, Ré MI, Guterres SS, Pohlmann AR. Controlling the size of poly(hydroxybutyrate-co-hydroxyvalerate) nanoparticles prepared by emulsification-diffusion technique using ethanol as surface agent. *Colloids Surf Physicochem Eng Asp* 2008;324:105–12.
- Powell TJ, Palath N, DeRome ME, Tang J, Jacobs A, Boyd JG. Synthetic nanoparticle vaccines produced by layer-by-layer assembly of artificial biofilms induce potent protective t-cell and antibody responses in vivo. *Vaccine* 2011;29:558–69.
- Prata AS, Garcia L, Tonon RV, Hubinger MD. Wall material selection for encapsulation by spray drying. *J Colloid Sci Biotechnol* 2013;2:86–92.
- Prior S, Gamazo C, Irache J, Merkle H, Gander B. Gentamicin encapsulation in pla/plga microspheres in view of treating brucella infections. *Int J Pharm* 2000;196:115–25.
- Qi W, Yan X, Fei J, Wang A, Cui Y, Li J. Triggered release of insulin from glucose-sensitive enzyme multilayer shells. *Biomaterials* 2009;30:2799–806.
- Quintanar-Guerrero D, Fessi H, Allémann E, Doelker E. Influence of stabilizing agents and preparative variables on the formation of poly(D,L-lactic acid) nanoparticles by an emulsification-diffusion technique. *Int J Pharm* 1996;143:133–41.
- Radtchenko IL, Sukhorukov GB, Möhwald H. Incorporation of macromolecules into polyelectrolyte micro- and nanocapsules via surface controlled precipitation on colloidal particles. *Colloids Surf Physicochem Eng Asp* 2002;202:127–33.
- Raffin RP, Colomé LM, Schapoval EES, Pohlmann AR, Guterres SS. Increasing sodium pantoprazole photostability by microencapsulation: effect of the polymer and the preparation technique. *Eur J Pharm Biopharm* 2008;69:1014–8.
- Rao JP, Geckeler KE. Polymer nanoparticles: preparation techniques and size-control parameters. *Prog Polym Sci* 2011;36:887–913.
- Rattes ALR, Oliveira WP. Spray drying conditions and encapsulating composition effects on formation and properties of sodium diclofenac microparticles. *Powder Technol* 2007;171:7–14.
- Raval JP, Naik DR, Amin KA, Patel PS. Controlled-release and antibacterial studies of doxycycline-loaded poly(ϵ -caprolactone) microspheres. *J Saudi Chem Soc*, 2011, epub ahead of print, <http://dx.doi.org/10.1016/j.jscs.2011.11.004>
- Rawat A, Burgess DJ. Effect of ethanol as a processing co-solvent on the plga microsphere characteristics. *Int J Pharm* 2010;394:99–105.
- Rees DA, Welsh EJ. Secondary and tertiary structure of polysaccharides in solutions and gels. *Angew Chem Int Ed Engl* 1977;16:214–24.
- Rosset V, Ahmed N, Zaanoun I, Stella B, Fessi H, Elaissari A. Elaboration of argan oil nanocapsules containing naproxen for cosmetic and transdermal local application. *J Colloid Sci Biotechnol* 2012;1:218–24.
- Ruan G, Feng S-S. Preparation and characterization of poly(lactic acid)-poly(ethylene glycol)-poly(lactic acid) (pla-peg-pla) microspheres for controlled release of paclitaxel. *Biomaterials* 2003;24:5037–44.
- Sacchetin PSC, Morales AR, Moraes ÂM, Rosa P de TV e. Formation of pla particles incorporating 17 α -methyltestosterone by supercritical fluid technology. *J Supercrit Fluids* 2013;77:52–62.
- Sajadi Tabassi SA, Tafaghodi M, Jaafari MR. Induction of high antitoxin titers against tetanus toxoid in rabbits by intranasal immunization with dextran microspheres. *Int J Pharm* 2008;360:12–7.
- Sane A, Limtrakul J. Formation of retinyl palmitate-loaded poly(L-lactide) nanoparticles using rapid expansion of supercritical solutions into liquid solvents (resolv). *J Supercrit Fluids* 2009;51:230–7.
- Sanli D, Bozbag SE, Erkey C. Synthesis of nanostructured materials using supercritical co₂: part i. physical transformations. *J Mater Sci* 2012;47:2995–3025.
- Santos DT, Albarelli JQ, Beppu MM, Meireles MAA. Stabilization of anthocyanin extract from jaboticaba skins by encapsulation using supercritical co₂ as solvent. *Food Res Int* 2013;50:617–24.
- Sastre RL, Olmo R, Tejjón C, Muñoz E, Tejjón JM, Blanco MD. 5-fluorouracil plasma levels and biodegradation of subcutaneously injected drug-loaded microspheres prepared by spray-drying poly(D,L-lactide) and poly(D,L-lactide-co-glycolide) polymers. *Int J Pharm* 2007;338:180–90.

- Seju U, Kumar A, Sawant KK. Development and evaluation of olanzapine-loaded plga nanoparticles for nose-to-brain delivery: in vitro and in vivo studies. *Acta Biomater* 2011;7:4169–76.
- Seo D-H, Jeong Y-I, Kim D-G, Jang M-J, Jang M-K, Nah J-W. Methotrexate-incorporated polymeric nanoparticles of methoxy poly(ethylene glycol)-grafted chitosan. *Colloids Surf B Biointerfaces* 2009;69:157–63.
- Seremeta KP, Chiappetta DA, Sosnik A. Poly(ϵ -caprolactone), eudragit® rs 100 and poly(ϵ -caprolactone)/eudragit® rs 100 blend submicron particles for the sustained release of the antiretroviral efavirenz. *Colloids Surf B Biointerfaces* 2013;102:441–9.
- Shao Y, Zhu B, Li J, Liu X, Tan X, Yang X. Novel chitosan microsphere-templated microcapsules suitable for spontaneous loading of heparin. *Mater Sci Eng C* 2009;29:936–41.
- Silva AL, Rosalia RA, Sazak A, Carstens MG, Osendorp F et al. Optimization of encapsulation of a synthetic long peptide in plga nanoparticles: low-burst release is crucial for efficient cd8(+) t cell activation. *Eur J Pharm Biopharm* 2013;83:338–45.
- Simşek S, Eroğlu H, Kurum B, Ulubayram K. Brain targeting of atorvastatin loaded amphiphilic plga-b-peg nanoparticles. *J Microencapsul* 2013;30:10–20.
- Singh M, Kazzaz J, Ugozzoli M, Chesko J, O'Hagan DT. Charged polylactide co-glycolide microparticles as antigen delivery systems. *Expert Opin Biol Ther* 2004;4:483–91.
- Sinha VR, Bansal K, Kaushik R, Kumria R, Trehan A. Poly-epsilon-caprolactone microspheres and nanospheres: an overview. *Int J Pharm* 2004;278:1–23.
- Siqueira-Moura MP, Primo FL, Espreafico EM, Tedesco AC. Development, characterization, and photocytotoxicity assessment on human melanoma of chloroaluminum phthalocyanine nanocapsules. *Mater Sci Eng C* 2013;33:1744–52.
- Soares ASP. A special issue on applications of microencapsulation. *J Colloid Sci Biotechnol* 2013;2:77.
- Sollohub K, Cal K. Spray drying technique: ii. current applications in pharmaceutical technology. *J Pharm Sci* 2010;99:587–97.
- Song KC, Lee HS, Choung IY, Cho KI, Ahn Y, Choi EJ. The effect of type of organic phase solvents on the particle size of poly(d,l-lactide-co-glycolide) nanoparticles. *Colloids Surf Physicochem Eng Asp* 2006;276:162–7.
- Song X, Zhao Y, Wu W, Bi Y, Cai Z et al. Plga nanoparticles simultaneously loaded with vincristine sulfate and verapamil hydrochloride: systematic study of particle size and drug entrapment efficiency. *Int J Pharm* 2008;350:320–9.
- Sou T, Meeusen EN, de Veer M, Morton DAV, Kaminskas LM, McIntosh MP. New developments in dry powder pulmonary vaccine delivery. *Trends Biotechnol* 2011;29:191–8.
- Souguir H, Salaün F, Douillet P, Vroman I, Chatterjee S. Nanoencapsulation of curcumin in polyurethane and polyurea shells by an emulsion diffusion method. *Chem Eng J* 2013;221:133–45.
- Stulzer HK, Tagliari MP, Parize AL, Silva MAS, Laranjeira MCM. Evaluation of cross-linked chitosan microparticles containing acyclovir obtained by spray-drying. *Mater Sci Eng C* 2009;29:387–92.
- Su Z, Sun F, Shi Y, Jiang C, Meng Q et al. Effects of formulation parameters on encapsulation efficiency and release behavior of risperidone poly(d,l-lactide-co-glycolide) microsphere. *Chem Pharm Bull (Tokyo)* 2009;57:1251–6.
- Sukhorukov GB, Donath E, Lichtenfeld H, Knippel E, Knippel M et al. Layer-by-layer self assembly of polyelectrolytes on colloidal particles. *Colloids Surf Physicochem Eng Asp* 1998;137:253–66.
- Suksiriworapong J, Sripha K, Kreuter J, Junyaprasert VB. Functionalized (poly(ϵ -caprolactone))₂-poly(ethyleneglycol) nanoparticles with grafting nicotinic acid as drug carriers. *Int J Pharm* 2012;423:562–70.
- Surassmo S, Min S-G, Bejrappa P, Choi M-J. Effects of surfactants on the physical properties of capsicum oleoresin-loaded nanocapsules formulated through the emulsion-diffusion method. *Food Res Int* 2010;43:8–17.
- Tawfeek HM. Evaluation of peg and mpeg-co-(pga-co-pdl) microparticles loaded with sodium diclofenac. *Saudi Pharm J* 2013;21:387–97.
- Tewa-Tagne P, Briançon S, Fessi H. Spray-dried microparticles containing polymeric nanocapsules: formulation aspects, liquid phase interactions and particles characteristics. *Int J Pharm* 2006;325:63–74.

- Thomas C, Gupta V, Ahsan F. Influence of surface charge of plga particles of recombinant hepatitis b surface antigen in enhancing systemic and mucosal immune responses. *Int J Pharm* 2009;379:41–50.
- Trapani A, Laquintana V, Denora N, Lopedota A, Cutrignelli A et al. Eudragit rs 100 microparticles containing 2-hydroxypropyl- β -cyclodextrin and glutathione: physicochemical characterization, drug release and transport studies. *Eur J Pharm Sci* 2007;30:64–74.
- Tse MT, Blatchford C, Oya Alpar H. Evaluation of different buffers on plasmid dna encapsulation into plga microparticles. *Int J Pharm* 2009;370:33–40.
- Valot P, Baba M, Nedelec J-M, Sintès-Zydowicz N. Effects of process parameters on the properties of biocompatible ibuprofen-loaded microcapsules. *Int J Pharm* 2009;369:53–63.
- Van de Ven H, Vermeersch M, Matheeußen A, Vandervoort J, Weyenberg W et al. Plga nanoparticles loaded with the antileishmanial saponin β -aescin: factor influence study and in vitro efficacy evaluation. *Int J Pharm* 2011;420:122–32.
- Van de Ven H, Paulussen C, Feijens PB, Matheeußen A, Rombaut P et al. Plga nanoparticles and nanosuspensions with amphotericin b: potent in vitro and in vivo alternatives to fungizone and ambisome. *J Control Release* 2012;161:795–803.
- Van der Graaf S, Schroën CGPH, Boom RM. Preparation of double emulsions by membrane emulsification—a review. *J Membr Sci* 2005;251:7–15.
- Vanderhoff JW, El-Aasser MS, Ugelstad J. Polymer emulsification process. U.S. Patent 4,177,177, December 1979.
- Vandervoort J, Ludwig A. Preparation and evaluation of drug-loaded gelatin nanoparticles for topical ophthalmic use. *Eur J Pharm Biopharm* 2004;57:251–61.
- Vauthier C, Bouchemal K. Methods for the preparation and manufacture of polymeric nanoparticles. *Pharm Res* 2009;26:1025–58.
- Vergaro V, Scarlino F, Bellomo C, Rinaldi R, Vergara D et al. Drug-loaded polyelectrolyte microcapsules for sustained targeting of cancer cells. *Adv Drug Deliv Rev* 2011;63:847–64.
- Vila A, Gill H, McCallion O, Alonso MJ. Transport of pla-peg particles across the nasal mucosa: effect of particle size and peg coating density. *J Control Release* 2004;98:231–44.
- Vila A, Sánchez A, Évora C, Soriano I, McCallion O, Alonso MJ. Pla-peg particles as nasal protein carriers: the influence of the particle size. *Int J Pharm* 2005;292:43–52.
- Vladislavljević GT, Williams RA. Recent developments in manufacturing particulate products from double-emulsion templates using membrane and microfluidic devices. In: Aserin A (ed.): Multiple emulsions: technology and applications (pp 121-64). New York: Wiley, 2008.
- Wang F-J, Wang C-H. Sustained release of etanidazole from spray dried microspheres prepared by non-halogenated solvents. *J Control Release* 2002;81:263–80.
- Wang G, Yu B, Wu Y, Huang B, Yuan Y, Liu CS. Controlled preparation and antitumor efficacy of vitamin e tpgs-functionalized plga nanoparticles for delivery of paclitaxel. *Int J Pharm* 2013;446:24–33.
- Wang S, Guo S, Cheng L. Disodium norcantharidate loaded poly(ϵ -caprolactone) microspheres: i. preparation and evaluation. *Int J Pharm* 2008;350:130–7.
- Xie H, Smith JW. Fabrication of plga nanoparticles with a fluidic nanoprecipitation system. *J Nanobiotechnol* 2010;8:18.
- Xin H, Chen L, Gu J, Ren X, Wei Z, et al. Enhanced anti-glioblastoma efficacy by ptx-loaded pegylated poly(ϵ -caprolactone) nanoparticles: in vitro and in vivo evaluation. *Int J Pharm* 2010;402:238–47.
- Yadav KS, Sawant KK. Formulation optimization of etoposide loaded plga nanoparticles by double factorial design and their evaluation. *Curr Drug Deliv* 2010;7:51–64.
- Yan S, Zhu J, Wang Z, Yin J, Zheng Y, Chen X. Layer-by-layer assembly of poly(l-glutamic acid)/chitosan microcapsules for high loading and sustained release of 5-fluorouracil. *Eur J Pharm Biopharm* 2011;78:336–45.
- Yang J, Park S-B, Yoon H-G, Huh Y-M, Haam S. Preparation of poly ϵ -caprolactone nanoparticles containing magnetite for magnetic drug carrier. *Int J Pharm* 2006;324:185–90.
- Yang Z, Birkenhauer P, Julmy F, Chickering D, Raniere JP et al. Sustained release of heparin from polymeric particles for inhibition of human vascular smooth muscle cell proliferation. *J Control Release* 1999;60:269–77.

- Yildiz A, John E, Özsoy Y, Araman A, Birchall JC et al. Inhaled extended-release microparticles of heparin elicit improved pulmonary pharmacodynamics against antigen-mediated airway hyper-reactivity and inflammation. *J Control Release* 2012;162:456–63.
- Yoncheva K, Gómez S, Campanero MA, Gamazo C, Irache JM. Bioadhesive properties of pegylated nanoparticles. *Expert Opin Drug Deliv* 2005;2:205–18.
- Yoo M-K, Kang S-K, Choi J-H, Park I-K, Na H-S et al. Targeted delivery of chitosan nanoparticles to peyer's patch using m cell-homing peptide selected by phage display technique. *Biomaterials* 2010;31:7738–47.
- Yordanov G. Influence of the preparation method on the physicochemical properties of econazole-loaded poly(butyl cyanoacrylate) colloidal nanoparticles. *Colloids Surf Physicochem Eng Asp* 2012;413:260–5.
- Youm I, Murowchick JB, Youan B-BC. Entrapment and release kinetics of furosemide from pegylated nanocarriers. *Colloids Surf B Biointerfaces* 2012;94:133–42.
- Young S, Wong M, Tabata Y, Mikos AG. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. *J Control Release* 2005;109:256–74.
- Zakeri-Milani P, Loveymi BD, Jelvehgari M, Valizadeh H. The characteristics and improved intestinal permeability of vancomycin plga-nanoparticles as colloidal drug delivery system. *Colloids Surf B Biointerfaces* 2013;103:174–81.
- Zambaux MF, Bonneaux F, Gref R, Maincent P, Dellacherie E et al. Influence of experimental parameters on the characteristics of poly(lactic acid) nanoparticles prepared by a double emulsion method. *J Control Release* 1998;50:31–40.
- Zandanel C, Vauthier C. Poly (isobutylcyanoacrylate) nanoparticles decorated with chitosan: effect of conformation of chitosan chains at the surface on complement activation properties. *J Colloid Sci Biotechnol* 2012;1:68–81.
- Zhang C, Li G, Wang Y, Cui F, Zhang J, Huang Q. Preparation and characterization of 5-fluorouracil-loaded plla-peg/peg nanoparticles by a novel supercritical co2 technique. *Int J Pharm* 2012;436:272–81.
- Zhang F, Wu Q, Chen Z-C, Zhang M, Lin X-F. Hepatic-targeting microcapsules construction by self-assembly of bioactive galactose-branched polyelectrolyte for controlled drug release system. *J Colloid Interface Sci* 2008a;317:477–84.
- Zhang F, Wu Q, Liu L-J, Chen Z-C, Lin X-F. Thermal treatment of galactose-branched polyelectrolyte microcapsules to improve drug delivery with reserved targetability. *Int J Pharm* 2008b;357:22–31.
- Zhang H, Cui W, Bei J, Wang S. Preparation of poly(lactide-co-glycolide-co-caprolactone) nanoparticles and their degradation behaviour in aqueous solution. *Polym Degrad Stab* 2006;91:1929–36.
- Zhao H, Gagnon J, Hafeli UO. Process and formulation variables in the preparation of injectable and biodegradable magnetic microspheres. *Biomagn Res Technol* 2007;5:2.
- Zheng X, Kan B, Gou M, Fu S, Zhang J et al. Preparation of mpeg-pla nanoparticle for honokiol delivery in vitro. *Int J Pharm* 2010;386:262–7.
- Zhou YZ, Alany RG, Chuang V, Wen J. Optimization of plga nanoparticles formulation containing l-dopa by applying the central composite design. *Drug Dev Ind Pharm* 2013;39:321–30.

2.2. La thérapie Génique et les techniques d'encapsulations

La thérapie génique est un traitement ou une tentative de traitement des maladies génétiques ou non par réparation de gène défectueux. Elle utilise le gène comme un médicament. Depuis sa découverte, la thérapie génique a ouvert un nouvel horizon pour traiter un grand nombre de maladies graves et incurables par les méthodes conventionnelles. Le succès de cette approche thérapeutique dépend des technologies avancées sur l'ADN et sur sa conservation sans dégradation pour cibler les noyaux des cellules. Mais, comme l'ADN est une grande molécule, polyanionique, hydrophile et dégradabile facilement, il ne peut pas franchir les barrières cellulaires sans l'aide d'un vecteur qui masque sa charge et le protège de toute dégradation possible. C'est pourquoi le développement d'une thérapie génique sûre et efficace passe par la connaissance et la mise au point de véhicules capables de protéger et de délivrer le gène réparateur.

Les chercheurs utilisent des virus modifiés pour le transfert de gène, en exploitant leur circulation dans l'organisme. Ces virus modifiés sont connus sous le nom de vecteurs viraux, mais ils souffrent de sévères inconvénients. En effet, l'intégration d'un exogène de grande taille est difficile et engendrent généralement des réactions immunitaires et d'autres peuvent redevenir pathogènes après mutagenèse. Enfin, leur production et leur manipulation sont compliquées et coûteuses, ce qui a conduit les scientifiques à chercher d'autres vecteurs qui ont la même efficacité mais sans limites. En revanche, les vecteurs synthétiques présentent de multiples avantages en terme de coût, de faciliter la mise en œuvre, de sécurité et d'éthique, par rapport aux vecteurs viraux. Malheureusement, ils présentent des limitations comme la non spécificité et l'élimination rapide de l'organisme par les macrophages et la faible efficacité d'encapsulation. Les vecteurs synthétiques sont nombreux : l'interaction électrostatique attractive conduit au complexe ADN/polycations (polyplex ou lipoplex), l'adsorption de l'ADN sur des particules cationiques, ou l'encapsulation de l'ADN qui est de nos jours, le procédé qui retient le plus d'attention.

Aujourd'hui, aucun de ces vecteurs ne présente l'efficacité et la sécurité requise pour une utilisation thérapeutique chez l'homme. Dans cette optique la recherche continue à développer et améliorer la synthèse et la formulation des vecteurs, mais aussi pour élaborer des vecteurs nouveaux plus sécurisés et efficaces, car le bon vecteur est la clé de la réussite de la thérapie génique.



Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm



Review

Gene therapy and DNA delivery systems

D. Ibraheem^{a,b}, A. Elaissari^{a,b}, H. Fessi^{a,b,*}

^aUniversity of Lyon, F-69622 Lyon, France
^bUMR 5007, LAGEP-CPE, 43 bd 11 Novembre 1918, F-69622 Villeurbanne, France

ARTICLE INFO

Article history:
Received 24 July 2013
Received in revised form 31 October 2013
Accepted 19 November 2013
Available online xxx

Keywords:
Gene therapy
Carriers
DNA
Viral vectors
Non-viral vectors

ABSTRACT

Gene therapy is a promising new technique for treating many serious incurable diseases, such as cancer and genetic disorders. The main problem limiting the application of this strategy *in vivo* is the difficulty of transporting large, fragile and negatively charged molecules like DNA into the nucleus of the cell without degradation. The key to success of gene therapy is to create safe and efficient gene delivery vehicles. Ideally, the vehicle must be able to remain in the bloodstream for a long time and avoid uptake by the mononuclear phagocyte system, in order to ensure its arrival at the desired targets. Moreover, this carrier must also be able to transport the DNA efficiently into the cell cytoplasm, avoiding lysosomal degradation. Viral vehicles are the most commonly used carriers for delivering DNA and have long been used for their high efficiency. However, these vehicles can trigger dangerous immunological responses. Scientists need to find safer and cheaper alternatives. Consequently, the non-viral carriers are being prepared and developed until techniques for encapsulating DNA can be found. This review highlights gene therapy as a new promising technique used to treat many incurable diseases and the different strategies used to transfer DNA, taking into account that introducing DNA into the cell nucleus without degradation is essential for the success of this therapeutic technique.

© 2013 Published by Elsevier B.V.

Contents

1. Introduction.....	00
2. Protein therapy and gene therapy.....	00
3. The harbingers of gene therapy.....	00
4. Classification of gene therapy.....	00
4.1. Germ line gene therapy.....	00
4.2. Somatic gene therapy.....	00
5. Clinical gene therapy trials.....	00
6. Gene therapy: principle and vectors.....	00
6.1. Viral vectors for transferring the gene.....	00
6.2. Non-viral carriers.....	00
6.2.1. Physical methods.....	00
6.2.2. Chemical vectors.....	00
7. Conclusion.....	00
Acknowledgments.....	00
References.....	00

1. Introduction

Gene therapy is a promising therapeutic strategy (Friedmann, 1996) based on using genes as a medicine (Mohsen, 2011). It can be employed effectively to cure a wide range of serious acquired and

inherited diseases (Gardlík et al., 2005), such as cancer (Rochlitz, 2001), acquired immunodeficiency syndrome (AIDS) (Yu et al., 1994), cardiovascular diseases (Dishart et al., 2003), infectious diseases (Bunnell and Morgan, 1998), cystic fibrosis (Davies et al., 2001), and X-linked severe combined immune deficiency (X-linked SCID) (Kohn et al., 2003). Theoretically, gene therapy is a simple therapeutic method depending on either replacing a distorted gene by healthy one, or completing a missing gene in order to express the required protein (Zhang et al., 2004b). However, in practice this is a

* Corresponding author.
E-mail address: Fessi@lagep.univ-lyon1.fr (H. Fessi).

complex operation (Tong et al., 2007), due to several obstacles that must be overcome by the transgene to reach the targeted human cell-nucleus, where it should be expressed correctly. Hence, for ensuring the arrival of a transgene into a cell nucleus without degradation, it is necessary to use gene delivery system that can protect the transgene from degradation and pass through the plasma membrane to the nucleus (Luo and Saltzman, 2000; Gao et al., 2007). At present, a perfect delivering system (carrier) capable of ensuring the success of gene therapy must satisfy the following criteria: (i) it must not interact with vascular endothelial cells and blood components (Schatzlein, 2001); (ii) it must be capable of avoiding uptake by the reticuloendothelial system (Mohsen, 2011); (iii) it must be small enough to pass through the cell-membrane and reach the nucleus (Labhasetwar, 2005). In fact, viruses were the first carriers to be used to deliver and protect the therapeutic gene, benefiting from the virus-life cycle. This type of carrier, known as viral vector, is one of the vectors used most in gene therapy, due to its ability to carry the gene efficiently and ensure long-term expression (Boulaiz et al., 2005). However, the risk of provoking immune response by using viruses as delivering vectors (Lv et al., 2006; El-Aneed, 2004), the high cost and difficulty relating to their preparation (Boulaiz et al., 2005), and the limited size of the genetic materials that can be inserted into human cells (Lv et al., 2006; El-Aneed, 2004), have restricted the use of these vectors in gene therapy, and led to research into safer and cheaper alternatives. Therefore non-viral vectors have appeared. Non-viral approaches for delivering transgenes can be divided into two groups:

- 1- Physical approaches: these depend on a physical force that weakens the cell membrane to facilitate the penetration of the gene into the nucleus. They include needle injection, electroporation, gene gun, ultrasound, and hydrodynamic delivery.
- 2- Chemical vectors: these can be prepared by electrostatic interaction between poly cationic derivatives that can be lipids or polymers and the anionic phosphate of DNA to form a particle called polyplex when the interaction occurs between the polymer and the DNA, and lipoplex when the DNA interacts with a lipid, or by encapsulation of DNA within biodegradable spherical structures that lead to micro and nanoparticles containing DNA, or by adsorption of DNA.

2. Protein therapy and gene therapy

Proteins have been used for treating various kinds of diseases for a long time (Goddard, 1991; Talmadge, 1993) in what is known as *protein therapy*, but using proteins for treating diseases is confronted by many obstacles such as low bioavailability in the body, short life in the blood stream due to high rates of hepatic and renal clearance and *in vivo* instability as it can degrade in the biological medium. The latter two constraints make it necessary to repeat recombinant protein injection using high doses that can increase its toxicity. In addition it is expensive to produce industrially (Han, 2000). Therefore scientists have found that using the gene which encodes the protein is easier and more efficient than using the protein itself. This then is the basic of *gene therapy*, since a single gene can produce many proteins when it enters into a cell.

At present, treatment using genes is preferable to protein therapy because (Ledley and Shapiro, 1998):

- Gene therapy ensures the lasting production of a stable quantity protein,
- Gene therapy localizes transgene-expression, which leads to avoiding the unwanted effects caused by the systematic presence of a protein.

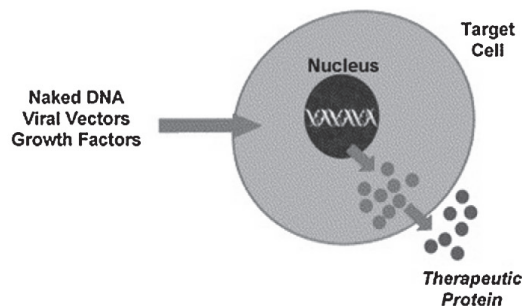


Fig. 1. Principle of gene therapy (Kendirci et al., 2006).

3. The harbingers of gene therapy

Since 1944, the year in which Avery, Mcleod and Mc Carthy, proved that DNA encodes human genetic information (Avery et al., 1944; Dahm, 2010), much valuable genetic information was published until, finally, Watson and Crick published their article on the double helix structure of DNA in 1953 (Watson and Crick, 1953). This led to a genuine genetic revolution that led to understanding the mechanisms of many diseases, and to the development of new treatment methods, such as gene therapy. The major problem faced by gene therapy is that of ensuring the DNA reaches its target inside the cell-nucleus without degradation by DNAase (Nam et al., 2009). At present, gene therapy can be defined as a strategy that ensures the introduction of an intact pharmaceutical gene into a human cell to treat diseases (Stribley et al., 2002) Fig. 1.

4. Classification of gene therapy

The main goal of gene therapy is to insert a functional gene that plays the role of drug into the cell targeted in order to cure a disease or to repair a dysfunction caused by a genetic defect. Gene therapy can be classified into two major categories according to the nature of targeted cell.

4.1. Germ line gene therapy

This depends on inserting the functional gene into the germ cells (reproductive cells) as sperm and zygote. This gene will be integrated into the individual genomes, causing a heritable modification in the patient's genetic characteristics (Stribley et al., 2002). This type of gene therapy has not been applied on human beings in France for ethical reasons, as the law on bioethics law of 29 July 1994 states that "*any eugenic practice tending towards the organization of the selection of human individuals is forbidden*" and that the treatment and/or prevention of genetic diseases must avoid making heritable changes in human genetic characteristics "*without prejudice to research tending to the prevention and treatment of genetic diseases, no transformation can be made to genetic characteristics in view to modifying the descendance of an individual*".

However, the Food and Drug Administration (FDA) has allowed the use of germ line gene therapy in the United States of America.

4.2. Somatic gene therapy

When therapeutic genes are transferred into the patient's somatic cells (non-reproductive cells), which means that the effect appears in only one generation and any modifications and effects are restricted to the individual, i.e. the resulting modifications in

Please cite this article in press as: Ibraheem, D., et al., Gene therapy and DNA delivery systems. Int J Pharmaceut (2013), <http://dx.doi.org/10.1016/j.ijpharm.2013.11.041>

Indications Addressed by Gene Therapy Clinical Trials

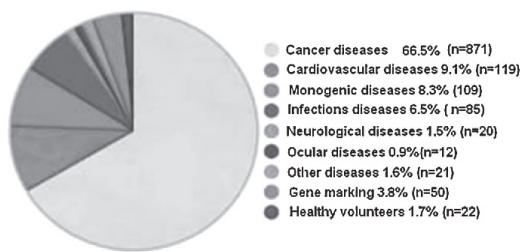


Fig. 2. Clinical targets for gene therapy (Edelstein et al., 2007).

the patient's genetic characteristics cannot be inherited (Stribley et al., 2002).

Actually, there are no Europe-wide regulations related to the gene therapy. Although experts argue that somatic-cell gene-therapy trials should be allowed for life-threatening disorders after careful risk/benefit evaluation.

5. Clinical gene therapy trials

The expression gene therapy owes its origin to the term "genetic engineering" which was employed for the first time at the Sixth International Congress of Genetics held at Ithaca in 1932 (Wolff and Lederberg, 1994). Though the idea of gene therapy existed already, concrete development in this field began in late 1960s and early 1970s (Friedmann, 1992) (Roemer and Friedmann, 1992) and gene therapy in humans was practiced in the late 1980s (Anderson, 1992) as a result of developments in the field of molecular biology of inventions and improvements in gene delivery systems (Miller, 1992).

Since the first human gene therapy trial performed in 1989 by Rosenberg and his team, who tried to treat advanced melanoma in five patients (Rosenberg et al., 1990), more than 1500 clinical gene therapy trials were performed up to 2010 (Herzog et al., 2010). More than 66% of them were to treat cancer while 9.1% of them were devoted to cardiovascular diseases, and only 8.3% were targeted at treating monogenic genetic disorders (Edelstein et al., 2007), see Fig. 2. This presentation of the targets of clinical gene therapy trials leads the conclusion that, potentially, gene therapy can be used to treat a wide range of serious acquired diseases such as cancer (Cho-Chung, 2005) and AIDS (Guo and Huang, 2012), in addition to the heritable genetic ones like cystic fibrosis (Davies et al., 2001), familial hypercholesterolemia and muscular dystrophy (Ropert, 1999).

6. Gene therapy: principle and vectors

Gene therapy is a technique employed recently to treat serious diseases (acquired or inherited) by correcting their genetic causes (Müller-Reible, 1994), either by replacing the deformed genes by healthy ones or by completing missing genes (Sandhu et al., 1997). Different types of genetic material are used in gene therapy; such as double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), plasmid DNA (Ferreira et al., 2000) and anti-sense oligonucleotides (ASON) (Knipe et al., 2013). The success of gene therapy essentially depends on ensuring that the therapeutic gene enters the targeted cell without any form of biodegradation (Nam et al., 2009). However, the sensitivity of DNA to the nuclease of the biological medium (Nam et al., 2009), the hydrophilic poly anionic nature of the DNA macromolecule (Nam et al., 2009) (Nagasaki and Shinkai, 2007) and its large size prevent it from penetrating passively through the cell

membrane (Ropert, 1999). Therefore DNA must be associated with the delivery system or vector that carries the therapeutic gene into the targeted cell, protecting it from degradation by nucleases (Nam et al., 2009; Gardlik et al., 2005), and making sure that it transcribes inside the cell (Gardlik et al., 2005).

The ideal transfer system, called vector, should satisfy several criteria (Somia and Verma, 2000):

- It must not trigger a strong immune response.
- It must be capable of transporting nucleic acids whatever their size.
- It must lead to the sustained and regular expression of its genetic cargo.
- The vector must deliver the gene to only certain types of cells, especially when the target cells are scattered throughout the body, or when they are part of a heterogeneous population.
- It must be able to infect both dividing and non-dividing cells.
- It must be easy to prepare, be inexpensive and available at high concentrations commercially.
- It must either remain in episomal position or integrate into a specific region of the genome, but not integrate randomly.

Gene transfer systems (vectors) are classified into two types:

6.1. Viral vectors for transferring the gene

A virus is biological entity that can penetrate into the cell nucleus of the host and exploit the cellular machinery to express its own genetic material and replicate it, then spread to the other cells (Kay et al., 2001). Researchers have used different viruses to deliver therapeutic genes into cell nuclei and exploit the virus life cycle. To use a virus as a vector to transfer a gene, it must be modified by genetic engineering. The pathogenic part of its genes is removed and replaced by the therapeutic gene (Bouard et al., 2009). At the same time, the virus retains its non-pathogenic structures (envelope proteins, fusogenic proteins, etc.) which allow it to infect the cell (Kay et al., 2001). The resulting non-pathogenic virus carrying the therapeutic gene is called a viral vector. To date, viral vectors are the vectors most often used to transfer genes, due their high transfection efficiency *in vivo* (Munier et al., 2005), in spite of their drawbacks. These can be summarized by:

- The acute immune response that may be caused (stimulated) by viral vectors which can be fatal (Templeton, 2002; Munier et al., 2005).
- The production of viral vectors in large quantities is very difficult and too expensive (Templeton, 2002; Nagasaki and Shinkai, 2007).
- The limited size of gene that can be delivered by the virus (Nagasaki and Shinkai, 2007).

The viruses used most as carrier vectors are retroviruses, adenoviruses, adeno-associated viruses (AAV), and simple herpes virus (Walther and Stein, 2000).

Table 1 presents the main viruses used as gene transfer systems with brief explanations of the advantages and disadvantages of each virus (Ratko et al., 2003).

6.2. Non-viral carriers

The drawbacks of viral vectors, especially severe immune response, have led scientists to find safer alternatives. Consequently, non-viral vectors have been designed for transferring DNA. Research in this field has attracted great attention (Roy et al., 2003; Zhang et al., 2004b) as a result of advantages that they offer in comparison to viral vectors. Non-viral vectors are relatively safe,

Please cite this article in press as: Ibraheem, D., et al., Gene therapy and DNA delivery systems. Int J Pharmaceut (2013), <http://dx.doi.org/10.1016/j.ijpharm.2013.11.041>

Table 1

The main viruses used as gene delivery systems, with their advantages and disadvantages (Ratko et al., 2003).

Vector	Advantages	Disadvantages
Adenovirus	Very high titers (10^{12} pfu/mL) High transduction efficiency ex vivo and in vivo Transduces many cell types Transduces proliferating and nonproliferating cells Production easy at high titers	Remains episomal Transient expression Requires packaging cell line Immune-related toxicity with repeated administration Potential replication competence No targeting Limited insert size: 4–5 kb
Adeno-associated virus	Integration on human chromosome 19 (wild-type only) to establish latent infection Prolonged expression Transduction does not require cell division Small genome, no viral genes	Not well characterized No targeting Requires packaging cell line Potential insertional mutagenesis High titers (10^{10} pfu/mL) but production difficult Limited insert size: 5 kb
Herpes simplex virus	Large insert size: 40–50 kb Neuronal tropism Latency expression Efficient transduction in vivo Replicative vectors available	Cytotoxic No targeting Requires packaging cell line Transient expression, does not integrate into genome Moderate titers (10^4 – 10^8 pfu/mL)
Lentivirus	Transduces proliferating and nonproliferating cells hematopoietic stem cells Prolonged expression Relatively high titers (10^6 – 10^7 pfu/mL)	Safety concerns: from human immunodeficiency virus origin Difficult to manufacture and store Limited insert size: 8 kb Clinical experience limited
Retrovirus	Integration into cellular genome Broad cell tropism Prolonged stable expression Requires cell division for transduction Relatively high titers (10^6 – 10^7 pfu/mL) Larger insert size: 9–12 kb	Inefficient transduction Insertional mutagenesis Requires cell division for transfection Requires packaging cell line No targeting Potential replication competence

generally causes low immune response, they can be prepared easily, at low cost and in large quantities. In addition, they can transfer different and large transgenes, and they can be stored for long periods due to their stability (Munier et al., 2005; Kircheis et al., 2001; Li and Huang, 2000). However, unfortunately, their low transfection efficiency limits their use on a large scale (Bergen et al., 2008). Non-viral DNA delivery systems are classed into two groups:

- Physical methods: DNA is delivered into its target without using any carrier, by using physical forces to weaken the membrane cell to make it more permeable to the transgene (Gao et al., 2007).
- Chemical methods: DNA is carried into the nucleus by a carrier which can be prepared by several types of chemical reactions which we will examine further on (Gao et al., 2007).

6.2.1. Physical methods

The principle of physical gene delivery systems is to use mechanical, ultrasonic, electric, hydrodynamic or laser-based energy in order to create temporary weak points in the membrane of the target cell, by causing transient injuries or defects in it and allows the DNA to enter the cell by diffusion.

6.2.1.1. Electroporation. This physical technique, which is termed electro-permeabilization in some references (Mehierhumbert and Guy, 2005), and electric pulse mediated gene transfer in others (Villemejane and Mir, 2009), was used for the first time in 1982 by Neumann and his team, who use electric pulses to insert DNA into viable mouse lymphoma cells (Neumann et al., 1982). The principle of electroporation is to induce the uptake of injected DNA into the cell, by increasing the permeability of the cell membrane through exposure to a controlled electric field (Niidome and Huang, 2002). Intense electric pulses affect the cell membrane and cause temporary localized destabilization, allowing DNA to pass easily into the cell (Mehierhumbert and Guy, 2005). This technique has been

applied successfully on various tissues (Heller et al., 2005), such as skin (Dujardin and Pr at, 2004), muscle (Aihara and Miyazaki, 1998), liver (Heller et al., 1996; Suzuki et al., 1998), and tumour (Jaroszeski et al., 2013; Rols et al., 1998). The efficacy of gene transfer with this method is affected by different factors that are both physical (pulse duration, field intensity and electrode geometry) and biological (cell size, shape and density) (Gehl, 2003). In spite of the advantages presented by electroporation as a physical DNA delivery system such as safety, efficiency and reproducibility (Al-Dosari and Gao, 2009), its use in vivo is still limited by the following drawbacks (Gao et al., 2007): (i) it is difficult to employ for transferring DNA into a large area of tissue because the effective range between the electrodes in this method is ~ 1 cm; (ii) it requires surgery to install the electrode in the internal organs; (iii) it can cause incurable harm and mutilation in the tissue treated due to the high voltage applied. However, these limitations can be overcome by improvements such as modifying the shape of the electrode, the field-strength applied and the duration and frequency of the electric pulses (Gehl, 2003).

6.2.1.2. Gene gun. Gene gun (ballistic DNA transfer or DNA-coated particle bombardment) technology was first employed in 1987 by Sanford and his colleagues to introduce genes into plant cells (Gehl, 2003). In the early nineties further development and improvement of this approach led to its use in mammalian cells and tissues (Yang et al., 1990; Williams et al., 1991). In this method transgene delivery into the target cell and tissue is carried out by using accelerated particle carriers biocompatible heavy metals such as gold, tungsten or silver. Ideally, particle carriers should be biocompatible, inert and have small diameters (usually 1 – 1.5 μm) (Mehierhumbert and Guy, 2005). These carriers are coated with plasmid DNA, and the required acceleration is provided either by vaporization water under high-voltage electric spark (Yang et al., 1990), or by using helium discharge (Wolff et al., 1991). The

efficiency of this method is determined by many factors: the size of the particles employed as DNA-carriers, the gas pressure used to accelerate the particles, and the dosage of the transgene blasted into the target (Uchida et al., 2002). This approach can be employed effectively in many fields such as genetic vaccination, immune therapy, and suicide gene therapy to treat cancer (Lin et al., 2000). Gene gun transfer method presents many advantages:

- i) High, level gene expression is achieved quickly (after only 3 h in cattle) (Loehr et al., 2000).
- ii) Long, lasting gene expression (extends more than 28–50 days after extrusion of the DNA coated particles onto muscle tissue) (Ajiki et al., 2003).
- iii) Numerous organs can be reached by this technology without injury to the surrounding tissues such as liver (Muangmoonchai et al., 2002; Watkins et al., 2005; Kuriyama et al., 2000), heart (Matsuno et al., 2003), brain (Zhang and Selzer, 2001; Sato et al., 2000) and muscle (Lauritzen et al., 2002).

However, its efficiency is poor when used to transfer the gene onto whole tissue due to low penetration by the metal particles and surgery is often necessary in order to use this approach for deep tissues.

6.2.1.3. Ultrasound. Ultrasound-mediated gene transfer or sonoporation depends on increasing the permeability of cell membrane by using ultrasound waves that create pores or acoustic cavitation in the exposed cell membrane (Al-Dosari and Gao, 2009) which is relatively recent. Ultrasound was first used to mediate the transfer of DNA *in vitro* in the mid-1990s (Kim et al., 1996) (Bao et al., 1997) and it was not used as DNA delivery system until the 2000s when it was used to transfer DNA into muscle (Lu et al., 2003; Wang et al., 2005), solid tumors (Miller and Song, 2003), liver (Miao et al., 2005), kidney (Koike et al., 2005), heart (Bekeredjian et al., 2003), and very recently for transdermal delivery (Smith, 2007). This approach presents many advantages as it is safe, non-invasive and it can reach internal organs without the need for surgery (Al-Dosari and Gao, 2009). The efficiency of this method is subject to numerous factors that include the intensity and frequency of ultrasound irradiation, the period of cell exposure to ultrasound radiation, DNA concentration, the use of a contrast factor (Huber et al., 1999; Al-Dosari and Gao, 2009; Mehierhumbert and Guy, 2005), which is considered the most important factor for improving the efficiency of this transfer technique. It has been demonstrated that the gene expression level (Taniyama et al., 2002) could be increased further by combining ultrasound irradiation with an ultrasound contrast agent or microbubble, which consists of compressible gas-filled microbubbles such as perfluorocarbon-filled microspheres of heat-denatured human albumin in the case of the commonly used Optison™.

6.2.1.4. Hydrodynamic injection. Hydrodynamic gene delivery is an easier approach used to insert DNA effectively into the internal organs, especially the liver (Zhang et al., 1999). It was first used in 1996 by Budker and his team who succeeded in inserting plasmid DNA into the skeletal muscle of rats by rapid injection into the femoral artery (Budker et al., 1998). Its simplicity makes this method that most commonly used to transfer genes in rodents to study various applications (Suda and Liu, 2007). Gene transfer is performed by this method by high-speed intravenous injection of a large volume of DNA solution (8–9% of the body weight) for only a few seconds (3–5 s) (Zhang et al., 2004a). Experiments showed that in a mouse model, the best gene expression was achieved by injecting 1.6–1.8 ml of saline solution of DNA for a 20 g mouse (i.e. 8–9% body weight) via the tail vein for 5–8 s (Liu et al., 1999). It is still considered to be the method employed most frequently to

transport genes in rats and mice, but it is non-applicable in humans since, in order to achieve high efficiency, a large volume of solution, equivalent to 8–9% body weight, must be injected at high speed. 8–9% of human body weight is equivalent to 7.5 L of saline solution, which is many times more than the volume of liquid that can be safely injected into the human body in only a few seconds. The result could be transient heart failure and cardiac congestion (Suda and Liu, 2007).

6.2.2. Chemical vectors

Chemical vectors are proposed as promising alternatives to viral ones to overcome the drawbacks of the latter. These vectors have three objectives that improve gene transfer into the cell nucleus. They: (i) mask DNA-negative charges, (ii) compress the DNA molecule to make it smaller, and (iii) protect it from degradation by intracellular nuclease. These objectives can be achieved through packing DNA either by electrostatic interaction between anionic DNA and polycations, or encapsulating it with biodegradable polymers, or by the adsorbing it.

6.2.2.1. Electrostatic interaction between DNA and polycations. This type of gene delivery system uses the electrostatic attraction between anionic DNA and a cationic lipid or polymer, leading to a positive complex known as lipoplex or polyplex, respectively. In this field, various works have been reported by Elaissari et al. by studying the interaction involved in the adsorption process (Elaissari et al., 1994; Elaissari et al., 1995; Ganachaud et al., 1997a,b).

Lipoplexes A cationic lipid consists of three parts: a hydrophobic anchor (two aliphatic chains (saturated/unsaturated), or a cholesterol derivative), a hydrophilic positively charged head, and a spacer (linker). The linker connects the anchor to the head and plays an important role in determining the lipid's biodegradability (Gao and Hui, 2001). It condenses with anionic DNA to form positively charged lipoplex. N[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), produced by Felgner et al. (1987), was the first non-natural lipid employed for gene transfer. Since then, many different lipids have been developed to form self-assembling nano-sized DNA delivery systems (lipoplexes). Fig. 3 shows the main lipids used to prepare lipoplexes (Pedroso de Lima et al., 2001). The major problem hindering the use of lipoplex *in vivo*, besides poor efficiency, is cytotoxicity resulting from the lipoplex-positive charge (Lv et al., 2006).

Polyplexes: A cationic polymer (at physiological pH) is used to condense anionic nucleic acid into a nano-sized complex called "polyplex" by self-assembly via electrostatic interactions. It can compress DNA molecules to relatively small size which facilitates cellular internalization and thus improves transfection efficacy (Zhang et al., 2004b). Unfortunately, the use of polyplexes as gene-transfer systems *in vivo* must overcome many obstacles, such as their toxicity, poor efficiency, polymer polydispersity, and the lack of information about the gene transfer mechanism involved in these methods (Edinger and Wagner, 2011). Recently, scientists have found effective solutions for some of these obstacles, by designing monodispersed biodegradable polymer structures that reduce the toxicity of the polyplex by masking the positive charge, and by working to understanding the mechanisms of polyplex delivery (Schaffert and Wagner, 2008). Indeed, poly-L-lysine (PLL) is considered as one of the first polymers to be used for gene transfer *in vivo* (Wu and Wu, 1988). In the following period, many cationic polymers were evaluated as gene carriers both *in vitro* and *in vivo*, such as Poly ethylenimine (PEI) (Boussif et al., 1995; Chemin et al., 1998), poly amido amine (PAA) (Rudolph et al., 2000) and others, as shown in Fig. 4 (Tong et al., 2007).

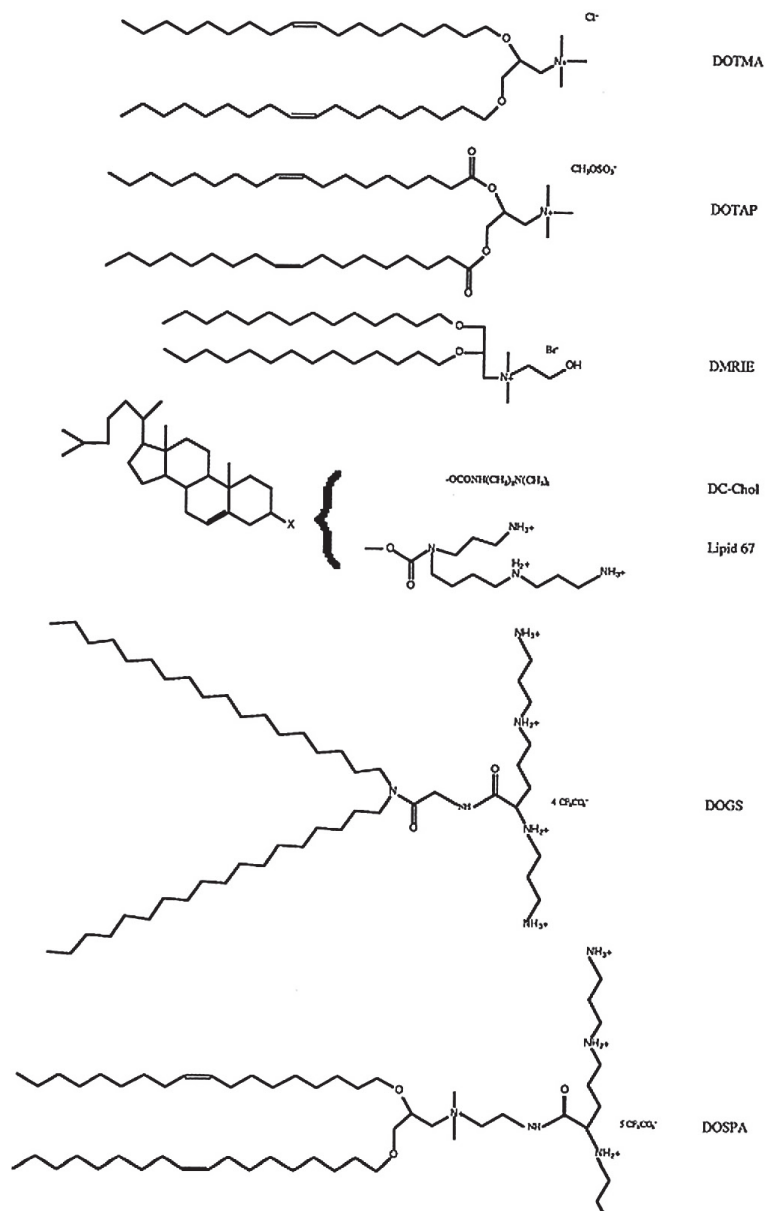


Fig. 3. The main lipids used for preparing lipoplex (Lv et al., 2006).

6.2.2.2. DNA encapsulation. In biomedical field, the encapsulation of active molecules, biomolecules and inorganic nanoparticles have been largely investigated using various approaches (Rahman and El-Helw, 2012; Delmas et al., 2012; Doustgani et al., 2012; Roveimiyab et al., 2012; Poletto et al., 2012; Khan et al., 2012; Rosset et al., 2012; Macková et al., 2012; Ahsan et al., 2013; Malacrida et al., 2013; Campos et al., 2013). DNA encapsulation using biodegradable

polymer is a good alternative to condensing DNA with polycations, and can overcome the obstacles and drawbacks that limit using the condensing strategy to form DNA protection and delivery systems. This strategy leads to nanometric or micrometric spherical structures of hydrolytically degradable polymers containing DNA. DNA encapsulation presents remarkable advantages such as ease of removal from the body due to polymer biodegradability, the

Please cite this article in press as: Ibraheem, D., et al., Gene therapy and DNA delivery systems. Int J Pharmaceut (2013), <http://dx.doi.org/10.1016/j.ijpharm.2013.11.041>

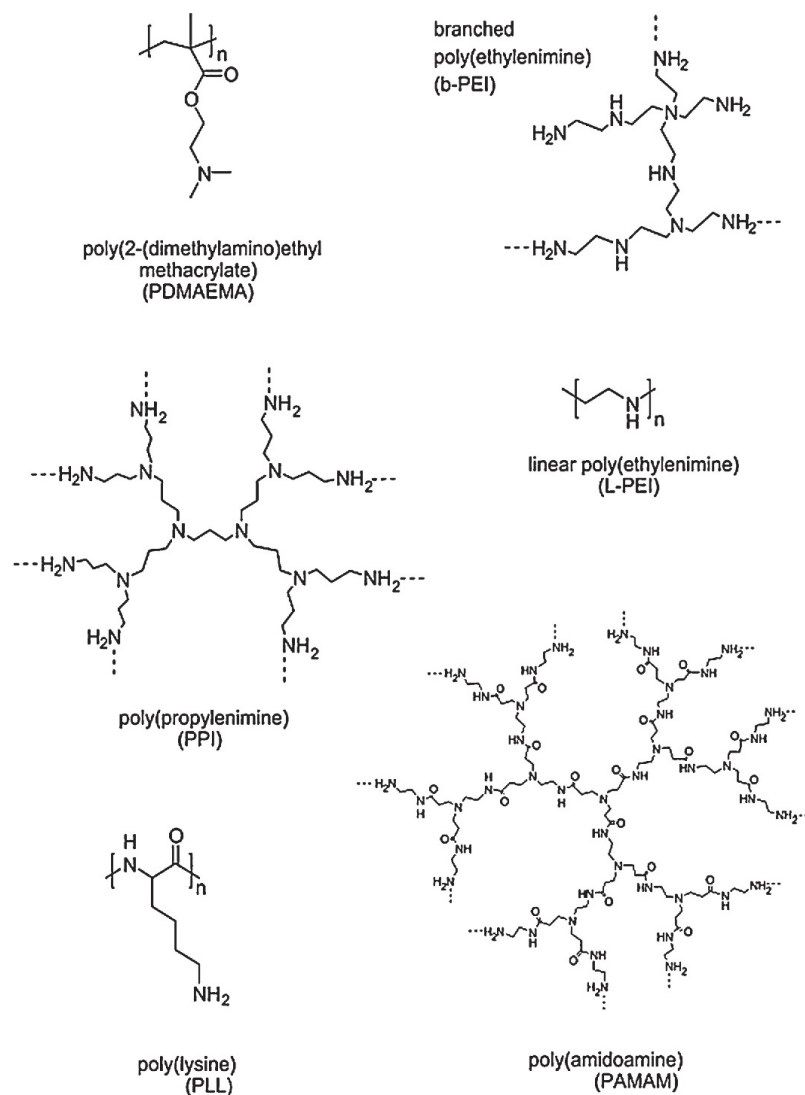


Fig. 4. The main polymers used for preparing a polyplex (Tong et al.).

458 possibility of controlling DNA release, and good DNA protection.
 459 However, it suffers from some drawbacks such as severe and draco-
 460 nian preparation conditions (organic solvents, high temperatures,
 461 high shear forces) that may destroy the transferred gene (Ando
 462 et al., 1999), low encapsulation efficiency, destructive environment
 463 created during the preparation of particles subsequent of polymer
 464 degradation, for example; acidic conditions inside the hydrolysed
 465 polyester particles that can induces DNA degradation (Vert et al.,
 466 1994; Mäder et al., 1998), and incomplete DNA release leading to
 467 low DNA bioavailability (Fu et al., 2000; Wang et al., 1999). Vari-
 468 ous strategies have been implemented to encapsulate DNA for use
 469 *in vivo*, including block-copolymer micelles (Kataoka et al., 2001),
 470 inversion emulsion, liposomes (Tsumoto et al., 2001; Edwards and

Baumner, 2007), and layer by layer (LbL) (Shchukin et al., 2004;
 Zelikin et al., 2006).

471
 472
 473 Many strategies have been developed and conducted to deliver
 474 nucleic acids for gene therapy application. Then various methods
 475 for DNA encapsulation or various approaches for DNA adsorption
 476 onto seed particles have been developed as reported in the liter-
 477 ature (Panyam and Labhasetwar, 2012; Luo and Saltzman, 2000;
 478 Cohen et al., 2000; Safinya, 2001; Ganachaud et al., 1997b). DNA
 479 can be transported using various kinds of organic and inorganic
 480 particles such as silica nanoparticles, carbon nanotubes (CNTs)
 481 and functionalised superparamagnetic iron oxide nanoparticles
 482 (FSPIONs). Silica based particles are generally homogeneous silica
 483 nanoparticles or magnetic core and silica shell are homogeneous

Please cite this article in press as: Ibraheem, D., et al., Gene therapy and DNA delivery systems. Int J Pharmaceut (2013), <http://dx.doi.org/10.1016/j.ijpharm.2013.11.041>

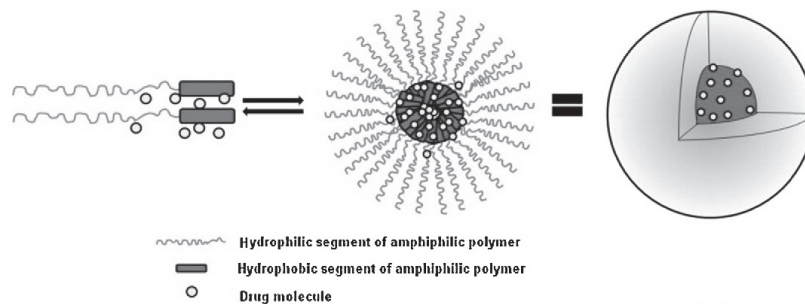


Fig. 5. forming polymeric micelles carrying system and locating the drug inside it (Lu and Park, 2013).

484 core/shell for DNA carrier as solid support (Tan et al., 2004;
485 Wang et al., 2008). Carbon nanotubes CNTs and principally ammo-
486 nium-functionalized CNTs (f-CNTs) are used for DNA transfer by
487 condensing the negatively charged plasmid DNA onto the surface
488 of the carrier f-CNTs via attractive electrostatic interactions. High
489 gene expression levels were achieved by using f-CNTs as gene deliv-
490 ery compared to free DNA (Bianco et al., 2005; Pantarotto et al.,
491 2004).

492 Functionalised superparamagnetic iron oxide nanoparticles
493 (FSPIONs) known as manofections were used as a carrier of
494 nucleic acid molecules in order to deliver nucleic acid molecules
495 such as (plasmids, small interfering siRNA, short hairpin shRNA,
496 and antisense oligonucleotides; Mykhaylyk et al., 2009; Krötz
497 et al., 2003). Manofections proved efficient nonviral transfection
498 *in vitro* and *in vivo* (Scherer et al., 2002; Prijic et al., 2012; Prijic
499 and Sersa, 2011). For gene delivering, the functionalized SPIONs
500 containing chemically grafted nucleic acids are controlled by an
501 external magnetic field to the targeted cells, which ensure more
502 rapid and controlled targeting. In this review, we are principally
503 focussing on biodegradable organic material for DNA encapsula-
504 tion, but various approaches using inorganic particles have been
505 also reported. The use of inorganic particles is for nucleic acids
506 adsorption and not for encapsulation as generally suitable for
507 *in vivo* gene therapy.

508 **Complex coacervation of gels:** Gel-based nanoparticles have
509 been prepared via complex coacervation technique with a view
510 to protect and transfer the gene. Chitosan and gelatine are widely
511 used to prepare hydrogels for *in vivo* applications (particularly for
512 gene delivery), thanks to their biocompatibility and biodegradabil-
513 ity (Leong et al., 1998; Mao et al., 2001; Young et al., 2005; Yuan
514 et al., 2009). The encapsulation of DNA (or attractive electrostatic
515 entrapment of nucleic acids) via such method depends on the coac-
516 cervation phenomenon which can be described as the separation of a
517 colloidal system (induced by different factors such as salt addition)
518 into two liquid phases: one is concentrated in colloid dispersion
519 known as coacervate and the second is the supernatant named
520 as the equilibrium phase (De Kruijff et al., 2004). When the DNA
521 solution was added in the contact with positively charged hydro-
522 gel dispersion, the resulting solution leads to two separate phases
523 as mentioned earlier. DNA loaded nanoparticles are then obtained
524 and mainly governed by attractive electrostatic interactions (Leong
525 et al., 1998; Truong-Le et al., 1998; Mao et al., 2001). This DNA
526 compaction method presents advantage to be performed in aque-
527 ous medium at low temperature necessary for fragile biomolecules
528 (Leong et al., 1998) and the disadvantage related to DNA release
529 from generally strong attractive electrostatic interaction in physi-
530 ological medium.

531 **Block copolymer micelles:** In the aqueous phase, amphiphilic
532 block copolymers self-assemble into spherical core-shell

533 polymeric micelles of mesoscopic size (Moffitt et al., 1996; Munk
534 et al., 2013). These hydrophobic segments of block copolymers
535 accumulate away from the surrounding aqueous medium to form
536 the inner core enveloped by a palisade of hydrophilic segments,
537 as shown in Fig. 5. This structure is employed to entrap plasmid
538 DNA and oligonucleotides as non-viral vectors (Kataoka et al.,
539 1996; Katayose and Kataoka, 1997, 1998), by coating the polyion
540 complex consisting of polycation and polyanion DNA with a layer
541 of hydrophilic polymer (Vinogradov et al., 1998). The mechanism
542 underlying this operation can be explained by the following: the
543 block copolymer consists of a cationic segment and a hydrophilic
544 segment; its cationic segment reacts electrostatically with anionic
545 DNA to form the complex block copolymer-DNA which, in an
546 aqueous medium, forms a core-shell structure where the complex
547 segment cationic-DNA makes up the core and the hydrophilic
548 segment makes up the shell. They are promising for targeted drug
549 delivery due to their small size, ranging from 10 to 100 nm with a
550 very narrow distribution, high drug-loading capacity, low toxicity,
551 they are easy to manufacture and have good structural stability
552 (Nishiyama and Kataoka, 2004; Yokoyama, 2011). However, the
553 main problem handicapping polymeric micelle drug delivery
554 systems is their short life in the blood circulation due to non
555 specific uptake by the reticuloendothelial system (RES) (Kazunori
556 et al., 1993). The diffusion and propagation of drug-loaded micelles
557 mainly depends on their size and surface properties (Kazunori et al.,
558 1993). Several block copolymers are used in clinical applications
559 such as the block copolymer monomethoxy poly(ethylene glycol)
560 and poly(D,L-lactide) (MPEG-b-PDLLA) (Kim et al., 2004, 2007;
561 Lee et al., 2008), MPEG-b-poly(aspartic acid) (PAsp) (Matsumura
562 et al., 2004; Negishi et al., 2006), and Pluronic block copolymers
563 (Danson et al., 2004).

564 **Inversion emulsion:** In order to improve the properties of
565 lipoplexes with regard to increasing their *in vivo* stability, decreas-
566 ing their toxicity and improving their capacity to reach the targeted
567 cells. It is important to cover the lipoplex-positive charge by formu-
568 lating novel neutral particles. Lipid nanoparticles (LNs) are
569 good candidates as drug delivery systems since they satisfy the
570 previous requirements. They are submicronic-sized particles that
571 have a core-shell structure in which the core is an oily liquid
572 enclosed by a solid or semi-solid shell, and that can be prepared
573 without using organic solvents (Dulieu and Bazile, 2005). Indeed,
574 the structure of lipid nanoparticles is a cross between polymeric
575 nanocapsules and liposomes (Heurtault et al., 2002). Heurtault et al.
576 (2002) developed a new method for preparing lipidic nanocap-
577 sules (20–100 nm), based on the phase inversion of an emulsion
578 strategy, where the inversion of an emulsion phase is elicited and
579 activated by temperature variations and by a rapid cooling-dilution
580 process with deionized water. In 2009, Vonarbourg et al. reported a
581 method for DNA lipoplex encapsulation that relied on Heurtault's

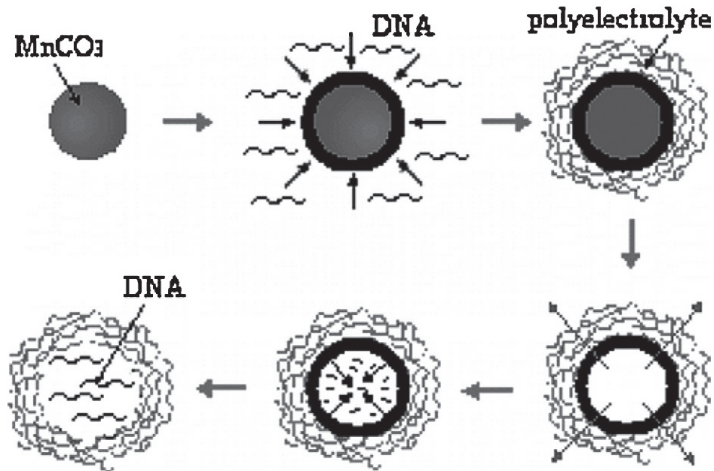


Fig. 6. Schematic illustration of the DNA encapsulation process using LBL strategy, in which DNA molecules were deposited as DNA/spermidine complex on the surface of template to form the core of the particle, followed by the formation of a large number of chondroitin sulfate/poly(-1-arginine) layers to form the microparticle shell (Shchukin et al., 2004).

582 technique. The resulting nanoparticles have an oily core consisting
583 of a mixture of triglycerides and polyglyceryl-6 dioleate (lipophilic
584 Labrafac/Oleic Plurol), and a shell composed of the association of
585 free PEG and HS-PEG (Solutol). The resulting lipid nanocapsules can
586 incorporate DOTAP-DOPE/DNA lipoplexes with a high level of effi-
587 ciency (Vonarbourg et al., 2009). The lipid nanocapsules obtained
588 are non-toxic due since they are prepared without using an organic
589 solvent (Harris, 1985). They present good stability in vitro for more
590 than one year (Heurtault et al., 2002) and they have relatively good
591 circulation time (Cahouet et al., 2002). LNs can be introduced into
592 the human body by oral, parenteral and ocular routes (Dulieu and
593 Bazile, 2005).

594 Layer by layer (LbL): Layer by layer (LbL) technique was devel-
595 oped and explored in order to obtain capsules since 1998 by
596 Sukhorukov et al. (1998). It consists in preparing hollow polyelec-
597 trolyte capsules that can be employed as drug delivery systems
598 as they can be formulated and manufactured with controlled size,
599 structure and functionality, in addition to the fact that they can

600 be loaded with model therapeutic substances (proteins) and other
601 drugs having low molecular weight (Zelikin et al., 2006). The layer
602 by layer technique depends on the sequential adsorption of oppo-
603 sately charged polymers layers on a sacrificial template that is
604 removed at the end of preparation (Geest et al., 2007). Regarding
605 nucleic acids, DNA can be encapsulated using LbL strategy by DNA
606 deposition on a substrate with spermidine, followed by the forma-
607 tion of a large number of polyelectrolyte layers. Finally, when the
608 core particle dissolves the DNA is released inside the capsule
609 (Shchukin et al., 2004), as shown in Fig. 6. Recently, Alexander
610 and his team encapsulated DNA using the polycation-free method.
611 This method comprises four steps that are illustrated in Diagram 3
612 (Zelikin et al., 2007) in Fig. 7:

613 Positively charged amino-functionalized silica (SiO_2^+) is used as
614 a template (sacrificial colloidal particles) to adsorb the negatively
615 charged DNA.

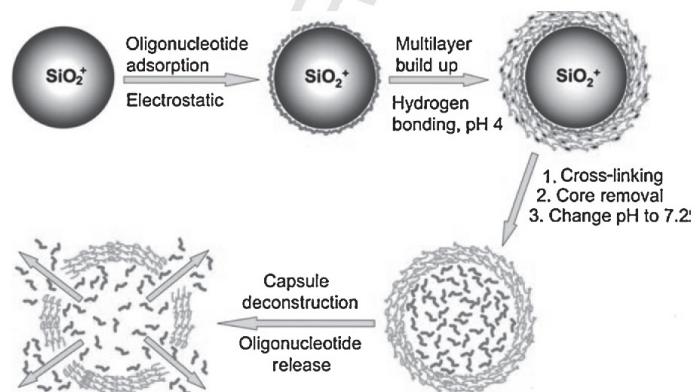


Fig. 7. Schematic illustration of the main steps of DNA encapsulation using the polycation-free method (Zelikin et al., 2007).

Please cite this article in press as: Ibraheem, D., et al., Gene therapy and DNA delivery systems. Int J Pharmaceut (2013), <http://dx.doi.org/10.1016/j.ijpharm.2013.11.041>

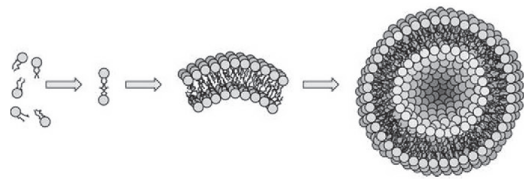


Fig. 8. Formation of liposome by the self-assembly of amphiphilic lipids placed in aqueous phase (Balazs and Godbey, 2010).

- The second step is the assembly of a thin polymer film prepared by the alternative deposition of PMA_{SH} {thiolated poly(methacrylic acid)} and PVPON {poly(vinylpyrrolidone)}, which interacts with covalent or hydrogen bonding forces on sacrificial colloidal particles.
- This step is followed by the oxidation of the PMA_{SH} thiol group to form disulfide bonds.
- Finally, the template is removed.

This technique presents many advantages: the capsules can be prepared without using severe mechanical forces liable to damage the nucleic acids, the resulting capsules can incorporate oligonucleotides efficiently which should be released inside the cells during the degradation of the polymer forming the shell of the capsules (Zelikin et al., 2006).

Liposomes: Liposomes are structures that were described for the first time in 1961 by Dr. Alec D Bangham (Bangham and Horne, 1964, 1962; Finean and Rumsby, 1963; Laouini et al., 2012). They can be identified as artificial vesicles consisting of one or more bilayers of amphiphilic lipid that trap an equal number of internal aqueous compartments (Bagasra et al., 1999; Date et al., 2007; Yang et al., 2011; Felnerova et al., 2004) Fig. 8. These structures are formed spontaneously when certain lipids are placed in aqueous phase, through the self-assembly of these lipids in such a way as to orient their hydrophobic parts away from the water, while the hydrophilic parts are oriented towards the aqueous phase surrounding the hydrophobic ones (Torchilin, 1996; Huang, 2008). Due to the structure of liposome, it can encapsulate a wide range of hydrophilic and hydrophobic drugs, in the phospholipid bilayer, in the internal aqueous compartments or at the bilayer interface (Sharma and Sharma, 1997) as shown in Fig. 9. Depending on the composition and method of preparation, liposome can take various forms (Templeton, 2002). According to the New York Academy of Science, liposomes are classified into three types (Papahadjopoulos and New York Academy of Sciences, 1978), small unilamellar vesicles (SUV), with a diameter less than 50 nm, large unilamellar vesicles (LUV) with a diameter of 50–500 nm, and multilamellar liposomes (MLV) with a large diameter up to 10,000 nm. SUV can condense DNA on its surface (Sternberg, 1996), making it

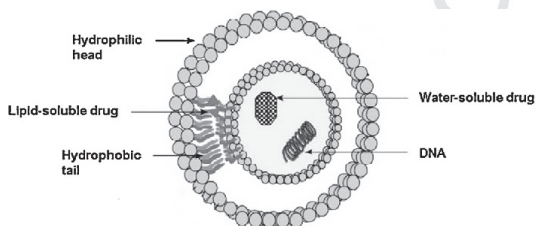


Fig. 9. Schematic illustration representing the structure of liposome and the potential position of lipophilic and hydrophilic drugs (Malam et al., 2009).

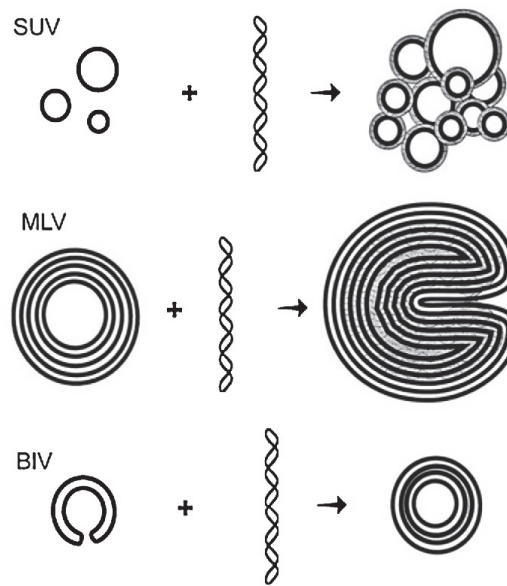


Fig. 10. Liposome formulations used as DNA delivery systems, and the position of DNA in each DNA-liposome complex (Templeton, 2002).

vulnerable to the enzymes in the medium. This ineffective protection, in addition to the short half-life of the DNA- SUV complex in the blood circulation, limits its use as a DNA delivery system. The large size of multilamellar liposomes (MLV) hinders their use for the systematic administration or transport of DNA into cells (Templeton, 2002). In order to overcome these obstacles that limit the use of liposomes for gene delivering, another liposome formulation has been developed called bilamellar invaginated structures (BIV) (Templeton et al., 1997), that provide many advantages for DNA encapsulation as they can encapsulate large amounts of DNA of any size efficiently. Fig. 10 shows the reaction of DNA with different types of liposome. The characteristics of liposomes used as DNA delivery systems include: (i) low immunogenic response; (ii) large amounts of DNA can be loaded easily; (iii) they increase the stability of DNA in the body; and (iv) lack of clearance that improves the DNA blood circulation (Huang, 2008). However, there are many drawbacks that limit their application *in vivo*, i.e. liposomes at high concentrations may form large aggregates, they suffer from instability in the presence of serum (Litzinger et al., 1996), in addition to low encapsulation efficiency achieved when hydrophilic molecules (such as DNA, protein) are encapsulated via liposomes (Xu et al., 2012; Soppimath et al., 2001).

6.2.2.3. DNA adsorption. This technique is the result of combining the two previous techniques (electrostatic interaction and encapsulation), where the positively charged polymers adsorb on the surface of biodegradable particles to which DNA can be electrostatically linked (Singh et al., 2000). It has been demonstrated to have many advantages including those of improving DNA bioavailability and augmenting loading efficiency (Kasturi et al., 2005). In this method microparticles can be prepared without the harsh encapsulation conditions that may degrade the DNA. However, the presence of DNA adsorbed on the surface of particles exposes it to enzymes that may damage or degrade it, despite the protection afforded by

Please cite this article in press as: Ibraheem, D., et al., Gene therapy and DNA delivery systems. Int J Pharmaceut (2013), <http://dx.doi.org/10.1016/j.ijpharm.2013.11.041>

the polymer against enzymatic degradation and by the degree of electrostatic interaction with DNA (Munier et al., 2005; Oster et al., 2005).

7. Conclusion

The main purpose of all of these pharmaceutical developments is to increase the desired medical impacts of a drug, and to decrease the side effects related to its use. This is the main objective now in gene therapy, in which when DNA is the drug to be administered. Gene therapy has become a promising strategy for treating many incurable diseases, whether acquired or heritable. However, the main challenge facing gene therapy, which prevents its widespread use *in vivo* is to find efficient delivery systems capable of protecting and delivering the transgene to target cells in which it can successfully express itself. Viruses have been used for a long time to deliver transgenes in what is called “viral vectors”, however, the severe killer immune response caused by these carriers, in addition to the fact that the size of recombinant viruses limits their use as vectors to deliver genes and emphasizes the need to search for alternative vectors to satisfy conditions of safety and efficacy, thereby raising the issue of whether non-viral vectors can be used. This includes physical and chemical techniques. The earliest chemical vectors depended on the electrostatic complexation of DNA polyanions with polycations to form polyplexes (with polymers) and lipoplexes (with lipids). The need to improve these vectors, in order to increase their capacity to protect the genes and decrease their size, since small size has positive effects on intracellular delivery, encourages scientists to focus their research on DNA encapsulation and improve the properties of the resulting capsules.

Acknowledgments

The authors thank and appreciate the research grant from Syrian government. The authors also thank MILADI Karim for his technical help and discussions.

References

- Ahsan, A., Aziz, A., Arshad, M.A., Ali, O., Nauman, M., Ahmad, N.M., Elaissari, A., 2013. Smart magnetically engineering colloids and biothin films for diagnostics applications. *J. Colloid Sci. Biotechnol.* 2, 19–26.
- Aihara, H., Miyazaki, J., 1998. Gene transfer into muscle by electroporation *in vivo*. *Nat. Biotechnol.* 16, 867–870.
- Ajiki, T., Murakami, T., Kobayashi, Y., Hakamata, Y., Wang, J., Inoue, S., Ohtsuki, M., Nakagawa, H., Kariya, Y., Hoshino, Y., et al., 2003. Long-lasting gene expression by particle-mediated intramuscular transfection modified with bupivacaine: combinatorial gene therapy with IL-12 and IL-18 cDNA against rat sarcoma at a distant site. *Cancer Gene Ther.* 10, 318–329.
- Anderson, W.F., 1992. Human gene therapy. *Science* 256, 808–813.
- Ando, S., Putnam, D., Pack, D.W., Langer, R., 1999. PLGA microspheres containing plasmid DNA: preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization. *J. Pharm. Sci.* 88, 126–130.
- Avery, O.T., MacLeod, C.M., McCarty, M., 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a deoxyribonucleic acid fraction isolated from pneumococcus type III. *J. Exp. Med.* 79, 137–158.
- Al-Dosari, M.S., Gao, X., 2009. Nonviral gene delivery: principle, limitations, and recent progress. *Aaps J.* 11, 671–681.
- Bagasra, O., Amjad, M., Mukhtar, M., 1999. Liposomes in gene therapy. In: Blankenstein, P.D.T. (Ed.), *Gene Therapy*. Birkhäuser Basel, pp. 61–71.
- Balazs, D.A., Godbey, W.T., 2010. Liposomes for use in gene delivery. *J. Drug Deliv. Bangham, A.D., Home, R.W., 1964. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. J. Mol. Biol.* 8, 660–1010.
- Bao, S., Thrall, B.D., Miller, D.L., 1997. Transfection of a reporter plasmid into cultured cells by sonoporation *in vitro*. *Ultrasound Med. Biol.* 23, 953–959.
- Bekeredjian, R., Chen, S., Frenkel, P.A., Grayburn, P.A., Shohet, R.V., 2003. Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart. *Circulation* 108, 1022–1026.
- Bergen, J.M., Park, I.-K., Horner, P.J., Pun, S.H., 2008. Nonviral approaches for neuronal delivery of nucleic acids. *Pharm. Res.* 25, 983–998.
- Bianco, A., Kostarelos, K., Prato, M., 2005. Applications of carbon nanotubes in drug delivery. *Curr. Opin. Chem. Biol.* 9, 674–679.

- Bouard, D., Alazard-Dany, N., Cosset, F.-L., 2009. Viral vectors: from virology to transgene expression. *Br. J. Pharmacol.* 157, 153–165.
- Boulaiz, H., Marchal, J.A., Prados, J., Melgizo, C., Aránega, A., 2005. Non-viral and viral vectors for gene therapy. *Cell. Mol. Biol. Noisy-Gd. Fr* 51, 3–22.
- Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc. Natl. Acad. Sci.* 92, 7297–7301.
- Budker, V., Zhang, G., Danko, I., Williams, P., Wolff, J., 1998. The efficient expression of intravascularly delivered DNA in rat muscle. *Gene Ther.* 5, 272–276.
- Bunnell, B.A., Morgan, R.A., 1998. Gene therapy for infectious diseases. *Clin. Microbiol. Rev.* 11, 42–56.
- Cahouet, A., Denizot, B., Hindré, F., Passirani, C., Heurtault, B., Moreau, M., Le Jeune, J., Benoît, J., 2013. Biodistribution of dual radiolabeled lipid nanocapsules in the rat using scintigraphy and γ counting. *Int. J. Pharm.* 242, 367–371.
- Campos, E.V.R., de Melo, N.F.S., de Paula, E., Rosá, A.H., Fraceto, L.F., 2013. Screening of conditions for the preparation of poly(ϵ -caprolactone) nanocapsules containing the local anesthetic articaine. *J. Colloid Sci. Biotechnol.* 2, 106–111.
- Chemin, I., Moradpour, D., Wieland, S., Offensperger, W.-B., Walter, E., Behr, J.-P., Blum, H.E., 1998. Liver-directed gene transfer: a linear polyethylenimine derivative mediates highly efficient DNA delivery to primary hepatocytes *in vitro* and *in vivo*. *J. Viral Hepat.* 5, 369–375.
- Cho-Chung, Y.S., 2005. DNA drug design for cancer therapy. *Curr. Pharm. Des.* 11, 2811–2823.
- Cohen, H., Levy, R.J., Gao, J., Fishbein, I., Kousav, V., Sosnowski, S., Slomkowski, S., Golomb, G., 2000. Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. *Gene Ther.* 7, 1896–1905.
- Dahm, R., 2010. From discovering to understanding. *EMBO Rep.* 11, 153–160.
- Danson, S., Ferry, D., Alakhov, V., Margison, J., Kerr, D., Jowle, D., Brampton, M., Halbert, G., Ranson, M., 2004. Phase I dose escalation and pharmacokinetic study of pluronic polymer-bound doxorubicin (SP1049C) in patients with advanced cancer. *Br. J. Cancer* 90, 2085–2091.
- Date, A.A., Joshi, M.D., Patravale, V.B., 2007. Parasitic diseases: liposomes and polymeric nanoparticles versus lipid nanoparticles. *Adv. Drug Deliv. Rev.* 59, 505–521.
- Davies, J.C., Geddes, D.M., Alton, E.W.F.W., 2001. Gene therapy for cystic fibrosis. *J. Gene Med.* 3, 409–417.
- Delmas, T., Fraichard, A., Bayle, P.-A., Texier, I., Bardet, M., Baudry, J., Bibette, J., Couffin, A.-C., 2012. Encapsulation and release behavior from lipid nanoparticles: model study with Nile red fluorophore. *J. Colloid Sci. Biotechnol.* 1, 16–25.
- Dishart, K.L., Work, L.M., Denby, L., Baker, A.H., 2003. Gene therapy for cardiovascular disease. *J. Biomed. Biotechnol.* 2003, 138–148.
- Doustgani, A., Farahani, E.V., Imani, M., Doulabi, A.H., 2012. Dexamethasone sodium phosphate release from chitosan nanoparticles prepared by ionic gelation method. *J. Colloid Sci. Biotechnol.* 1, 42–50.
- Dujardin, N., Pr at, V., 2004. Delivery of DNA to skin by electroporation. In: Heiser, W.C. (Ed.), *Gene Delivery to Mammalian Cells*. Humana Press, pp. 215–226.
- Dulieu, C., Bazile, D., 2005. Influence of lipid nanocapsules composition on their aptness to freeze-drying. *Pharm. Res.* 22, 285–292.
- Edelstein, M.L., Abedi, M.R., Wixon, J., 2007. Gene therapy clinical trials worldwide to 2007—an update. *J. Gene Med.* 9, 833–842.
- Efinger, D., Wagner, E., 2011. Bioresponsive polymers for the delivery of therapeutic nucleic acids. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 3, 33–46.
- Edwards, K.A., Baumner, A.J., 2007. DNA, oligonucleotide encapsulating liposomes and secondary signal amplification means. *Anal. Chem.* 79, 1806–1815.
- Elaissari, A., Pichot, C., Delair, T., Cros, P., Kuerfurst, R., 1995. Adsorption and desorption studies of polyadenylic acid onto positively charged latex particles. *Langmuir* 11, 1261–1267.
- Elaissari, A., Cros, P., Pichot, C., Laurent, V., Mandrand, B., 1994. Adsorption of oligonucleotides onto negatively and positively charged latex particles. *Colloids Surfaces Physicochem. Eng. Asp.* 83, 25–31.
- El-Anead, A., 2004. An overview of current delivery systems in cancer gene therapy. *J. Control. Release Off. J. Control. Release Soc.* 94, 1–14.
- Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci.* 84, 7413–7417.
- Felnerova, D., Viret, J.-F., Gl uck, R., Moser, C., 2004. Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs. *Curr. Opin. Biotechnol.* 15, 518–529.
- Ferreira, G.N.M., Monteiro, G.A., Prazeres, D.M.F., Cabral, J.M.S., 2000. Downstream processing of plasmid DNA for gene therapy and DNA vaccine applications. *Trends Biotechnol.* 18, 380–388.
- Finean, J.B., Rumsby, M.G., 1963. Negatively stained lipoprotein membranes. *Nature* 200, 1340.
- Friedmann, T., 1992. A brief history of gene therapy. *Nat. Genet.* 2, 93–98.
- Friedmann, T., 1996. Human gene therapy—an immature genie, but certainly out of the bottle. *Nat. Genet.* 2, 144–147.
- Fu, K., Pack, D.W., Klibanov, A.M., Langer, R., 2000. Visual evidence of acidic environment within degrading poly(lactic-co-glycolic acid) (PLGA) microspheres. *Pharm. Res.* 17, 100–106.
- Ganachaud, F., Elaissari, A., Pichot, C., 1997a. Effect of Triton X-405 on the adsorption and desorption of single-stranded DNA fragments onto positively charged latex particles. *Langmuir* 13, 7021–7029.
- Ganachaud, F., Elaissari, A., Pichot, C., Laayoun, A., Cros, P., 1997b. Adsorption of single-stranded DNA fragments onto cationic aminated latex particles. *Langmuir* 13, 701–707.

- Gao, H., Hui, K.M., 2001. Synthesis of a novel series of cationic lipids that can act as efficient gene delivery vehicles through systematic heterocyclic substitution of cholesterol derivatives. *Gene Ther.* 8, 855–863.
- Gao, X., Kim, K.-S., Liu, D., 2007. Nonviral gene delivery: what we know and what is next. *Aaps J.* 9, E92–E104.
- Gardlik, R., Pálffy, R., Hodossy, J., Lukács, J., Turna, J., Celec, P., 2005. Vectors and delivery systems in gene therapy. *Med. Sci. Monit. Int. Med. J. Exp. Clin. Res.* 11, RA110–RA121.
- Geest, B.G.D., Sanders, N.N., Sukhorukov, G.B., Demeester, J., Smedt, S.C.D., 2007. Release mechanisms for polyelectrolyte capsules. *Chem. Soc. Rev.* 36, 636–649.
- Gehl, J., 2003. Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. *Acta Physiol. Scand.* 177, 437–447.
- Goddard, P., 1991. Therapeutic proteins—a pharmaceutical perspective. *Adv. Drug Deliv. Rev.* 6, 103–131.
- Guo, X., Huang, L., 2012. Recent advances in nonviral vectors for gene delivery. *Accounts Chem. Res.* 45, 971–979.
- Han, S., 2000. Development of biomaterials for gene therapy. *Mol. Ther.* 2, 302–317.
- Harris, J.M., 1985. Laboratory synthesis of polyethylene glycol derivatives. *J. Macromol. Sci. Part C Polym. Rev.* 25, 325–373.
- Heller, L.C., Ugen, K., Heller, R., 2005. Electroporation for targeted gene transfer. *Expert Opin. Drug Deliv.* 2, 255–268.
- Heller, R., Jaroszeski, M., Atkin, A., Moradpour, D., Gilbert, R., Wands, J., Nicolau, C., 1996. In vivo gene electroinjection and expression in rat liver. *FEBS Lett.* 389, 225–228.
- Herzog, R.W., Cao, O., Srivastava, A., 2010. Two decades of clinical gene therapy—success is finally mounting. *Discov. Med.* 9, 105–111.
- Heurtault, B., Saulnier, P., Pech, B., Proust, J.-E., Benoit, J.-P., 2002. A novel phase inversion-based process for the preparation of lipid nanocarriers. *Pharm. Res.* 19, 875–880.
- Huang, S.-L., 2008. Liposomes in ultrasonic drug and gene delivery. *Adv. Drug Deliv. Rev.* 60, 1167–1176.
- Huber, P.E., Jenne, J., Debus, J., Wannemacher, M.F., Pfisterer, P., 1999. A comparison of shock wave and sinusoidal-focused ultrasound-induced localized transfection of HeLa cells. *Ultrasound Med. Biol.* 25, 1451–1457.
- Wolff, J.A., Williams, P., Acasadi, G., Jiao, S., Jani, A., Chong, W., 1991. Conditions affecting direct gene transfer into rodent muscle in vivo. *Biotechniques* 11, 474–485.
- Jaroszeski, M.J., Heller, L.C., Gilbert, R., Heller, R., 2013. Electrically mediated plasmid DNA delivery to solid tumors in vivo. In: *Gene Delivery to Mammalian Cells*. Humana Press, New Jersey, pp. 237–244.
- Kasturi, S.P., Sachapibulkij, K., Roy, K., 2005. Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. *Biomaterials* 26, 6375–6385.
- Kataoka, K., Togawa, H., Harada, A., Yasugi, K., Matsumoto, T., Katayose, S., 1996. Spontaneous formation of polyion complex micelles with narrow distribution from antisense oligonucleotide and cationic block copolymer in physiological saline. *Macromolecules* 29, 8556–8557.
- Kataoka, K., Harada, A., Nagasaki, Y., 2001. Block copolymer micelles for drug delivery: design, characterization and biological significance. *Adv. Drug Deliv. Rev.* 47, 113–131.
- Katayose, S., Kataoka, K., 1997. Water-soluble polyion complex associates of DNA and poly(ethylene glycol)-poly(L-lysine) block copolymer. *Bioconjug. Chem.* 8, 702–707.
- Katayose, S., Kataoka, K., 1998. Remarkable increase in nucleic acid resistance of plasmid DNA through supramolecular assembly with poly(ethylene glycol)-poly(L-lysine) block copolymer. *J. Pharm. Sci.* 87, 160–163.
- Kay, M.A., Glorioso, J.C., Naldini, L., 2001. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat. Med.* 7, 33–40.
- Kazunori, K., Glenn, S., Masayuki, Y., Teruo, O., Yasuhisa, S., 1993. Block copolymer micelles as vehicles for drug delivery. *J. Control. Release* 24, 119–132.
- Kendirci, M., Teloken, P.E., Champion, H.C., Hellstrom, W.J.G., Bivalacqua, T.J., 2006. Gene therapy for erectile dysfunction: fact or fiction? *Eur. Urol.* 50, 1208–1222.
- Khan, M.S., Vishakante, G.D., Bathool, A., 2012. Development and characterization of brimonidine tartrate loaded eudragit nanosuspensions for ocular drug delivery. *J. Colloid Sci. Biotechnol.* 1, 122–128.
- Kim, D.-W., Kim, S.-Y., Kim, H.-K., Kim, S.-W., Shin, S.W., Kim, J.S., Park, K., Lee, M.Y., Heo, D.S., 2007. Multicenter phase II trial of Genexol-PM, a novel Cremophor-free, polymeric micelle formulation of paclitaxel, with cisplatin in patients with advanced non-small-cell lung cancer. *Ann. Oncol.* 18, 2009–2014.
- Kim, H.J., Greenleaf, J.F., Kinnick, R.R., Bronk, J.T., Bolander, M.E., 1996. Ultrasound-mediated transfection of mammalian cells. *Hum. Gene Ther.* 7, 1339–1346.
- Kim, T.-Y., Kim, D.-W., Chung, J.-Y., Shin, S.G., Kim, S.-C., Heo, D.S., Kim, N.K., Bang, Y.-J., 2004. Phase I and pharmacokinetic study of genexol-PM, a cremophor-free, polymeric micelle-formulated paclitaxel, in patients with advanced malignancies. *Clin. Cancer Res.* 10, 3708–3716.
- Kirchheis, R., Wightman, L., Wagner, E., 2001. Design and gene delivery activity of modified polyethylenimines. *Adv. Drug Deliv. Rev.* 53, 341–358.
- Knipe, J.M., Peters, J.T., Peppas, N.A., 2013. Theranostic agents for intracellular gene delivery with spatiotemporal imaging. *Nano Today* 8, 21–38.
- Kohn, D.B., Sadelain, M., Glorioso, J.C., 2003. Occurrence of leukaemia following gene therapy of X-linked SCID. *Nat. Rev. Cancer* 3, 477–488.
- Koike, H., Tomita, N., Azuma, H., Taniyama, Y., Yamasaki, K., Kunugiza, Y., Tachibana, K., Ogihara, T., Morishita, R., 2005. An efficient gene transfer method mediated by ultrasound and microbubbles into the kidney. *J. Gene Med.* 7, 108–116.
- Krötz, F., de Wit, C., Sohn, H.Y., Zahler, S., Gloe, T., Pohl, U., Plank, C., 2003. Magneto-fecton—a highly efficient tool for antisense oligonucleotide delivery in vitro and in vivo. *Mol. Ther. J. Am. Soc. Gene Ther.* 7, 700–710.
- De Kruijff, C.G., Weinbreck, F., de Vries, R., 2004. Complex coacervation of proteins and anionic polysaccharides. *Curr. Opin. Colloid Interface Sci.* 9, 340–349.
- Kuriyama, S., Mitoro, A., Tsujinoue, H., Nakatani, T., Yoshiji, H., Tsujimoto, T., Yamazaki, M., Fukui, H., 2000. Particle-mediated gene transfer into murine livers using a newly developed gene gun. *Gene Ther.* 7, 1132–1136.
- Labhasetwar, V., 2005. Nanotechnology for drug and gene therapy: the importance of understanding molecular mechanisms of delivery. *Curr. Opin. Biotechnol.* 16, 674–680.
- Laouini, A., Jaafar-Maalej, C., Limayem-Blouza, I., Sfar, S., Charcosset, C., Fessi, H., 2012. Preparation, characterization and applications of liposomes: state of the art. *J. Colloid Sci. Biotechnol.* 1, 147–168.
- Lauritzen, H.P., Reynet, C., Schjerling, P., Ralston, E., Thomas, S., Galbo, H., Ploug, T., 2002. Gene gun bombardment-mediated expression and translocation of EGFP-tagged GLUT4 in skeletal muscle fibres in vivo. *Pflügers Arch.* 444, 710–721.
- Ledley, F.D., Shapiro, T., 1998. Pharmacokinetic considerations in somatic gene therapy. *Adv. Drug Deliv. Rev.* 30, 133–150.
- Lee, K.S., Chung, H.C., Im, S.A., Park, Y.H., Kim, C.S., Kim, S.-B., Rha, S.Y., Lee, M.Y., Ro, J., 2008. Multicenter phase II trial of Genexol-PM, a Cremophor-free, polymeric micelle formulation of paclitaxel, in patients with metastatic breast cancer. *Breast Cancer Res. Treat.* 108, 241–250.
- Leong, K., Mao, H.-Q., Truong-Le, V., Roy, K., Walsh, S., August, J., 1998. DNA-polycation nanospheres as non-viral gene delivery vehicles. *J. Control. Release* 53, 183–193.
- Li, S., Huang, L., 2000. Nonviral gene therapy: promises and challenges. *Gene Ther.* 7, 31–34.
- Lin, M.T.S., Pulkkinen, L., Uitto, J., Yoon, K., 2000. The gene gun: current applications in cutaneous gene therapy. *Int. J. Dermatol.* 39, 161–170.
- Litzinger, D.C., Brown, J.M., Wala, I., Kaufman, S.A., Han, G.Y., Farrell, C.L., Collins, D., 1996. Fate of cationic liposomes and their complex with oligonucleotide in vivo. *Biochim. Biophys. Acta Biomembr.* 1281, 139–149.
- Liu, F., Song, Y., Liu, D., 1999. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* 6, 1258–1266.
- Loehr, B.L., Willson, P., Babjuk, L.A., Hurk, S., van den, D.L., 2000. Gene gun-mediated DNA immunization primes development of mucosal immunity against bovine herpesvirus 1 in cattle. *J. Virol.* 74, 6077–6086.
- Lu, Y., Park, K., 2013. Polymeric micelles and alternative nanonized delivery vehicles for poorly soluble drugs. *Int. J. Pharm.* 453, 198–214.
- Lu, Q.L., Liang, H.-D., Partridge, T., Blomley, M.J.K., 2003. Microbubble ultrasound improves the efficiency of gene transduction in skeletal muscle in vivo with reduced tissue damage. *Gene Ther.* 10, 396–405.
- Luo, D., Saltzman, W.M., 2000. Synthetic DNA delivery systems. *Nat. Biotechnol.* 18, 33–37.
- Lv, H., Zhang, S., Wang, B., Cui, S., Yan, J., 2006. Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Control. Release* 114, 100–109.
- Macková, H., Horák, D., Trachtová, Š., Řitich, B., Španová, A., 2012. The use of magnetic poly(N-isopropylacrylamide) microspheres for separation of DNA from probiotic dairy products. *J. Colloid Sci. Biotechnol.* 1, 235–240.
- Mäder, K., Bittner, B., Li, Y., Wohlauf, W., Kissel, T., 1998. Monitoring microviscosity and microacidity of the albumin microenvironment inside degrading microparticles from poly(lactide-co-glycolide) (PLG) or ABA-triblock polymers containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethyleneoxide) B blocks. *Pharm. Res.* 15, 787–793.
- Malacrida, C.R., Ferreira, S., Nicoletti Telis, V.R., 2013. Stability at different temperatures of turmeric oleoresin encapsulated in maltodextrin/gelatin matrices by freeze-drying. *J. Colloid Sci. Biotechnol.* 2, 100–105.
- Malam, Y., Loizidou, M., Seifalian, A.M., 2009. Liposomes and nanoparticles: nano-sized vehicles for drug delivery in cancer. *Trends Pharmacol. Sci.* 30, 592–599.
- Mao, H.-Q., Roy, K., Truong-Le, V.L., Janes, K.A., Lin, K.Y., Wang, Y., August, J.T., Leong, K.W., 2001. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. *J. Control. Release* 70, 399–421.
- Matsumura, Y., Hamaguchi, T., Ura, T., Muro, K., Yamada, Y., Shimada, Y., Shira, K., Okusaka, T., Ueno, H., Ikeda, M., et al., 2004. Phase I clinical trial and pharmacokinetic evaluation of NK911, a micelle-encapsulated doxorubicin. *Br. J. Cancer* 91, 1775–1781.
- Matsumo, Y., Iwata, H., Umeda, Y., Takagi, H., Mori, Y., Miyazaki, J., Kosugi, A., Hirose, H., 2003. Nonviral gene gun mediated transfer into the beating heart. *Asaio J. Am. Soc. Artif. Intern. Organs* 1992 (49), 641–644.
- Mehierhumbert, S., Guy, R., 2005. Physical methods for gene transfer: improving the kinetics of gene delivery into cells. *Adv. Drug Deliv. Rev.* 57, 733–753.
- Miao, C.H., Brayman, A.A., Loeb, K.R., Ye, P., Zhou, L., Mourad, P., Crum, L.A., 2005. Ultrasound enhances gene delivery of human factor IX plasmid. *Hum. Gene Ther.* 16, 893–905.
- Miller, A.D., 1992. Human gene therapy comes of age. *Nature* 357, 455–460.
- Miller, D.L., Song, J., 2003. Tumor growth reduction and DNA transfer by cavitation-enhanced high-intensity focused ultrasound in vivo. *Ultrasound Med. Biol.* 29, 887–893.
- Moffitt, M., Khougaz, K., Eisenberg, A., 1996. Micellization of anionic block copolymers. *Accounts Chem. Res.* 29, 95–102.
- Mohsen, M.M., 2011. Cationic liposomes as gene delivery system. *Afr. J. Pharm. Pharmacol.* 5.
- Muangmoonchai, R., Wong, S.C., Smirlis, D., Phillips, I.R., Shephard, E.A., 2002. Transfection of liver in vivo by biolistic particle delivery. *Mol. Biotechnol.* 20, 145–151.
- Müller-Reible, C.R., 1994. Principles, possibilities and limits of gene therapy. *Z. Für Kardiologie* 83 (Suppl. 6), 5–8.
- Munier, S., Messali, I., Delair, T., Verrier, B., Ataman-Onal, Y., 2005. Cationic PLA nanoparticles for DNA delivery: comparison of three surface polycations for DNA

Please cite this article in press as: Ibraheem, D., et al., Gene therapy and DNA delivery systems. *Int J Pharmaceut* (2013), <http://dx.doi.org/10.1016/j.ijpharm.2013.11.041>

- binding, protection and transfection properties. *Colloids Surf. B Biointerfaces* 43, 163–173.
- Munk, P., Prochazka, K., Tuzar, Z., Webber, S.E., 2013. Exploiting polymer micelle technology. *Chemtech* 28, 20–28.
- Mykhaylyk, O., Zelpathi, O., Hammerschmid, E., Anton, M., Rosenacker, J., Plank, C., 2009. Recent advances in magnetofection and its potential to deliver siRNAs in vitro. *Methods Mol. Biol. Clifton Nj* 487, 111–146.
- Nagasaki, T., Shinkai, S., 2007. The concept of molecular machinery is useful for design of stimuli-responsive gene delivery systems in the mammalian cell. *J. Incl. Phenom. Macrocyt. Chem.* 58, 205–219.
- Nam, H.Y., Park, J.H., Kim, K., Kwon, I.C., Jeong, S.Y., 2009. Lipid-based emulsion system as non-viral gene carriers. *Arch. Pharm. Res.* 32, 639–646.
- Negishi, T., Koizumi, F., Uchino, H., Kuroda, J., Kawaguchi, T., Naito, S., Matsumura, Y., 2006. NK105, a paclitaxel-incorporating micellar nanoparticle, is a more potent radiosensitizing agent compared to free paclitaxel. *Br. J. Cancer* 95, 601–606.
- Neumann, E., Schaefer-Ridder, M., Wang, Y., Hofschneider, P.H., 1982. Gene transfer into mouse lymphoma cells by electroporation in high electric fields. *Embo J.* 1, 841–845.
- Niidome, T., Huang, L., 2002. Gene therapy progress and prospects: nonviral vectors. *Gene Ther.* 9, 1647–1652.
- Nishiyama, N., Kataoka, K., 2004. Polymeric micelle drug carrier systems: PEG-PAsp(Dox) and second generation of micellar drugs. In: Maeda, H., Kabanov, A., Kataoka, K., Okano, T. (Eds.), *Polymer Drugs in the Clinical Stage*. Springer, US, pp. 155–177.
- Oster, C.G., Kim, N., Grode, L., Barbu-Tudoran, L., Schaper, A.K., Kaufmann, S.H.E., Kissel, T., 2005. Cationic microparticles consisting of poly(lactide-co-glycolide) and polyethylenimine as carriers systems for parental DNA vaccination. *J. Control. Release* 104, 359–377.
- Pantaro, D., Singh, R., McCarthy, D., Erhardt, M., Briand, J.-P., Prato, M., Kostarelos, K., Bianco, A., 2004. Functionalized carbon nanotubes for plasmid DNA gene delivery. *Angew. Chem.* 116, 5354–5358.
- Panyam, J., Labhasetwar, V., 2012. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv. Drug Deliv. Rev.* 64 (Suppl.), 61–71.
- Papahadjopoulos, D., New York Academy of Sciences, 1978. *Liposomes and Their Uses in Biology and Medicine*. New York Academy of Sciences.
- Pedroso de Lima, M.C., Simões, S., Pires, P., Faneça, H., Düzgünes, N., 2001. Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. *Adv. Drug Deliv. Rev.* 47, 277–294.
- Poletto, F.S., Fiel, L.A., Lopes, M.V., Schaab, G., Gomes, A.M.O., Guterres, S.S., Rossi-Bergmann, B., Pohlmann, A.R., 2012. Fluorescent-labeled poly(ϵ -caprolactone) lipid-core nanocapsules: synthesis, physicochemical properties and macrophage uptake. *J. Colloid Sci. Biotechnol.* 1, 89–98.
- Prijic, S., Sersa, G., 2011. Magnetic nanoparticles as targeted delivery systems in oncology. *Radiol. Oncol.* 45, 1–16.
- Prijic, S., Prosen, L., Cemazar, M., Scancar, J., Romih, R., Lavrencak, J., Bregar, V.B., Coer, A., Krzan, M., Znidarsic, A., et al., 2012. Surface modified magnetic nanoparticles for immuno-gene therapy of murine mammary adenocarcinoma. *Biomaterials* 33, 4379–4391.
- Rahman, M.M., Elaissari, A., 2012. Multi-stimuli responsive magnetic core-shell particles: synthesis, characterization and specific RNA recognition. *J. Colloid Sci. Biotechnol.* 1, 3–15.
- Ratko, T.A., Cummings, J.P., Blebea, J., Matuszewski, K.A., 2003. Clinical gene therapy for nonmalignant disease. *Ann. N. Y. Acad. Sci.* 1000, 560–569.
- Rochlitz, C.F., 2001. Gene therapy of cancer. *Swiss Med. Wkly.* 131, 4–9.
- Roemer, K., Friedmann, T., 1992. Concepts and strategies for human gene therapy. *Eur. J. Biochem.* 208, 211–225.
- Rols, M.P., Delteil, C., Goltz, M., Dumond, P., Cros, S., Teissie, J., 1998. In vivo electrically mediated protein and gene transfer in murine melanoma. *Nat. Biotechnol.* 16, 168–171.
- Ropert, C., 1999. Liposomes as a gene delivery system. *Braz. J. Med. Biol. Res.* 32, 207–219.
- Rosenberg, S.A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R.A., Moen, R., Carson, E.M., Lotze, M.T., Yang, J.C., Topalian, S.L., 1990. Gene transfer into humans—immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N. Engl. J. Med.* 323, 570–578.
- Rosset, V., Ahmed, N., Zaanoun, I., Stella, B., Fessi, H., Elaissari, A., 2012. Elaboration of argan oil nanocapsules containing naproxen for cosmetic and transdermal local application. *J. Colloid Sci. Biotechnol.* 1, 218–224.
- Roveimiyab, Z., Mahdavian, A.R., Biazar, E., Heidari, K.S., 2012. Preparation of magnetic chitosan nanocomposite particles and their susceptibility for cellular separation applications. *J. Colloid Sci. Biotechnol.* 1, 82–88.
- Roy, I., Mitra, S., Maitra, A., Mozumdar, S., 2003. Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery. *Int. J. Pharm.* 250, 25–33.
- Rudolph, C., Lousier, J., Naundorf, S., Müller, R.H., Rosenacker, J., 2000. In vivo gene delivery to the lung using polyethylenimine and fractured polyamidoamine dendrimers. *J. Gene Med.* 2, 269–278.
- Safinya, C.R., 2001. Structures of lipid-DNA complexes: supramolecular assembly and gene delivery. *Curr. Opin. Struct. Biol.* 11, 440–448.
- Sandhu, J.S., Keating, A., Hozumi, N., 1997. Human gene therapy. *Crit. Rev. Biotechnol.* 17, 307–326.
- Sato, H., Hattori, S., Kawamoto, S., Kudoh, I., Hayashi, A., Yamamoto, I., Yoshinari, M., Minami, M., Kanno, H., 2000. In vivo gene gun-mediated DNA delivery into rodent brain tissue. *Biochem. Biophys. Res. Commun.* 270, 163–170.
- Schaffert, D., Wagner, E., 2008. Gene therapy progress and prospects: synthetic polymer-based systems. *Gene Ther.* 15, 1131–1138.
- Schatzlein, A.G., 2001. Non-viral vectors in cancer gene therapy: principles and progress. *Anticancer Drugs* 12, 275–304.
- Scherer, F., Anton, M., Schillinger, U., Henke, J., Bergemann, C., Krüger, A., Gänzbacher, B., Plank, C., 2002. Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. *Gene Ther.* 9, 102–109.
- Sharma, A., Sharma, U.S., 1997. Liposomes in drug delivery: progress and limitations. *Int. J. Pharm.* 154, 123–140.
- Shchukin, D.G., Patel, A.A., Sukhorukov, G.B., Lvov, Y.M., 2004. Nanoassembly of biodegradable microcapsules for DNA encasing. *J. Am. Chem. Soc.* 126, 3374–3375.
- Singh, M., Briones, M., Ott, G., O'Hagan, D., 2000. Cationic microparticles: a potent delivery system for DNA vaccines. *Proc. Natl. Acad. Sci.* 97, 811–816.
- Smith, N.B., 2007. Perspectives on transdermal ultrasound mediated drug delivery. *Int. J. Nanomed.* 2, 585–594.
- Somia, N., Verma, I.M., 2000. Gene therapy: trials and tribulations. *Nat. Rev. Genet.* 1, 91–99.
- Soppimath, K.S., Aminabhavi, T.M., Kulkarni, A.R., Ruzinski, W.E., 2001. Biodegradable polymeric nanoparticles as drug delivery devices. *J. Control. Release* 70, 1–20.
- Stemberg, B., 1996. Morphology of cationic liposome/DNA complexes in relation to their chemical composition. *J. Liposome Res.* 6, 515–533.
- Stribley, J.M., Rehman, K.S., Niu, H., Christman, G.M., 2002. Gene therapy and reproductive medicine. *Fertil. Steril.* 77, 645–657.
- Suda, T., Liu, D., 2007. Hydrodynamic gene delivery: its principles and applications. *Mol. Ther.* 15, 2063–2069.
- Sukhorukov, G.B., Donath, E., Lichtenfeld, H., Knippel, E., Knippel, M., Budde, A., Möhwald, H., 1998. Layer-by-layer self-assembly of polyelectrolytes on colloidal particles. *Colloids Surfaces Physicochem. Eng. Asp.* 137, 253–266.
- Suzuki, T., Shin, B.C., Fujikura, K., Matsuzaki, T., Takata, K., 1998. Direct gene transfer into rat liver cells by in vivo electroporation. *FEBS Lett.* 425, 436–440.
- Talmadge, J.E., 1993. The pharmacokinetics and delivery of therapeutic polypeptides and proteins. *Adv. Drug Deliv. Rev.* 10, 247–299.
- Tan, W., Wang, K., He, X., Zhao, X.J., Drake, T., Wang, L., Bagwe, R.P., 2004. Bionanotechnology based on silica nanoparticles. *Med. Res. Rev.* 24, 621–638.
- Taniyama, Y., Tachibana, K., Hiraoka, K., Namba, T., Yamasaki, K., Hashiya, N., Aoki, M., Ogihara, T., Yasufumi, K., Morishita, R., 2002. Local delivery of plasmid DNA into rat carotid artery using ultrasound. *Circulation* 105, 1233–1239.
- Templeton, N.S., 2002. Cationic liposome-mediated gene delivery in vivo. *Biosci. Rep.* 22, 283–295.
- Templeton, N.S., Lasic, D.D., Frederik, P.M., Strey, H.H., Roberts, D.D., Pavlakis, G.N., 1997. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat. Biotechnol.* 15, 647–652.
- Tong, S.Y., Pelet, J.M., Putnam, D., 2007. Polymer systems for gene delivery—past, present, and future. *Prog. Polym. Sci.* 32, 799–837.
- Torchilin, V.P., 1996. Liposomes as delivery agents for medical imaging. *Mol. Med. Today* 2, 242–249.
- Truong-Le, V.L., August, J.T., Leong, K.W., 1998. Controlled gene delivery by DNA-gelatin nanospheres. *Hum. Gene Ther.* 9, 1709–1717.
- Tsumoto, K., Nomura, S.M., Nakatani, Y., Yoshikawa, K., 2001. Giant liposome as a biochemical reactor: transcription of DNA and transportation by laser tweezers. *Langmuir* 17, 7225–7228.
- Uchida, M., Natsume, H., Kobayashi, D., Sugibayashi, K., Morimoto, Y., 2002. Effects of particle size, helium gas pressure and microparticle dose on the plasma concentration of indomethacin after bombardment of indomethacin-loaded poly-L-lactic acid microspheres using a Helios™ gun system. *Biol. Pharm. Bull.* 25, 690–693.
- Vert, M., Mauduit, J., Li, S., 1994. Biodegradation of PLA/GA polymers: increasing complexity. *Biomaterials* 15, 1209–1213.
- Villemejeane, J., Mir, L.M., 2009. Physical methods of nucleic acid transfer: general concepts and applications. *Br. J. Pharmacol.* 157, 207–219.
- Vinogradov, S.V., Bronich, T.K., Kabanov, A.V., 1998. Self-assembly of polyamine-poly(ethylene glycol) copolymers with phosphorothioate oligonucleotides. *Bioconjug. Chem.* 9, 805–812.
- Vonarboug, A., Passirani, C., Desigaux, L., Allard, E., Saulnier, P., Lambert, O., Benoit, J.-P., Pitard, B., 2009. The encapsulation of DNA molecules within biomimetic lipid nanocapsules. *Biomaterials* 30, 3197–3204.
- Walther, W., Stein, U., 2000. Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs* 60, 249–271.
- Wang, D., Robinson, D.R., Kwon, G.S., Samuel, J., 1999. Encapsulation of plasmid DNA in biodegradable poly(D,L-lactide-co-glycolic acid) microspheres as a novel approach for immunogene delivery. *J. Control. Release* 57, 9–18.
- Wang, L., Zhao, W., Tan, W., 2008. Bioconjugated silica nanoparticles: development and applications. *Nano Res.* 1, 99–115.
- Wang, X., Liang, H.-D., Dong, B., Lu, Q.-L., Blomley, M.J.K., 2005. Gene transfer with microbubble ultrasound and plasmid DNA into skeletal muscle of mice: comparison between commercially available microbubble contrast agents. *Radiology* 237, 224–229.
- Watkins, C., Hopkins, J., Harkiss, G., 2005. Reporter gene expression in dendritic cells after gene gun administration of plasmid DNA. *Vaccine* 23, 4247–4256.
- Watson, J.D., Crick, F.H., 1953. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* 171, 737–738.
- Williams, R.S., Johnston, S.A., Riedy, M., DeVit, M.J., McElligott, S.G., Sanford, J.C., 1991. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. *Proc. Natl. Acad. Sci.* 88, 2726–2730.
- Wolff, J.A., Lederberg, J., 1994. An early history of gene transfer and therapy. *Hum. Gene Ther.* 5, 469–480.

Please cite this article in press as: Ibraheem, D., et al., Gene therapy and DNA delivery systems. *Int J Pharmaceut* (2013), <http://dx.doi.org/10.1016/j.ijpharm.2013.11.041>

- 1185 Wu, G.Y., Wu, C.H., 1988. Receptor-mediated gene delivery and expression in vivo. 1203
1186 J. Biol. Chem. 263, 14621-14624. 1204
- 1187 Xu, X., Costa, A., Burgess, D.J., 2012. Protein encapsulation in unilamellar liposomes: 1205
1188 high encapsulation efficiency and a novel technique to assess lipid-protein inter- 1206
1189 action. Pharm. Res. 29, 1919-1931. 1207
- 1190 Yang, F., Jin, C., Jiang, Y., Li, J., Di, Y., Ni, Q., Fu, D., 2011. Liposome based delivery 1208
1191 systems in pancreatic cancer treatment: from bench to bedside. Cancer Treat. 1209
1192 Rev. 37, 633-642. 1210
- 1193 Yang, N.S., Burkholder, J., Roberts, B., Martinell, B., McCabe, D., 1990. In vivo and 1211
1194 in vitro gene transfer to mammalian somatic cells by particle bombardment. 1212
1195 Proc. Natl. Acad. Sci. 87, 9568-9572. 1213
- 1196 Yokoyama, M., 2011. Clinical applications of polymeric micelle carrier systems 1214
1197 in chemotherapy and image diagnosis of solid tumors. J. Exp. Clin. Med. 3, 1215
1198 151-158. 1216
- 1199 Young, S., Wong, M., Tabata, Y., Mikos, A.G., 2005. Gelatin as a delivery vehi- 1217
1200 cle for the controlled release of bioactive molecules. J. Control. Release 109, 1218
1201 256-274. 1219
- 1202 Yu, M., Poeschla, E., Wong-Staal, F., 1994. Progress towards gene therapy for HIV 1220
infection. Gene Ther. 1, 13-26. 1221
- Yuan, Y., Tan, J., Wang, Y., Qian, C., Zhang, M., 2009. Chitosan nanoparticles as 1203
non-viral gene delivery vehicles based on atomic force microscopy study. Acta 1204
Biochim. Biophys. Sin. 41, 515-526. 1205
- Zelikin, A.N., Li, Q., Caruso, F., 2006. Degradable polyelectrolyte capsules filled with 1206
oligonucleotide sequences. Angew. Chem. Int. Ed. 45, 7743-7745. 1207
- Zelikin, A.N., Becker, A.L., Johnston, A.P.R., Wark, K.L., Turatti, F., Caruso, F., 2007. A 1208
general approach for DNA encapsulation in degradable polymer microcapsules. 1209
ACS Nano 1, 63-69. 1210
- Zhang, G., Selzer, M.E., 2001. In vivo transfection of lamprey brain neurons by gene 1211
gun delivery of DNA. Exp. Neurol. 167, 304-311. 1212
- Zhang, G., Budker, V., Wolff, J.A., 1999. High levels of foreign gene expression in 1213
hepatocytes after tail vein injections of naked plasmid DNA. Hum. Gene Ther. 1214
10, 1735-1737. 1215
- Zhang, G., Gao, X., Song, Y.K., Vollmer, R., Stolz, D.B., Gasiorowski, J.Z., Dean, D.A., 1216
Liu, D., 2004a. Hydroporation as the mechanism of hydrodynamic delivery. Gene 1217
Ther. 11, 675-682. 1218
- Zhang, S., Xu, Y., Wang, B., Qiao, W., Liu, D., Li, Z., 2004b. Cationic compounds used 1219
in lipoplexes and polyplexes for gene delivery. J. Control. Release Off. J. Control. 1220
Release Soc. 100, 165-180. 1221

Please cite this article in press as: Ibraheem, D., et al., Gene therapy and DNA delivery systems. Int J Pharmaceut (2013), <http://dx.doi.org/10.1016/j.ijpharm.2013.11.041>

2.3. Protéines et peptides pour applications in vivo

La protéine est une partie vitale du corps humain puisqu'elle est directement impliquée dans toutes les étapes importantes des processus biologiques et physiologiques. Récemment, les protéines et les peptides ont fait l'objet d'une attention particulière comme actif pour des traitements potentiels de plusieurs maladies dangereuses traditionnellement incurables comme le cancer, le sida, les maladies auto-immunes, et autres. En plus, les protéines peuvent être utilisées dans le domaine de diagnostique.

Actuellement, la plupart des protéines thérapeutiques sont administrées par voie parentérale ce qui provoque de nombreux inconvénients (douleur, coût, toxicité...). Trouver des voies d'administration plus efficaces, plus faciles et plus sûrs pour les protéines et les peptides est la clé du succès de l'immunothérapie. Dans ce but, de nombreuses recherches ont été concentrées sur les voies d'administration non-invasives telles que la voie nasale, pulmonaire, orale, oculaire, et rectale.

Malheureusement, une large utilisation des protéines et des peptides comme médicaments est encore confrontée à de nombreux obstacles tels que la faible biodisponibilité, la demi-vie courte dans le sang, l'instabilité dans le milieu biologique et de nombreux autres problèmes. Afin de surmonter ces problèmes et d'améliorer l'efficacité du médicament protéique, diverses stratégies ont été développées telles que l'amélioration de la perméabilité, les enzymes de l'inhibition, et la modification de la structure de la protéine ou de la protection par encapsulation.

Cette revue illustre modestement tous les points précédents, afin de mettre en lumière l'utilisation des protéines et des peptides comme actif moléculaire et médicament et également dans l'immunothérapie.

Proteins and peptides for *in vivo* applications

D. Ibraheem, A. Elaissari, H. Fessi*

University of Lyon, F-69622, Lyon, France; Université Lyon-1, Villeurbanne; CNRS, UMR 5007, LAGEP- CPE; 43 bd 11 Novembre 1918, F-69622 Villeurbanne, France

Correspondance to: Fessi@lagep.univ-lyon1.fr

Abstract

Proteins are a vital constituent of the body as they perform many of its major physiological and biological processes. Recently, proteins and peptides have attracted much attention as potential treatments for various dangerous and traditionally incurable diseases such as cancer, AIDS, dwarfism and autoimmune disorders. Furthermore, proteins could be used for diagnostics.

At present, most therapeutic proteins are administered via parenteral routes that have many drawbacks, for example, they are painful, expensive and may cause toxicity. Finding more effective, easier and safer alternative routes for administering proteins and peptides is the key to therapeutic and commercial success. In this context, much research has been focused on non-invasive routes such as nasal, pulmonary, oral, ocular, and rectal for administering proteins and peptides.

Unfortunately, the widespread use of proteins and peptides as drugs is still faced by many obstacles such as low bioavailability, short half-life in the blood stream, *in vivo* instability and numerous other problems. In order to overcome these problems and improve protein drug efficacy, various strategies have been developed such as permeability enhancement, enzyme inhibition, protein structure modification and protection by encapsulation.

This review provides a detailed description of all the previous points in order to highlight the importance and potential of proteins and peptides as drugs.

1. Introduction

The revolution of biotechnology has led to the creation of various types of therapeutic proteins (Brown, 2005)(Xie et al., 2008)(Almeida and Souto, 2007)(Tan and Danquah, 2012)(Stevenson et al., 2012)(Ye et al., 2010)(Torchilin and Lukyanov, 2003), with the potential to provide treatment for certain dangerous diseases that have long been thought incurable (Brown, 2005). Biotherapy now has the potential to target chronic and malignant diseases such as cancer (Dougan and Dranoff, 2012)(Jaffee, 1999)(Chen et al., 2013)(Rossi et al., 2013a), dwarfism

(Matiasevic and Gershberg, 1966), AIDS (Tomasselli and Henrikson, 2000), autoimmune disorders (Almeida and Souto, 2007), etc. Moreover, proteins can be used to detect and diagnose diseases (Gelfand, 2001)(Hj et al., 1983) in addition to using proteins as vaccines in order to ensure protection from various diseases (Rossi et al., 2013b)(Chura-Chambi et al.).

The chemical structure of proteins allows them to perform specific reactions in the body, increasing efficacy and decreasing undesirable side effects (Russell and Clarke, 1999)(Morishita and Peppas, 2006)(Moeller and Jorgensen, 2008). However, employing proteins for therapeutic purposes is confronted by many obstacles such as their short half-life in the bloodstream, making it necessary to repeat administrations (Putney and Burke, 1998)(Putney, 1998), their chemical and physical instability (Bilati et al., 2005), their rapid denaturation in the stomach and intestinal environment, and their retention by the impermeable mucosal tissues in the intestine that hinder oral protein administration (Lee, 2002)(Pettit and Gombotz, 1998).

Recently, recombinant proteins have been developed and prepared on a large scale, and they play a dominant role in improving protein-based therapy (Overton). Somatostatin was the first recombinant human protein fabricated by Genentech in 1977 (Itakura et al., 1977), whereas human insulin was the first recombinant protein to be marketed by Genentech (Buckel, 1996). Since then various recombinant proteins have been prepared using different methods. Most therapeutic proteins are prepared either by using mammalian cells (especially Chinese hamster ovary cells CHO), or *Escherichia coli* (Chu and Robinson, 2001)(Swartz, 2001)(Russell and Clarke, 1999).

Synthesizing recombinant proteins could solve many problems that have hindered wider clinical applications for therapeutic proteins (Morishita and Peppas, 2006), making it possible to prepare proteins of interest in sufficient quantity, and avoiding the presence of human pathogenic viruses (Buckel, 1996)(Swiech et al., 2012). However, many drawbacks restrict the use of recombinant proteins *in vivo*, such as their instability and incapacity to reach intracellular targets (Russell and Clarke, 1999).

Various techniques have been developed to improve the physicochemical properties of proteins, protect them in biological media and deliver them to their target (Torchilin and Lukyanov, 2003). In this review we illustrate the techniques that have been used to achieve the objectives described above, such as protein structure modification, using special adjuvants that can enhance protein absorption, inhibit protein degradation, or using different protein encapsulation techniques.

This review also highlights the potential therapeutic applications of proteins and peptides and the obstacles that limit their widespread clinical use. It presents a full description of the protein administration routes most frequently used.

2. Potential uses of proteins in the medical field

The development achieved in the field of biotechnology, in addition to better understanding of diseases and pathogenesis, has opened new horizons for using proteins to prevent and treat of various kinds of dangerous diseases (Drews, 2000) (Russell and Clarke, 1999).

At present, medical biotechnology has proved its efficacy in treating and discovering many diseases and disorders, as shown in Fig.1.

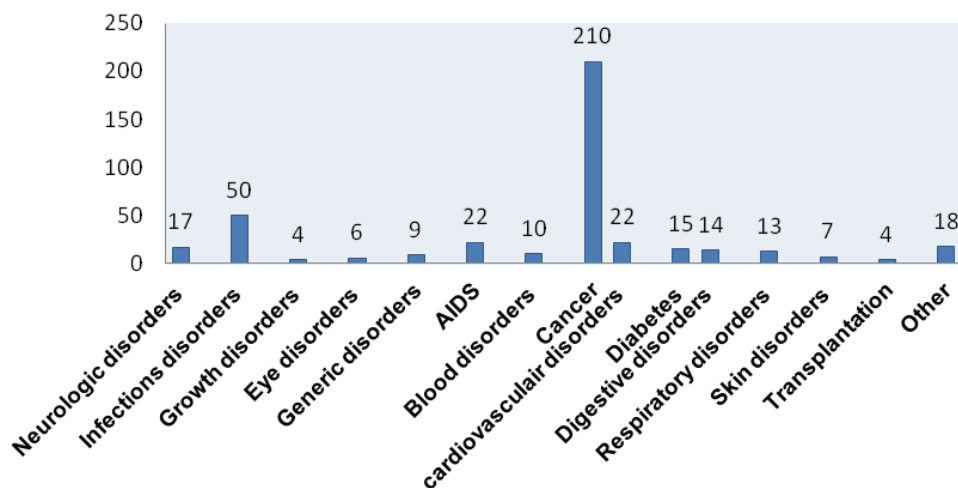


Fig.1. Medical biotechnology in development by therapeutic category (Almeida and Souto, 2007)

In the following paragraph we highlight some of the therapeutic applications of proteins. Since the advent of monoclonal antibodies in 1975 (Köhler and Milstein, 2005), many diagnostic and therapeutic antibodies have found their way to clinical applications after being approved by the Food and Drug Administration (FDA). Examples of these applications are the detection of rheumatoid arthritis (Larsen, 1991)(Vasiliauskiene et al., 2001)(Agrawal et al., 2007), the treatment of non-Hodgkin's lymphoma (Fanale and Younes, 2007), and protection against respiratory syncytial virus infection (Weltzin et al., 1994) (Taylor et al., 1984)(Walsh et al., 1984).

The recombinant soluble form of glycoprotein CD4 (sCD4) has been used to treat AIDS, with promising results (Schooley et al., 1990) (Deen et al., 1988). The growth hormone (GH) is another therapeutic protein that has long been employed since for treating growth disorders. GH was extracted on a small scale from cadaveric pituitaries in the late 1950s and early 1960s

(Grumbach et al., 1998), though recombinant GH has been available in sufficient amounts since 1985 (Bioethics, 1997). In some cases, the growth response obtained using GH is satisfactory (Wacharasindhu et al., 2007)(Hintz et al., 1999). Human insulin, now produced by genetic engineering techniques, saves the lives of millions of diabetics around the world (2002a)(Katsilambros et al., 2006). Work to develop use the immune system to fight cancer has been carried out for more than a century (Kumar and Mason, 2012), and it plays a key role in cancer therapy. The principle of cancer immunotherapy is to provoke the patient's immune system to recognize and attack malignant tumor cells quickly enough (Durrant and Scholefield, 2003)(Kumar and Mason, 2012).

3. The challenges and obstacles confronting the use of protein drugs

Drug efficacy, which reflects the ability of a drug to produce the desired therapeutic effect, is determined by the drug's capacity to reach the target of interest in a therapeutic concentration (Posner, 2012).

Obtaining sufficient therapeutic response to protein drugs is hindered by many obstacles. A protein is a complicated hydrophilic macromolecule. These physicochemical protein-characteristics complicate its transit through biological membranes (Zuzana Antosova, 2009)(Sinha and Trehan, 2003)(Salmaso and Caliceti, 2011). Proteins are easily degraded by the proteases of the gastrointestinal tract, hindering protein drug administration via the oral route (Morishita and Peppas, 2006)(Sinha and Trehan, 2003). The biological half life of proteins in the blood is short due to clearance from the bloodstream, making it necessary to repeat administration in order to maintain therapeutic levels in the blood (Sarciaux et al., 1995)(Zuzana Antosova, 2009). Furthermore, using protein as a drug may trigger dangerous immune response (Sauerborn et al., 2010).

Therefore, efforts of scientists focus on improving protein properties by exploring ways to protect proteins from the effects of enzymes in the biological environment, and thus prolong their *in vivo* half-life, increase absorption and decrease metabolic rate.

4. Protein administration routes

4.1. Parenteral routes

Until recently, therapeutic proteins and peptides were delivered by subcutaneous (SC), intramuscular (IM), and intravenous (IV) injections (Cleland et al., 2001)(Almeida and Souto, 2007)(Siddiqui and Chien, 1987)(Shantha Kumar et al., 2006). However, using intravenous injection for protein administration raises many problems as it is painful and poorly tolerated by patients. In addition, therapeutic proteins are rapidly cleared from the blood stream making it

necessary for patients to take repeated and high doses of drugs which may lead to toxic effects (Almeida and Souto, 2007)(Ye et al., 2000). Protein administration via subcutaneous injection may lead to bioavailability up to 100%, though this may be much lower depending on many factors, such as drug molecular weight, injection site, muscular activity and pathological conditions (Crommelin et al., 2003). Generally, intramuscular (IM), subcutaneous (SC) and intravenous (IV) injections are uncomfortable for patients; moreover they are expensive and may cause toxic and undesirable side effects. Consequently, scientists have focused their efforts to find more effective, easier and safer alternative routes for protein and peptide administration, thus non-invasive protein administration routes have emerged.

4.2. Non-invasive administration routes

The route of administration of a drug has a significant impact on its therapeutic result (Benet, 1978). Hence protein and peptide drug delivery routes have been the subject of intensive research in order to find patient-friendly, painless and effective methods of administration. These routes that are known as non-invasive and will be explained in the following paragraphs:

4.2.1. Nasal route of protein administration

Nasal drug delivery may be used for both local and systemic effects. It has recently gained importance as a non-invasive drug administration route due to the various advantages it provides:

- I. The anatomical structure of the nose helps in increasing protein bioavailability (Zheng et al., 2013)(Kissel and Werner, 1998)(Ridley et al.):
 - The large number of microvilli that cover the nose-epithelial surface creates a wide surface area of nasal mucosa. This means a wider surface area available for protein absorption.
 - The thin porous endothelial basement membrane of the nasal epithelium facilitates and accelerates drug absorption.
 - Due to the highly vascularized nasal epithelium, the blood of the nose venous system passes directly into the systematic circulation without passing by the liver; consequently, the hepatic first pass metabolism is avoided.
- II. Protein administration by the nasal route saves avoids degradation by the enzymes of gastrointestinal tract (Zheng et al., 2013).

III. The nasal protein delivery route is painless and requires no special instruments, so it is easy for the patient to administer the drug alone (Mygind and Dahl, 1998)(Ritthidej, 2011).

Insulin is a good example of proteins that have been studied and prepared for the nasal administration route (Orive et al., 2003)(Khafagy et al., 2007)(Sintov et al., 2010). This route for insulin administration has proved to be highly efficacious. Furthermore, the pharmacokinetics of the intranasal insulin delivery route resembles the pulsatile pattern of endogenous insulin secretion giving it an advantage over traditional subcutaneous administration (Hinchcliffe and Illum, 1999)(Duan and Mao, 2010).

However, the nasal drug administration route is limited by various drawbacks (Illum, 2003)(Ritthidej, 2011):

- The physical barrier of the nasal epithelium hinders the absorption of drug molecules, especially if they are large hydrophilic molecules such as proteins and peptides.
- Rapid mucociliary clearance that shortens the available time for drug absorption.
- The relatively small amount of drug that can be administered via the nasal route.

4.2.2. Oral route of protein administration

It has been mentioned in the literature that the oral bioavailability of protein drugs is normally less than 1-2% (Pauletti et al., 1996)(Carino and Mathiowitz, 1999). This fact is essentially due to protein degradation by the enzymes of the gastrointestinal tract, in addition to poor penetration of proteins through the intestinal membrane (Hamman et al., 2005)(Mahato et al., 2003)(Moeller and Jorgensen, 2008) owing to the large particle size of proteins and their negative charges. In spite of the problems that have to be solved, oral protein administration, is still an interesting and attractive alternative to traditional parenteral protein delivery, because it is a painless and very easy delivery route (des Rieux et al., 2006). However, achieving successful and satisfactory therapeutic results by oral protein delivery requires overcoming the challenges mentioned earlier, by increasing the permeability of the intestinal epithelial membrane, by inhibiting the protein degrading enzymes or by protecting therapeutic proteins by encapsulation (Li et al., 2013)(des Rieux et al., 2006)(Park et al., 2011)(Krishnankutty et al., 2009). Indeed, when the drug of interest must be used repeatedly and constantly, such as insulin, the oral route is considered the route of choice for patients. For this reason, insulin is foremost among the peptides that have

gained much attention in terms of developing an oral form (Krishnankutty et al., 2009) (Mukhopadhyay et al., 2013) (Chaturvedi et al., 2013).

4.2.3. Pulmonary route of protein administration

The lungs provide advantages for drug administration and could represent a promising protein delivery route for both local and systemic treatment (Adjei and Gupta, 1994)(Patton and Platz, 1992).

The lungs have a wide surface area (about 100m²) enabling them to absorb macromolecular drugs such as proteins and peptides (Kwon et al., 2007)(Wan et al., 2012). The thin alveolar epithelium (thickness 0.1-0.5µm (Wan et al., 2012)) enhances the permeability of the alveoli region membrane (Labiris and Dolovich, 2003)(Patton, 1996). Furthermore, pulmonary drug administration spares it from metabolic modification by avoiding the hepatic first-pass (Kwon et al., 2007). The low enzymatic activity in the lungs limits the metabolism of pulmonary administered proteins and peptides (Kwon et al., 2007)(Wan et al., 2012)(Codrons et al., 2003). In spite of all these advantages, the pulmonary delivery of protein drugs is still hindered by many obstacles, such as the barriers (epithelial lining fluid, epithelial cell layer and the endothelial membrane of capillary cells) that obstruct the passage of proteins and peptides through the alveoli to the general circulation (Wan et al., 2012). The proteins and peptides may also be subjected to phagocytosis by the macrophages in the lungs (Patton and Byron, 2007).

4.2.4. Ocular route of protein administration

Due to its unique characteristics, the eye is considered to be an effective drug delivery route (Vyas et al., 2011). The ocular route of protein delivery presents many advantages as it is easier and faster than traditional injection routes (Khafagy et al., 2007). Ocular protein drug administration protects proteins by avoiding gastrointestinal and hepatic first-pass metabolism which leads to the low bioavailability of proteins and peptides administered through the oral route (Lee et al., 2002)(Khafagy et al., 2007). However, many drawbacks limit the widespread use of ocular protein delivery route, such as low bioavailability due to poor eye membrane permeability regarding hydrophilic macromolecules like proteins (Vyas et al., 2011) In addition, the ocular tissue contains many enzymes (e.g., protease and aminopeptidase) that can degrade the proteins and peptides administered (Zhou and Po, 1991)(Davson, 1984). Although, the ocular

route could be used in order to deliver protein drugs for local and systemic treatment, it is not the preferred route for systemic protein administration (Vyas et al., 2011). In spite of the early promising results which have been obtained when insulin was administered via the ocular route(Christie and Hanzal, 1931)(Chiou, 1994)(Bartlett et al., 1994), wider use of this route for protein and peptide delivery needs more research and improvement.

4.2.5. Rectal route of protein administration

The rectal route has been used for both local and systematic treatment (Boer et al., 1982)(Mackay et al., 1997) and it could be a route of considerable importance for protein administration (De Boer et al., 1990), because protein drugs administrated rectally avoid first-pass elimination (de Boer et al., 1980). However, it is still limited by several drawbacks like poor protein and peptide absorption through the rectal epithelium, leading to low bioavailability (De Boer et al., 1990); moreover, many patients consider it distasteful (Yamamoto and Muranishi, 1997). Hence, it is very important to improve rectal absorption of proteins and peptides by using compounds that increase the permeability of the rectal epithelium (absorption enhancers), or decrease the enzymatic degradation of rectally administrated proteins (protease inhibitors) (Yamamoto and Muranishi, 1997)(De Boer et al., 1990).

5. Strategies used to improve the bioavailability of proteins and peptides

As mentioned previously, wider and successful use of proteins and peptides as drugs are faced by many obstacles and challenges such as protein *in vivo* instability, poor absorption, and short half-life.

Many strategies have been developed to overcome these challenges and to improve protein drug efficacy, such as permeability enhancement, enzyme inhibition, and protein structure modification and protection (Torchilin and Lukyanov, 2003) (Wearley, 1991) (Lee and Yamamoto, 1989) (Xin Hua Zhou, 1994). The following section will provide an overview of these strategies with a detailed explanation of the latter.

5.1. Penetration enhancers

Penetration enhancers can be identified as substances that are usually used to facilitate the passage of a pharmacologically active quantity of protein or peptide through the mucosal membranes (Lee, 1990) (Lee and Yamamoto, 1989).

Penetration enhancers are numerous and can be classified into five categories (Lee, 1990):

1. Chelators such as EDTA, citric acid, salicylates, N-acyl derivatives of collagen, and enamines (N-amino acyl derivatives of P-diketones).
2. Surfactants such as sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether.
3. Bile salts such as sodium deoxycholate, sodium glycocholate and sodium taurocholate, and their derivatives like sodium taurodihydrofusidate and sodium glycodihydrofusidate.
4. Fatty acids such as oleic acid, caprylic acid and capric acid, and their derivatives such as acylcarnitines, acylcholines and mono- and diglycerides.
5. Non-surfactants such as unsaturated cyclic ureas and 1-alkyl- and lalkenylazacycloalkanone derivatives.

The mechanism of penetration enhancement remains unclear. However, the literature mentions various mechanisms that point to three major orientations (Zhou and Li Wan Po, 1991)(Lee, 1990)(Lee and Yamamoto, 1989):

- Reducing the barrier functions of mucosal membranes by changing the structure or properties of these membranes;
- Changing the thermodynamic activity of proteins and peptides;
- Protecting proteins and peptides from proteolytic activity.

Most penetration enhancers function by causing a perturbation of membrane integrity (Lee, 1990)(Lee and Yamamoto, 1989). However, to date, there is not enough information about the relation between the efficacy of penetration enhancers and the degree of change of, or even damage to, membrane cells (Lee and Yamamoto, 1989). Using penetration enhancers may cause undetermined biochemical change in the site of effect. Practically, the effects of their chronic use on barrier functions are still obscure (Lee, 1990) as the the possible toxicity that may be caused by absorbing the penetration enhancers themselves (Lee and Yamamoto, 1989). There is lack of technical solutions to accelerate the repair of membrane damage (Lee, 1990).

Generally, the efficacy of penetration enhancers is determined by various factors such as the type and characteristics of the protein and penetration enhancer, the administration site and the delivery system (Lee and Yamamoto, 1989).

5.2. Enzymatic inhibitors

Whatever the route used to administer protein drugs, the enzymatic barrier is considered to be one of the dominant factors controlling the bioavailability of given proteins and peptides. This is

due to the high sensitivity of proteins and peptides to the different types of enzymes, and to the ubiquitous nature of these enzymes in the body(Xin Hua Zhou, 1994).

Many protease inhibitors have been investigated and used. In 1990, Lee reported some of the protease inhibitors that had been tested on the suppression of proteolytic activity (Lee, 1990):

- bacitracin (a non-specific protease inhibitor);
- 1,10-phenanthroline and phosphoramidon (metalloprotease inhibitors);
- pepstatylglutamic acid (an aspartylprotease inhibitor);
- p-hydroxymercuribenzoate (a cysteine proteinase inhibitor);
- diisopropyl fluorophosphate, aprotinin and soybean trypsin inhibitor (serine proteinase inhibitors);
- bestatin, puromycin and α -aminoboronic acid derivatives (aminopeptidase inhibitors).

A suitable protease inhibitor is chosen according to the type of protease of interest and the site of its distribution (Lee, 1990).

In fact long term use of enzymes inhibitors is not desirable as it may disrupt the protein absorption system and leads to the absorption of unwanted proteins, disturb the digestion of nutritive proteins, and incite the secretion of protease in the body as a result of feedback regulation (Salamat-Miller and Johnston, 2005).

5.3. Protein structure modification by polymer conjugation

Fast elimination of proteins from the blood stream and their degradation by enzymes limit their routine use as a drug.

Fast protein elimination may be due to the rapid renal clearance of proteins having a low molecular weight of 40kDa or less. Indeed, increasing the molecular weight of these therapeutic molecules to over 40kDa by conjugating them with water soluble polymers can effectively slow down renal clearance, thereby prolonging the survival of the drug in the bloodstream (Torchilin, 1991)(Harris and Chess, 2003)(Veronese and Mero, 2008)(Roberts et al., 2002).

The *in vivo* short half-life of proteins may result from the immune response mechanism. The combination of proteins and polymers may hinder opsonin and antigen processing cells from recognizing these proteins. Hence the expulsion of proteins from the general circulation by phagocytosis is prevented (Veronese and Mero, 2008)(Roberts et al., 2002).

Protein degradation by biological enzymes such as proteases is another cause of the short-half life of proteins. Polymers conjugation with proteins offers a hindrance to binding of protein to active sites of proteases thus preventing earlier protein degradation. (Torchilin, 2007)(Roberts et al., 2002).

Various polymers have been used for increasing protein stabilization. Foremost among these is poly (ethylene glycol) because it is biocompatible and inexpensive. Moreover, it has been approved for internal application by drug regulatory agencies (Roberts et al., 2012) (Roberts et al., 2002). Generally, the reaction of polyethylene glycol with proteins and peptides improves the properties of these biomolecules (Bailon and Won, 2009) (Torchilin and Lukyanov, 2003).

5.6. Protein encapsulation

Protecting proteins and peptides in polymeric reservoirs is an applicable and promising method for protein delivery (Xie et al., 2008). These particulate drug delivery systems provide several advantages, such as protecting the encapsulated protein against the effect of enzymes, and controlling the site and speed of protein release which help in avoiding undesirable side effects (Tan et al., 2010). Different techniques have been investigated and used for encapsulating therapeutic proteins. These approaches will be described in the next overview.

5.6.1. Liposomes

Liposomes are particulate protein carriers that have been studied extensively (Swaminathan and Ehrhardt, 2012). These delivery systems are characterized by properties that make them suitable vectors for transferring proteins and peptides: they are biocompatible, biologically inert, weakly immunogenic and possess limited toxicity (Torchilin and Lukyanov, 2003), in addition to their size (Akbarzadeh et al., 2013) and their aqueous core(Lee and Yuk, 2007) .

Liposomes are spherical vesicles consisting of an aqueous core entrapped by one or more lipid bilayers (Tan et al., 2010) (Lasic&dagger and Papahadjopoulos, 1998) (Sessa and Weissmann, 1968). They can be formed by various methods such as thin film hydration, reversed-phase evaporation, detergent dialysis, and solvent injection (Laouini et al., 2012). According to the preparation technique used, liposomes can be multi-, oligo- or unilamellar (Martins et al., 2007), with a size varying from several micrometers (when multilamellar) to 20nm (when unilamellar) (Yang and Alexandridis, 2000) (Walde and Ichikawa, 2001). In spite of the advantages presented

by liposomes, which make them promising drug delivery systems, they suffer from several disadvantages that limit them from being widely used for clinical application, such as their instability in biological media, leading to rapid and uncontrolled drug release, and short half-life due to the action of the reticuloendothelial system (RES) (Sharma and Sharma, 1997). In order to prolong the dwell time of liposomes in the bloodstream, their surface has been modified by grafting them with hydrophilic polymers such as poly ethylene glycol, Fig. 2. Covering the surface of liposomes with polymers hinders reaction with blood components and retards their degradation. Furthermore, it prevents the adsorption of plasma-proteins (opsonins) on the surface of liposomes, which helps liposomes to avoid recognition and uptake by the RES (Torchilin and Trubetskoy, 1995)(Lee and Yuk, 2007).

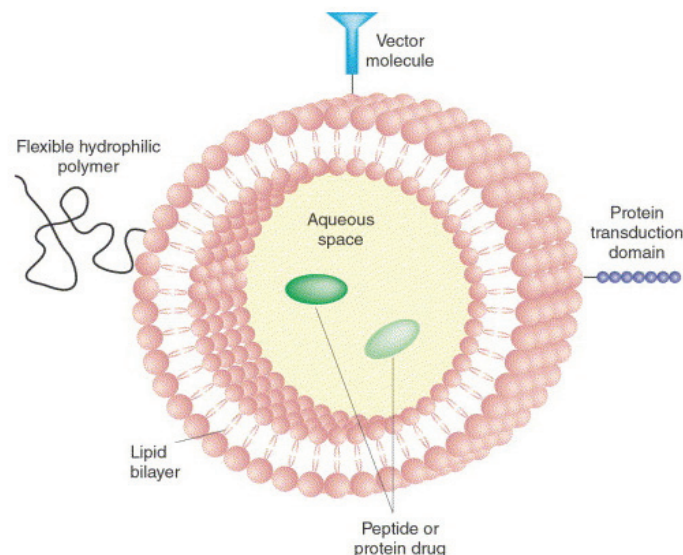


Fig. 2. Liposome grafted with hydrophilic polymers (poly(ethylene glycol) to increase its circulation half-life(Torchilin and Lukyanov, 2003). Many peptides and proteins have been associated with liposomes to improve their properties (stability, bioavailability), such as adamantyltripeptides, bovine serum albumin, insulin (Goto et al., 2006)(Zhang et al., 2005), human gamma-globulin (García-Santana et al., 2006).

5.6.2. Double emulsion

Double emulsions were described for the first time in 1925 by William Seifriz (Florence and Whitehill, 1982)(Garti, 1997)., This technology was used by Engel and his coworkers in the late 1960s for the encapsulation of insulin to better facilitate gastrointestinal absorption and enhance its efficiency when administered orally (Engel et al., 1968).

The encapsulation of proteins using the double emulsion /evaporation technique is performed in two stages (Tan and Danquah, 2012)(van der Graaf et al., 2005)(Nihant et al., 1994):

In the first stage the primary emulsion, i.e. water in oil (W/O), is prepared by adding the aqueous protein solution to the polymer organic solution under high shear conditions (ultrasonification or homogenization). Double emulsion (W/O/W) is produced during the second stage by dispersing, again by homogenization, the primary emulsion (W/O) in an external aqueous phase containing the chosen stabilizer. Protein-loaded particles are obtained after eliminating the organic solvent either by evaporation, or by extraction.

Although the double emulsion technique is considered a complex process, it has been widely used to encapsulate proteins in aqueous solution, resulting in high yields and encapsulation efficiencies (Sinha and Trehan, 2003).

This method is still limited by many drawbacks such as its sensitivity to the characteristics of polymers and difficulties in modifying the release rates of the drug from the particles obtained (Sinha and Trehan, 2003).

There are many examples of proteins which have been encapsulated using this method, such as bovine serum albumin (Yang et al., 2001), lysozyme (Nam et al., 2000), and recombinant human epidermal growth factor (Han et al., 2001).

5.6.3. Solid lipid nanoparticles (SLN)

Solid lipid nanoparticles (SLN) were synthesized for the first time by Muller et al in 1991 (Müller, 1991). Due to the considerable advantages they present, SLNs have attracted growing attention in recent years as promising alternatives to traditional colloidal carriers (Mehnert and Mäder, 2001) (Cavalli et al., 1997)(Yang et al., 1999)(Müller et al., 1996). SLNs combine the advantages of other carrier systems (polymeric nanoparticles, fat emulsions, and liposomes) while simultaneously minimizing the associated problems (Yang et al., 1999)(Müller et al., 1997). They are also non toxic drug carriers (Mehnert and Mäder, 2001)(Müller et al., 1997) that can efficiently control the release of the drug incorporated in them (Mehnert and Mäder, 2001) (Yang et al., 1999). Both hydrophilic and hydrophobic drugs can be incorporated in SLNs (Wissing et al., 2004) which are prepared without using organic solvents that may be harmful for the drug of interest (Almeida and Souto, 2007)(Mehnert and Mäder, 2001). Also, they can be easily produced on a large scale (Mehnert and Mäder, 2001) (Müller et al., 1997). The colloidal dimensions and controlled release properties of SLNs aid in achieving not only drug protection

but also permit their use in parenteral and non-parenteral (oral, dermal, ocular, pulmonary, rectal) drug administration routes (Yang et al., 1999) (Wissing et al., 2004) (Wissing and Müller, 2002) (Cavalli et al., 2002)(Jenning et al., 2000) (Sznitowska et al., 2001) (Videira et al., 2002).

The literature has been reported three different models for loading protein drugs into SLNs:

I) High pressure homogenization (HPH)

This can be performed either at high temperature (hot HPH) or at, or below, room temperature (cold HPH) (Müller et al., 2002) (Miglietta et al., 2000) (Fundarò et al., 2000). In the hot HPH technique, the bioactive ingredient is added to the molten lipid (heated at 5-10C° above its melting point). Then a hot pre-emulsion is produced by using high-speed stirring. The pre-emulsion obtained is transformed into a nanoemulsion by high pressure homogenization (usually 3-5 homogenization cycles at 500-1500 bar). The nanoemulsion is then cooled to room temperature, resulting in the formation of bioactive loaded SLNs (Rudolph et al., 2004). Using high temperature results in particles of small size due to the reduction of inner phase viscosity (Lander et al., 2000), but it can damage the drug to be encapsulated (Mehnert and Mäder, 2001). However, with the cold HPH process, the active moiety and lipid are first melted together and then quickly cooled (using either liquid nitrogen or dry ice) to produce solid lipid particles. The particles obtained are then dispersed in a cold solution of emulsifier to prepare the pre-emulsion that undergoes high pressure homogenization at, or below, room temperature (usually 5 cycles at 500-800 bars) (Almeida et al., 1997) (Mehnert and Mäder, 2001). Cold HPH minimises exposing the sample to high temperature. However, the particles obtained are larger and have a wider size distribution compared with particles prepared by hot HPH (Mehnert and Mäder, 2001).

III) Solvent diffusion method

Lipid is dissolved in an organic solvent (acetone or ethanol), then an acidic aqueous phase is added to control the zeta potential necessary to induce the coacervation of the lipid, leading to the formation of SLNs. Finally, the resulting particles are separated by centrifugation (Hu et al., 2002).

III) Double emulsion evaporation method

SLNs can be prepared by the double emulsion evaporation method by dissolving the hydrophilic bioactive ingredient in an aqueous phase, then emulsifying it in an organic phase containing biodegradable polymer under high speed stirring to prepare a W/O emulsion which will be poured into another aqueous phase containing an emulsifier. Finally, the organic solvent is eliminated from the particles (Garcia-Fuentes et al., 2005)(Zhang et al., 2006a). This method avoids exposing the sample to high temperature and pressure, so it is a good approach for

encapsulating heat sensitive materials such as proteins (Saraf et al., 2006). However, using organic solvent may create toxicity problems (Mehnert and Mäder, 2001).

Various examples of proteins and peptides that can be encapsulated in SLNs include insulin (Zhang et al., 2006a), bovine serum albumin (Schubert and Müller-Goymann, 2005) and cyclosporine A (Ugazio et al., 2002).

5.6.4. Spray drying

The spray drying technique is commonly used in the pharmaceutical sector (León-Martínez et al., 2010) (Sollohub and Cal, 2010) and in the food industry (León-Martínez et al., 2010) (Medina-Torres et al., 2013) (Saéñz et al., 2009). This technique guarantees the microbiological stability of the products, as the material of interest is protected against chemical and biological degradations. The products obtained have very interesting and useful characteristics such as fast and immediate solubility. What is more storage and transportation costs are minimized (Gharsallaoui et al., 2007). An additional advantage of this technology in the pharmaceutical sector is that thermolabile fluid extracts can be dried without causing decomposition (Sollohub and Cal, 2010).

Spray drying is a unit operation by which a liquid product can be instantaneously transformed into powder by atomizing it in a current of hot gas (air or more rarely an inert gas such as nitrogen) (León-Martínez et al., 2010) (Gharsallaoui et al., 2007)(Cal and Sollohub, 2010).

The spray drying technique can be summarized by three main steps (Sinha and Trehan, 2003):

- The selected polymer is dissolved in a volatile organic solvent.
- The solid form of the drug of interest (proteins or peptides) is dispersed in the polymer solution under high speed homogenization.
- The solution obtained is dispersed into a stream of hot air. The particles, ranging in size from 1 to 100µm, are dried by evaporating the solvent.

The operating conditions and the dryer design are chosen according to the characteristics of the material to be dried and the desired powder specifications (León-Martínez et al., 2010).

The essential advantage of this method is that drug loaded particles can be prepared and dried in only one step (Vemmer and Patel, 2013). It is also possible to make modifications to the polymers used in this technique (Sinha and Trehan, 2003).

This technique requires high temperature and fast drying rates which make heat sensitive moieties such as proteins susceptible to damage. In order to resolve this problem, the proteins have to be protected (for example by lyophilisation (Sinha and Trehan, 2003)) before or during the drying process (Vemmer and Patel, 2013).

This technique has been used successfully to encapsulate recombinant human erythropoietin (Bittner et al., 1998) and bromocriptine mesylate (Parlodel) (Kissel et al., 1991).

5.6.5. Phase separation technique (coacervation)

The solid form of the protein is dispersed into a solution of polymer in organic solvent. The solubility of the polymer in its solvent is decreased by adding a defined rate of silicon, causing phase separation. The drug is encapsulated in the polymer rich phase (coacervation). Finally, the microspheres obtained undergo hardening and washing with heptane (Sinha and Trehan, 2003).

This method is preferable for encapsulating hydrophilic molecules like proteins, vaccines and peptides due to its high encapsulation efficiency, as it minimizes the loss of hydrophilic drugs by avoiding their exposure to the continuous aqueous phase (Yeo et al., 2001). An additional advantage is that it is easy to control particle size and improve particle homogeneity by changing the variables of the components (the amount and/or the viscosity of the non-solvent, molecular weight of the polymer used, etc.) (Yeo et al., 2001). However, the microspheres prepared by this method tend to aggregate (Yeo et al., 2001). The residual solvents present a risk for safety (drug stability, drug release rates) and a problem of microsphere morphology stability (Sinha and Trehan, 2003)(Yeo et al., 2001).

Diphtheria toxoid is an example of the proteins that are encapsulated using this method (Johansen et al., 1999).

5.6.6. Co-axial electrospray method

The process of packaging proteins with a polymeric envelope provides many advantages, such as protecting the protein of interest and ensuring its sustained release (Blanco and Alonso, 1997) (Lee and Yuk, 2007) (Bock et al., 2012). However, it is limited by several drawbacks, as the methods most commonly used to encapsulate proteins in polymer-based microparticles are usually based on preparing a water in oil W/O emulsion (Tamber et al., 2005). This step may cause degradation and aggregation of the protein of interest (Sah, 1999). Another important limit of protein polymeric-based encapsulation techniques is the possibility of protein denaturation or damage during microcapsule preparation (normally microcapsules are prepared using organic solvent and under high shear conditions), storage and protein release (Blanco and Alonso, 1998) (Determan et al., 2006) (Bock et al., 2012). In order to avoid these unsuitable protein microparticle fabrication steps, the co-axial electrospray technique was developed to be applicable for protein-based polymer encapsulation. Firstly, nanofibers consisting of a poly(ϵ -

caprolactone) (PCL) shell and a protein-containing PEG core were synthesized and used to achieve continuous and controlled protein release (Jiang et al., 2005) (Zhang et al., 2006b). The fibers obtained are only applicable for transdermal use or as implant materials. Afterwards, biodegradable microparticles for protein delivery suitable for pharmaceutical uses were prepared using the co-axial electro spray method by electrostatic extrusion of a suspension of proteins in a solution of polymer in organic solvent (Amsden and Goosen, 1997) (Loscertales et al., 2002). The principle of the electro spray approach is to apply a high voltage on the liquid that is forced through a capillary nozzle. The electric charge generated on the droplet competes with its surface tension, leading the droplet to separate into nano or micro-droplets that turn into particles after evaporation of the solvent (Bock et al., 2012).

J. Xie and coworkers (Xie et al., 2008) used the co-axial electro spray technique to prepare biodegradable microparticles including a protein with diameters ranging from several tens of microns to 100 microns. The experimental setup they used consisted of two nozzles, a smaller one (core needle) located inside a larger one (spray nozzle). It was connected to a high voltage generator that generated an electrostatic field for electro spraying. An aqueous protein solution was forced through the core needle while the organic polymer solution was introduced in the annulus flow. When the nozzle-ground voltage was applied to the liquid introduced, it separated into highly charged droplets. The particles were obtained after the evaporation of the solvent, Fig.3.

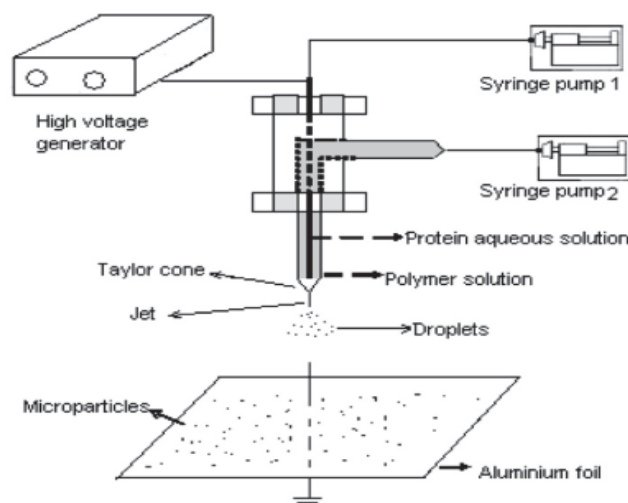


fig.3. Diagram of the co-axial electro spray apparatus for microencapsulation (Xie et al., 2008)

The electro spraying method presents many advantages, for example, the size of the droplets obtained is homogenous and ranges from a few hundred micrometers to several tens of nanometers, depending on the rate of the liquid flow and the voltage applied on the capillary nozzle (Jaworek and Sobczyk, 2008).

Regarding examples of proteins that have been encapsulated using this method, mention can be made of bovine serum albumin (BSA) (Xie and Wang, 2007) (Xu and Hanna, 2006), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF)(Ekaputra et al., 2011).

5.6.7. Layer by layer assembly (LbL)

1998 saw the first fabrication of particles using the LBL technique (Caruso et al., 1998) (Donath et al., 1998). This technique relies on the electrostatic attraction between two opposite charges (Balabushevitch et al., 2001)(Ai et al., 2003). LBL assembly is a simple, cheap, and versatile technique (Ariga et al., 2007)(Caruso et al., 2000) (Tang et al., 2006). It has been widely used for biomedical applications (Ai et al., 2003)(Tang et al., 2006) (Srivastava and Kotov, 2008).

Many research teams have encapsulated proteins using this technique (Shu et al., 2010) (De Temmerman et al., 2011). Briefly, anionic proteins are adsorbed on the positively charged surface of a solid medium (sacrificial template), that turns its charges into negative. This step is followed by the adsorption of a cationic polyelectrolyte; the surface charge obtained becomes positive. This alteration of surface charge allows continuous assembly layer after layer. The sacrificial template is removed to leave hollow polyelectrolyte capsules. Fig. 4. summarizes the work of Shu et al., who encapsulated bovine serum albumin (BSA) utilizing the LBL method.

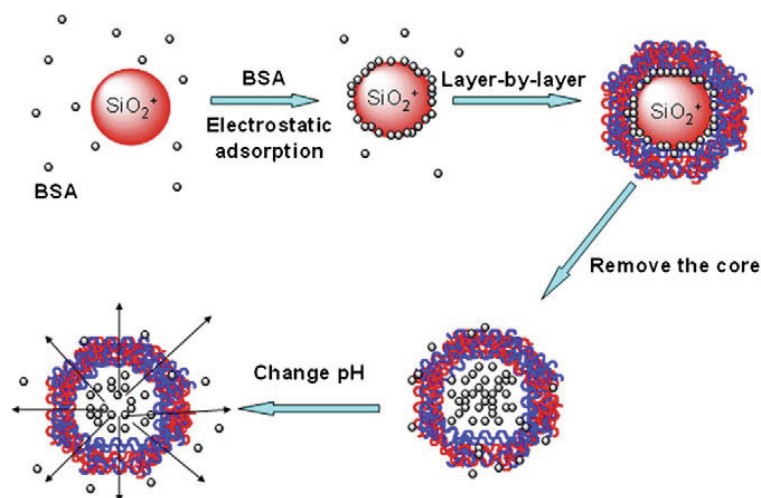


Fig. 4. The encapsulation of a

protein (BSA) within polyelectrolyte capsules using the layer by layer technique (Shu et al., 2010).

The particles fabricated using this method are hollow capsules possessing engineered characteristics like size, shape, composition and function (Johnston et al., 2006)(Shu et al., 2010).

However, this technique suffers from the problem of poor encapsulation efficiency and sudden and fast encapsulated drug release (Shu et al., 2010).

This method is used for encapsulating various proteins such as insulin (Fan et al., 2006), and is employed to encapsulate different enzymes such as catalase (Caruso et al., 2000).

Conclusion

Peptides, proteins and antibodies have now become important biopharmaceuticals that are used successfully to treat many diseases that were considered incurable until recently. They can be administered via various routes classified into two major classes: parenteral protein delivery routes (intravenous injection, subcutaneous injection and intramuscular injection) and non invasive protein delivery routes (nasal, oral, ocular, pulmonary and rectal routes). The formulation and specific characteristics of these molecules poses many problems when they are used *in vivo*, such as protein instability in the biological medium, short half-life in the bloodstream and the possibility of triggering dangerous immune response. Many technologies have been employed to solve these problems by increasing drug bioavailability, decreasing side effects, increasing ease of use by patients, and reducing the cost of fabrication as much as possible. In this context, account must be taken of the strategies developed to protect the proteins to be delivered and to enhance their absorption. These strategies include protein formulation modification, adjuvants and the encapsulation of therapeutic proteins and peptides, which can be implemented using various techniques such as double emulsion, solvent extraction or evaporation, coacervation, spray drying, co-axial electrospray process, layer by layer, and so forth.

References

- Adjei, A., and Gupta, P. (1994). Pulmonary delivery of therapeutic peptides and proteins. *J. Controlled Release* 29, 361–373.
- Agrawal, S., Misra, R., and Aggarwal, A. (2007). Autoantibodies in rheumatoid arthritis: association with severity of disease in established RA. *Clin. Rheumatol.* 26, 201–204.
- Ai, H., Jones, S.A., and Lvov, Y.M. (2003). Biomedical applications of electrostatic layer-by-layer nano-assembly of polymers, enzymes, and nanoparticles. *Cell Biochem. Biophys.* 39, 23–43.

- Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S.W., Zarghami, N., Hanifehpour, Y., Samiei, M., Kouhi, M., and Nejati-Koshki, K. (2013). Liposome: classification, preparation, and applications. *Nanoscale Res. Lett.* *8*, 102.
- Almeida, A.J., and Souto, E. (2007). Solid lipid nanoparticles as a drug delivery system for peptides and proteins. *Adv. Drug Deliv. Rev.* *59*, 478–490.
- Almeida, A.J., Runge, S., and Müller, R.H. (1997). Peptide-loaded solid lipid nanoparticles (SLN): Influence of production parameters. *Int. J. Pharm.* *149*, 255–265.
- Amsden, B.G., and Goosen, M.F.A. (1997). An examination of factors affecting the size, distribution and release characteristics of polymer microbeads made using electrostatics. *J. Controlled Release* *43*, 183–196.
- Ariga, K., Hill, J.P., and Ji, Q. (2007). Layer-by-layer assembly as a versatile bottom-up nanofabrication technique for exploratory research and realistic application. *Phys. Chem. Chem. Phys.* *Pccp* *9*, 2319–2340.
- Bailon, P., and Won, C.-Y. (2009). PEG-modified biopharmaceuticals. *Expert Opin. Drug Deliv.* *6*, 1–16.
- Balabushevitch, N.G., Sukhorukov, G.B., Moroz, N.A., Volodkin, D.V., Larionova, N.I., Donath, E., and Möhwald, H. (2001). Encapsulation of proteins by layer-by-layer adsorption of polyelectrolytes onto protein aggregates: Factors regulating the protein release. *Biotechnol. Bioeng.* *76*, 207–213.
- Bartlett, J.D., Turner-Henson, A., Atchison, J.A., Woolley, T.W., and Pillion, D.J. (1994). Insulin administration to the eyes of normoglycemic human volunteers. *J. Ocul. Pharmacol.* *10*, 683–690.
- Benet, L.Z. (1978). Effect of route of administration and distribution on drug action. *J. Pharmacokinet. Biopharm.* *6*, 559–585.
- Bilati, U., Allémann, E., and Doelker, E. (2005). Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles. *Eur. J. Pharm. Biopharm.* *59*, 375–388.
- Bioethics, C. on D. and C. on (1997). Considerations Related to the Use of Recombinant Human Growth Hormone in Children. *Pediatrics* *99*, 122–129.
- Bittner, B., Morlock, M., Koll, H., Winter, G., and Kissel, T. (1998). Recombinant human erythropoietin (rhEPO) loaded poly(lactide-co-glycolide) microspheres: influence of the encapsulation technique and polymer purity on microsphere characteristics. *Eur. J. Pharm. Biopharm.* *45*, 295–305.
- Blanco, D., and Alonso, M.J. (1998). Protein encapsulation and release from poly(lactide-co-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants. *Eur. J. Pharm. Biopharm.* *45*, 285–294.
- Blanco, M., and Alonso, M. (1997). Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres. *Eur. J. Pharm. Biopharm.* *43*, 287–294.
- Bock, N., Dargaville, T.R., and Woodruff, M.A. (2012). Electro spraying of polymers with therapeutic molecules: State of the art. *Prog. Polym. Sci.* *37*, 1510–1551.
- Boer, D.A.G. de, Moolenaar, F., Leede, L.G.J. de, and Breimer, D.D. (1982). Rectal Drug Administration. *Clin. Pharmacokinet.* *7*, 285–311.
- De Boer, A.G., Breimer, D.D., Pronk, J., and Gubbens-Stibbe, J.M. (1980). Rectal bioavailability of lidocaine in rats: Absence of significant first-pass elimination. *J. Pharm. Sci.* *69*, 804–807.
- De Boer, A.G., van Hoogdalem, E.J., Heijligers-Feijen, C.D., Verhoef, J., and Breimer, D.D. (1990). Rectal absorption enhancement of peptide drugs. *J. Controlled Release* *13*, 241–246.
- Brown, L.R. (2005). Commercial challenges of protein drug delivery. *Expert Opin. Drug Deliv.* *2*, 29–42.
- Buckel, P. (1996). Recombinant proteins for therapy. *Trends Pharmacol. Sci.* *17*, 450–456.
- Cal, K., and Sollohub, K. (2010). Spray drying technique. I: Hardware and process parameters. *J. Pharm. Sci.* *99*, 575–586.
- Carino, G.P., and Mathiowitz, E. (1999). Oral insulin delivery. *Adv. Drug Deliv. Rev.* *35*, 249–257.
- Caruso, F., Caruso, R.A., and Möhwald, H. (1998). Nanoengineering of Inorganic and Hybrid Hollow Spheres by Colloidal Templating. *Science* *282*, 1111–1114.
- Caruso, F., Trau, D., Möhwald, H., and Renneberg, R. (2000). Enzyme Encapsulation in Layer-by-Layer Engineered Polymer Multilayer Capsules. *Langmuir* *16*, 1485–1488.
- Cavalli, R., Caputo, O., Carlotti, M.E., Trotta, M., Scarnecchia, C., and Gasco, M.R. (1997). Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. *Int. J. Pharm.* *148*, 47–54.

- Cavalli, R., Gasco, M.R., Chetoni, P., Burgalassi, S., and Saettone, M.F. (2002). Solid lipid nanoparticles (SLN) as ocular delivery system for tobramycin. *Int. J. Pharm.* *238*, 241–245.
- Chaturvedi, K., Ganguly, K., Nadagouda, M.N., and Aminabhavi, T.M. (2013). Polymeric hydrogels for oral insulin delivery. *J. Controlled Release* *165*, 129–138.
- Chen, G., Jaffee, E.M., and Emens, L.A. (2013). Chapter 25 - Immunotherapy and Cancer Therapeutics: A Rich Partnership. In *Cancer Immunotherapy (Second Edition)*, G.C. Prendergast, and E.M. Jaffee, eds. (San Diego: Academic Press), pp. 415–432.
- Chiou, G.C.Y. (1994). Systemic Delivery of Polypeptide Drugs Through Ocular Route. *J. Ocul. Pharmacol. Ther.* *10*, 93–99.
- Christie, C.D., and Hanzal, R.F. (1931). INSULIN ABSORPTION BY THE CONJUNCTIVAL MEMBRANES IN RABBITS. *J. Clin. Invest.* *10*, 787–793.
- Chu, L., and Robinson, D.K. (2001). Industrial choices for protein production by large-scale cell culture. *Curr. Opin. Biotechnol.* *12*, 180–187.
- Chura-Chambi, R.M., Nakajima, E., de Carvalho, R.R., Miyasato, P.A., Oliveira, S.C., Morganti, L., and Martins, E.A.L. Refolding of the recombinant protein Sm29, a step toward the production of the vaccine candidate against schistosomiasis. *J. Biotechnol.*
- Cleland, J.L., Daugherty, A., and Mersny, R. (2001). Emerging protein delivery methods. *Curr. Opin. Biotechnol.* *12*, 212–219.
- Codrons, V., Vanderbist, F., Verbeeck, R.K., Arras, M., Lison, D., Pr eat, V., and Vanbever, R. (2003). Systemic delivery of parathyroid hormone (1-34) using inhalation dry powders in rats. *J. Pharm. Sci.* *92*, 938–950.
- Crommelin, D.J.A., Storm, G., Verrijck, R., de Leede, L., Jiskoot, W., and Hennink, W.E. (2003). Shifting paradigms: biopharmaceuticals versus low molecular weight drugs. *Int. J. Pharm.* *266*, 3–16.
- Davson, H. (1984). *The Eye Pt IB* (Elsevier).
- Deen, K.C., McDougal, J.S., Inacker, R., Folena-Wasserman, G., Arthos, J., Rosenberg, J., Maddon, P.J., Axel, R., and Sweet, R.W. (1988). Soluble form of CD4 (T4) protein inhibits AIDS virus infection. *Nature* *331*, 82–84.
- Determan, A.S., Wilson, J.H., Kipper, M.J., Wannemuehler, M.J., and Narasimhan, B. (2006). Protein stability in the presence of polymer degradation products: Consequences for controlled release formulations. *Biomaterials* *27*, 3312–3320.
- Donath, E., Sukhorukov, G.B., Caruso, F., Davis, S.A., and M ohwald, H. (1998). Novel Hollow Polymer Shells by Colloid-Templated Assembly of Polyelectrolytes. *Angew. Chem. Int. Ed.* *37*, 2201–2205.
- Dougan, M., and Dranoff, G. (2012). Immunotherapy of Cancer. In *Innate Immune Regulation and Cancer Immunotherapy*, R. Wang, ed. (Springer New York), pp. 391–414.
- Drews, J. (2000). Drug Discovery: A Historical Perspective. *Science* *287*, 1960–1964.
- Duan, X., and Mao, S. (2010). New strategies to improve the intranasal absorption of insulin. *Drug Discov. Today* *15*, 416–427.
- Durrant, L.G., and Scholefield, J.H. (2003). Principles of cancer treatment by immunotherapy. *Surg. Oxf.* *21*, 277–279.
- Ekaputra, A.K., Prestwich, G.D., Cool, S.M., and Hutmacher, D.W. (2011). The three-dimensional vascularization of growth factor-releasing hybrid scaffold of poly (ϵ -caprolactone)/collagen fibers and hyaluronic acid hydrogel. *Biomaterials* *32*, 8108–8117.
- Engel, R.H., Riggi, S.J., and Fahrenbach, M.J. (1968). Insulin: Intestinal Absorption as Water-in-Oil-in-Water Emulsions. *Nature* *219*, 856–857.
- Fan, Y.F., Wang, Y.N., Fan, Y.G., and Ma, J.B. (2006). Preparation of insulin nanoparticles and their encapsulation with biodegradable polyelectrolytes via the layer-by-layer adsorption. *Int. J. Pharm.* *324*, 158–167.
- Fanale, M.A., and Younes, A. (2007). Monoclonal antibodies in the treatment of non-Hodgkin's lymphoma. *Drugs* *67*, 333–350.
- Florence, A.T., and Whitehill, D. (1982). The formulation and stability of multiple emulsions. *Int. J. Pharm.* *11*, 277–308.

- Fundarò, A., Cavalli, R., Bargoni, A., Vighetto, D., Zara, G.P., and Gasco, M.R. (2000). Non-stealth and stealth solid lipid nanoparticles (SLN) carrying doxorubicin: pharmacokinetics and tissue distribution after i.v. administration to rats. *Pharmacol. Res. Off. J. Ital. Pharmacol. Soc.* *42*, 337–343.
- García-Fuentes, M., Prego, C., Torres, D., and Alonso, M.J. (2005). A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly(ethylene glycol) as carriers for oral calcitonin delivery. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* *25*, 133–143.
- García-Santana, M.A., Duconge, J., Sarmiento, M.E., Lanio-Ruiz, M.E., Becquer, M.A., Izquierdo, L., and Acosta-Domínguez, A. (2006). Biodistribution of liposome-entrapped human gamma-globulin. *Biopharm. Drug Dispos.* *27*, 275–283.
- Garti, N. (1997). Double emulsions — scope, limitations and new achievements. *Colloids Surfaces Physicochem. Eng. Asp.* *123–124*, 233–246.
- Gelfand, E.W. (2001). Antibody-directed therapy: past, present, and future. *J. Allergy Clin. Immunol.* *108*, S111–116.
- Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., and Saurel, R. (2007). Applications of spray-drying in microencapsulation of food ingredients: An overview. *Food Res. Int.* *40*, 1107–1121.
- Goto, T., Morishita, M., Nishimura, K., Nakanishi, M., Kato, A., Ehara, J., and Takayama, K. (2006). Novel mucosal insulin delivery systems based on fusogenic liposomes. *Pharm. Res.* *23*, 384–391.
- Van der Graaf, S., Schroën, C.G.P.H., and Boom, R.M. (2005). Preparation of double emulsions by membrane emulsification—a review. *J. Membr. Sci.* *251*, 7–15.
- Grumbach, M.M., Bin-Abbas, B.S., and Kaplan, S.L. (1998). The growth hormone cascade: progress and long-term results of growth hormone treatment in growth hormone deficiency. *Horm. Res.* *49 Suppl 2*, 41–57.
- Hamman, J.H., Enslin, G.M., and Kotzé, A.F. (2005). Oral delivery of peptide drugs: barriers and developments. *Biodrugs Clin. Immunother. Biopharm. Gene Ther.* *19*, 165–177.
- Han, K., Lee, K.-D., Gao, Z.-G., and Park, J.-S. (2001). Preparation and evaluation of poly(L-lactic acid) microspheres containing rhEGF for chronic gastric ulcer healing. *J. Controlled Release* *75*, 259–269.
- Harris, J.M., and Chess, R.B. (2003). Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug Discov.* *2*, 214–221.
- Hinchcliffe, M., and Illum, L. (1999). Intranasal insulin delivery and therapy. *Adv. Drug Deliv. Rev.* *35*, 199–234.
- Hintz, R.L., Attie, K.M., Baptista, J., and Roche, A. (1999). Effect of growth hormone treatment on adult height of children with idiopathic short stature. Genentech Collaborative Group. *N. Engl. J. Med.* *340*, 502–507.
- Hj, K., A, M., H, T., and R, B. (1983). Role of antibody to S100 protein in diagnostic pathology. *Am. J. Clin. Pathol.* *79*, 341–347.
- Hu, F.Q., Yuan, H., Zhang, H.H., and Fang, M. (2002). Preparation of solid lipid nanoparticles with clobetasol propionate by a novel solvent diffusion method in aqueous system and physicochemical characterization. *Int. J. Pharm.* *239*, 121–128.
- Illum, L. (2003). Nasal drug delivery—possibilities, problems and solutions. *J. Controlled Release* *87*, 187–198.
- Itakura, K., Hirose, T., Crea, R., Riggs, A.D., Heyneker, H.L., Bolivar, F., and Boyer, H.W. (1977). Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. *Science* *198*, 1056–1063.
- Jaffee, E.M. (1999). Immunotherapy of Cancer. *Ann. N. Y. Acad. Sci.* *886*, 67–72.
- Jaworek, A., and Sobczyk, A.T. (2008). Electro spraying route to nanotechnology: An overview. *J. Electrostat.* *66*, 197–219.
- Jenning, V., Schäfer-Korting, M., and Gohla, S. (2000). Vitamin A-loaded solid lipid nanoparticles for topical use: drug release properties. *J. Controlled Release* *66*, 115–126.
- Jiang, H., Hu, Y., Li, Y., Zhao, P., Zhu, K., and Chen, W. (2005). A facile technique to prepare biodegradable coaxial electrospun nanofibers for controlled release of bioactive agents. *J. Controlled Release* *108*, 237–243.

- Johansen, P., Moon, L., Tamber, H., Merkle, H.P., Gander, B., and Sesardic, D. (1999). Immunogenicity of single-dose diphtheria vaccines based on PLA/PLGA microspheres in guinea pigs. *Vaccine* 18, 209–215.
- Johnston, A.P.R., Cortez, C., Angelatos, A.S., and Caruso, F. (2006). Layer-by-layer engineered capsules and their applications. *Curr. Opin. Colloid Interface Sci.* 11, 203–209.
- Katsilambros, N., Diakoumopoulou, E., Ioannidis, I., Liatis, S., Makrilakis, K., Tentolouris, N., and Tsapogas, P. (2006). Treatment of Diabetes with Insulin. In *Diabetes in Clinical Practice*, (John Wiley & Sons, Ltd), pp. 371–408.
- Khafagy, E.-S., Morishita, M., Onuki, Y., and Takayama, K. (2007). Current challenges in non-invasive insulin delivery systems: A comparative review. *Adv. Drug Deliv. Rev.* 59, 1521–1546.
- Kissel, T., and Werner, U. (1998). Nasal delivery of peptides: an in vitro cell culture model for the investigation of transport and metabolism in human nasal epithelium. *J. Controlled Release* 53, 195–203.
- Kissel, T., Brich, Z., Bantle, S., Lancranjan, I., Nimmerfall, F., and Vit, P. (1991). Parenteral depot-systems on the basis of biodegradable polyesters. *J. Controlled Release* 16, 27–41.
- Köhler, G., and Milstein, C. (2005). Continuous cultures of fused cells secreting antibody of predefined specificity. 1975. *J. Immunol. Baltim. Md* 1950 174, 2453–2455.
- Krishnankutty, R.K., Mathew, A., Sedimbi, S.K., Suryanarayanan, S., and Sanjeevi, C.B. (2009). Alternative routes of insulin delivery. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 34, 933–948.
- Kumar, S., and Mason, M. (2012). Principles of cancer treatment by immunotherapy. *Surg. Oxf.* 30, 198–202.
- Kwon, M.J., Bae, J.H., Kim, J.J., Na, K., and Lee, E.S. (2007). Long acting porous microparticle for pulmonary protein delivery. *Int. J. Pharm.* 333, 5–9.
- Labiris, N.R., and Dolovich, M.B. (2003). Pulmonary drug delivery. Part I: Physiological factors affecting therapeutic effectiveness of aerosolized medications. *Br. J. Clin. Pharmacol.* 56, 588–599.
- Lander, R., Manger, W., Scouloudis, M., Ku, A., Davis, C., and Lee, A. (2000). Gaulin Homogenization: A Mechanistic Study. *Biotechnol. Prog.* 16, 80–85.
- Laouini, A., Jaafar-Maalej, C., Limayem-Blouza, I., Sfar, S., Charcosset, C., and Fessi, H. (2012). Preparation, Characterization and Applications of Liposomes: State of the Art. *J. Colloid Sci. Biotechnol.* 1, 147–168.
- Larsen, H.L. (1991). [Antikeratin antibodies in rheumatoid arthritis]. *Ugeskr. Laeger* 153, 1567–1571.
- Lasic&dagger, D.D., and Papahadjopoulos, D. (1998). *Medical Applications of Liposomes* (Elsevier).
- Lee, H.J. (2002). Protein drug oral delivery: The recent progress. *Arch. Pharm. Res.* 25, 572–584.
- Lee, V.H.L. (1990). Protease inhibitors and penetration enhancers as approaches to modify peptide absorption. *J. Controlled Release* 13, 213–223.
- Lee, K.Y., and Yuk, S.H. (2007). Polymeric protein delivery systems. *Prog. Polym. Sci.* 32, 669–697.
- Lee, V.H.L., and Yamamoto, A. (1989). Penetration and enzymatic barriers to peptide and protein absorption. *Adv. Drug Deliv. Rev.* 4, 171–207.
- Lee, Y.-C., Simamora, P., Pinsuwan, S., and Yalkowsky, S.H. (2002). Review on the systemic delivery of insulin via the ocular route. *Int. J. Pharm.* 233, 1–18.
- León-Martínez, F.M., Méndez-Lagunas, L.L., and Rodríguez-Ramírez, J. (2010). Spray drying of nopal mucilage (*Opuntia ficus-indica*): Effects on powder properties and characterization. *Carbohydr. Polym.* 81, 864–870.
- Li, X., Guo, S., Zhu, C., Zhu, Q., Gan, Y., Rantanen, J., Rahbek, U.L., Hovgaard, L., and Yang, M. (2013). Intestinal mucosa permeability following oral insulin delivery using core shell corona nanolipoparticles. *Biomaterials* 34, 9678–9687.
- Loscertales, I.G., Barrero, A., Guerrero, I., Cortijo, R., Marquez, M., and Gañán-Calvo, A.M. (2002). Micro/Nano Encapsulation via Electrified Coaxial Liquid Jets. *Science* 295, 1695–1698.
- Mackay, M., Phillips, J., and Hastewell, J. (1997). Peptide drug delivery: Colonic and rectal absorption. *Adv. Drug Deliv. Rev.* 28, 253–273.
- Mahato, R.I., Narang, A.S., Thoma, L., and Miller, D.D. (2003). Emerging trends in oral delivery of peptide and protein drugs. *Crit. Rev. Ther. Drug Carrier Syst.* 20, 153–214.

- Martins, S., Sarmiento, B., Ferreira, D.C., and Souto, E.B. (2007). Lipid-based colloidal carriers for peptide and protein delivery - liposomes versus lipid nanoparticles. *Int. J. Nanomedicine* 2, 595–607.
- Matiasevic, D., and Gershberg, H. (1966). Studies on hydroxyproline excretion and corticosteroid-induced dwarfism: Treatment with human growth hormone. *Metabolism* 15, 720–729.
- Medina-Torres, L., García-Cruz, E.E., Calderas, F., González Laredo, R.F., Sánchez-Olivares, G., Gallegos-Infante, J.A., Rocha-Guzmán, N.E., and Rodríguez-Ramírez, J. (2013). Microencapsulation by spray drying of gallic acid with nopal mucilage (*Opuntia ficus indica*). *Lwt - Food Sci. Technol.* 50, 642–650.
- Mehnert, W., and Mäder, K. (2001). Solid lipid nanoparticles: Production, characterization and applications. *Adv. Drug Deliv. Rev.* 47, 165–196.
- Miglietta, A., Cavalli, R., Bocca, C., Gabriel, L., and Gasco, M.R. (2000). Cellular uptake and cytotoxicity of solid lipid nanospheres (SLN) incorporating doxorubicin or paclitaxel. *Int. J. Pharm.* 210, 61–67.
- Moeller, E.H., and Jorgensen, L. (2008). Alternative routes of administration for systemic delivery of protein pharmaceuticals. *Drug Discov. Today Technol.* 5, e89–e94.
- Morishita, M., and Peppas, N.A. (2006). Is the oral route possible for peptide and protein drug delivery? *Drug Discov. Today* 11, 905–910.
- Mukhopadhyay, P., Sarkar, K., Chakraborty, M., Bhattacharya, S., Mishra, R., and Kundu, P.P. (2013). Oral insulin delivery by self-assembled chitosan nanoparticles: In vitro and in vivo studies in diabetic animal model. *Mater. Sci. Eng. C* 33, 376–382.
- Müller, R.H. (1991). *Colloidal Carriers for Controlled Drug Delivery and Targeting: Modification, Characterization and in Vivo Distribution* (Taylor & Francis).
- Müller, R., Maaben, S., Weyhers, H., and Mehnert, W. (1996). Phagocytic Uptake and Cytotoxicity of Solid Lipid Nanoparticles (SLN) Sterically Stabilized with Poloxamine 908 and Poloxamer 407. *J. Drug Target.* 4, 161–170.
- Müller, R.H., Maassen, S., Schwarz, C., and Mehnert*, W. (1997). Solid lipid nanoparticles (SLN) as potential carrier for human use: interaction with human granulocytes. *J. Controlled Release* 47, 261–269.
- Müller, R.H., Radtke, M., and Wissing, S.A. (2002). Nanostructured lipid matrices for improved microencapsulation of drugs. *Int. J. Pharm.* 242, 121–128.
- Mygind, N., and Dahl, R. (1998). Anatomy, physiology and function of the nasal cavities in health and disease. *Adv. Drug Deliv. Rev.* 29, 3–12.
- Nam, Y.S., Song, S.H., Choi, J.Y., and Park, T.G. (2000). Lysozyme microencapsulation within biodegradable PLGA microspheres: Urea effect on protein release and stability. *Biotechnol. Bioeng.* 70, 270–277.
- Nihant, N., Schugens, C., Grandfils, C., Jérôme, R., and Teyssié, P. (1994). Polylactide Microparticles Prepared by Double Emulsion/Evaporation Technique. I. Effect of Primary Emulsion Stability. *Pharm. Res.* 11, 1479–1484.
- Orive, G., Hernández, R.M., Gascón, A.R., Domínguez-Gil, A., and Pedraz, J.L. (2003). Drug delivery in biotechnology: present and future. *Curr. Opin. Biotechnol.* 14, 659–664.
- Overton, T.W. Recombinant protein production in bacterial hosts. *Drug Discov. Today*.
- Park, K., Kwon, I.C., and Park, K. (2011). Oral protein delivery: Current status and future prospect. *React. Funct. Polym.* 71, 280–287.
- Patton, J.S. (1996). Mechanisms of macromolecule absorption by the lungs. *Adv. Drug Deliv. Rev.* 19, 3–36.
- Patton, J.S., and Byron, P.R. (2007). Inhaling medicines: delivering drugs to the body through the lungs. *Nat. Rev. Drug Discov.* 6, 67–74.
- Patton, J.S., and Platz, R.M. (1992). (D) Routes of delivery: Case studies: (2) Pulmonary delivery of peptides and proteins for systemic action. *Adv. Drug Deliv. Rev.* 8, 179–196.
- Pauletti, G.M., Gangwar, S., Knipp, G.T., Nerurkar, M.M., Okumu, F.W., Tamura, K., Siahaan, T.J., and Borchardt, R.T. (1996). Structural requirements for intestinal absorption of peptide drugs. *J. Controlled Release* 41, 3–17.
- Pettit, D.K., and Gombotz, W.R. (1998). The development of site-specific drug-delivery systems for protein and peptide biopharmaceuticals. *Trends Biotechnol.* 16, 343–349.

- Posner, J. (2012). Clinical pharmacology: the basics. *Surg. Oxf.* *30*, 174–180.
- Putney, S.D. (1998). Encapsulation of proteins for improved delivery. *Curr. Opin. Chem. Biol.* *2*, 548–552.
- Putney, S.D., and Burke, P.A. (1998). Improving protein therapeutics with sustained-release formulations. *Nat. Biotechnol.* *16*, 153–157.
- Ridley, D., Perkins, A.C., Washington, N., Wilson, C.G., Wastie, M.L., O'flynn, P., Blattman, A., Ponchel, G., and Duchene, D. The effect of posture on nasal clearance of bioadhesive starch microspheres. *Stp Pharma Sci.* *5*, 442–446.
- Des Rieux, A., Fievez, V., Garinot, M., Schneider, Y.-J., and Pr eat, V. (2006). Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. *J. Controlled Release* *116*, 1–27.
- Ritthidej, G.C. (2011). Chapter 3 - Nasal Delivery of Peptides and Proteins with Chitosan and Related Mucoadhesive Polymers. In *Peptide and Protein Delivery*, C.V.D. Walle, ed. (Boston: Academic Press), pp. 47–68.
- Roberts, M.J., Bentley, M.D., and Harris, J.M. (2002). Chemistry for peptide and protein PEGylation. *Adv. Drug Deliv. Rev.* *54*, 459–476.
- Roberts, M.J., Bentley, M.D., and Harris, J.M. (2012). Chemistry for peptide and protein PEGylation. *Adv. Drug Deliv. Rev.* *64*, *Supplement*, 116–127.
- Rossi, G.R., Vahanian, N.N., Ramsey, W.J., and Link, C.J. (2013a). Chapter 29 - HyperAcute Vaccines: A Novel Cancer Immunotherapy. In *Cancer Immunotherapy (Second Edition)*, G.C. Prendergast, and E.M. Jaffee, eds. (San Diego: Academic Press), pp. 497–516.
- Rossi, R., Granoff, D.M., and Beernink, P.T. (2013b). Meningococcal factor H-binding protein vaccines with decreased binding to human complement factor H have enhanced immunogenicity in human factor H transgenic mice. *Vaccine* *31*, 5451–5457.
- Rudolph, C., Schillinger, U., Ortiz, A., Tabatt, K., Plank, C., M uller, R.H., and Rosenecker, J. (2004). Application of novel solid lipid nanoparticle (SLN)-gene vector formulations based on a dimeric HIV-1 TAT-peptide in vitro and in vivo. *Pharm. Res.* *21*, 1662–1669.
- Russell, C.S., and Clarke, L.A. (1999). Recombinant proteins for genetic disease. *Clin. Genet.* *55*, 389–394.
- Sa enz, C., Tapia, S., Ch avez, J., and Robert, P. (2009). Microencapsulation by spray drying of bioactive compounds from cactus pear (*Opuntia ficus-indica*). *Food Chem.* *114*, 616–622.
- Sah, H. (1999). Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation. *J. Controlled Release* *58*, 143–151.
- Salamat-Miller, N., and Johnston, T.P. (2005). Current strategies used to enhance the paracellular transport of therapeutic polypeptides across the intestinal epithelium. *Int. J. Pharm.* *294*, 201–216.
- Salmaso, S., and Caliceti, P. (2011). Chapter 11 - Peptide and Protein Bioconjugation: A Useful Tool to Improve the Biological Performance of Biotech Drugs. In *Peptide and Protein Delivery*, C.V.D. Walle, ed. (Boston: Academic Press), pp. 247–290.
- Saraf, S., Mishra, D., Asthana, A., Jain, R., Singh, S., and Jain, N.K. (2006). Lipid microparticles for mucosal immunization against hepatitis B. *Vaccine* *24*, 45–56.
- Sarciaux, J.M., Acar, L., and Sado, P.A. (1995). Using microemulsion formulations for oral drug delivery of therapeutic peptides. *Int. J. Pharm.* *120*, 127–136.
- Sauerborn, M., Brinks, V., Jiskoot, W., and Schellekens, H. (2010). Immunological mechanism underlying the immune response to recombinant human protein therapeutics. *Trends Pharmacol. Sci.* *31*, 53–59.
- Schooley, R.T., Merigan, T.C., Gaut, P., Hirsch, M.S., Holodniy, M., Flynn, T., Liu, S., Byington, R.E., Henochowicz, S., Gubish, E., et al. (1990). Recombinant Soluble CD4 Therapy in Patients with the Acquired Immunodeficiency Syndrome (AIDS) and AIDS-Related ComplexA Phase I-II Escalating Dosage Trial. *Ann. Intern. Med.* *112*, 247–253.
- Schubert, M.A., and M uller-Goymann, C.C. (2005). Characterisation of surface-modified solid lipid nanoparticles (SLN): influence of lecithin and nonionic emulsifier. *Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft F ur Pharm. Verfahrenstechnik Ev* *61*, 77–86.
- Sessa, G., and Weissmann, G. (1968). Phospholipid spherules (liposomes) as a model for biological membranes. *J. Lipid Res.* *9*, 310–318.

- Shantha Kumar, T.R., Soppimath, K., and Nachaegari, S.K. (2006). Novel Delivery Technologies for Protein and Peptide Therapeutics. *Curr. Pharm. Biotechnol.* 7, 261–276.
- Sharma, A., and Sharma, U.S. (1997). Liposomes in drug delivery: Progress and limitations. *Int. J. Pharm.* 154, 123–140.
- Shu, S., Sun, C., Zhang, X., Wu, Z., Wang, Z., and Li, C. (2010). Hollow and degradable polyelectrolyte nanocapsules for protein drug delivery. *Acta Biomater.* 6, 210–217.
- Siddiqui, O., and Chien, Y.W. (1987). Nonparenteral administration of peptide and protein drugs. *Crit. Rev. Ther. Drug Carrier Syst.* 3, 195–208.
- Sinha, V.R., and Trehan, A. (2003). Biodegradable microspheres for protein delivery. *J. Controlled Release* 90, 261–280.
- Sintov, A.C., Levy, H.V., and Botner, S. (2010). Systemic delivery of insulin via the nasal route using a new microemulsion system: In vitro and in vivo studies. *J. Controlled Release* 148, 168–176.
- Sollohub, K., and Cal, K. (2010). Spray drying technique: II. Current applications in pharmaceutical technology. *J. Pharm. Sci.* 99, 587–597.
- Srivastava, S., and Kotov, N.A. (2008). Composite Layer-by-Layer (LBL) Assembly with Inorganic Nanoparticles and Nanowires. *Accounts Chem. Res.* 41, 1831–1841.
- Stevenson, C.L., Rhodes, C.A., and Prestrelski, S.J. (2012). Delivery of Peptides and Proteins via Long Acting Injections and Implants. In *Long Acting Injections and Implants*, J.C. Wright, and D.J. Burgess, eds. (Springer US), pp. 409–427.
- Swaminathan, J., and Ehrhardt, C. (2012). Liposomal delivery of proteins and peptides. *Expert Opin. Drug Deliv.* 9, 1489–1503.
- Swartz, J.R. (2001). Advances in Escherichia coli production of therapeutic proteins. *Curr. Opin. Biotechnol.* 12, 195–201.
- Swiech, K., Picanço-Castro, V., and Covas, D.T. (2012). Human cells: New platform for recombinant therapeutic protein production. *Protein Expr. Purif.* 84, 147–153.
- Sznitowska, M., Gajewska, M., Janicki, S., Radwanska, A., and Lukowski, G. (2001). Bioavailability of diazepam from aqueous-organic solution, submicron emulsion and solid lipid nanoparticles after rectal administration in rabbits. *Eur. J. Pharm. Biopharm.* 52, 159–163.
- Tamber, H., Johansen, P., Merkle, H.P., and Gander, B. (2005). Formulation aspects of biodegradable polymeric microspheres for antigen delivery. *Adv. Drug Deliv. Rev.* 57, 357–376.
- Tan, M.X.L., and Danquah, M.K. (2012). Drug and Protein Encapsulation by Emulsification: Technology Enhancement Using Foam Formulations. *Chem. Eng. Technol.* 35, 618–626.
- Tan, M.L., Choong, P.F.M., and Dass, C.R. (2010). Recent developments in liposomes, microparticles and nanoparticles for protein and peptide drug delivery. *Peptides* 31, 184–193.
- Tang, Z., Wang, Y., Podsiadlo, P., and Kotov, N.A. (2006). Biomedical Applications of Layer-by-Layer Assembly: From Biomimetics to Tissue Engineering. *Adv. Mater.* 18, 3203–3224.
- Taylor, G., Stott, E.J., Bew, M., Fernie, B.F., Cote, P.J., Collins, A.P., Hughes, M., and Jebbett, J. (1984). Monoclonal antibodies protect against respiratory syncytial virus infection in mice. *Immunology* 52, 137–142.
- De Temmerman, M.-L., Demeester, J., De Vos, F., and De Smedt, S.C. (2011). Encapsulation Performance of Layer-by-Layer Microcapsules for Proteins. *Biomacromolecules* 12, 1283–1289.
- Tomasselli, A.G., and Heinrikson, R.L. (2000). Targeting the HIV-protease in AIDS therapy: a current clinical perspective. *Biochim. Biophys. Acta Bba - Protein Struct. Mol. Enzym.* 1477, 189–214.
- Torchilin, V.P. (1991). *Immobilized enzymes in medicine* (Springer-Verlag Berlin And Heidelberg Gmbh & Company K).
- Torchilin, V.P. (2007). Targeted pharmaceutical nanocarriers for cancer therapy and imaging. *Aaps J.* 9, E128–E147.
- Torchilin, V.P., and Lukyanov, A.N. (2003). Peptide and protein drug delivery to and into tumors: challenges and solutions. *Drug Discov. Today* 8, 259–266.
- Torchilin, V.P., and Trubetskoy, V.S. (1995). Which polymers can make nanoparticulate drug carriers long-circulating? *Adv. Drug Deliv. Rev.* 16, 141–155.

- Ugazio, E., Cavalli, R., and Gasco, M.R. (2002). Incorporation of cyclosporin A in solid lipid nanoparticles (SLN). *Int. J. Pharm.* *241*, 341–344.
- Vasiliauskiene, L., Wiik, A., and Hoier-Madsen, M. (2001). Prevalence and clinical significance of antikeratin antibodies and other serological markers in Lithuanian patients with rheumatoid arthritis. *Ann. Rheum. Dis.* *60*, 459–466.
- Vemmer, M., and Patel, A.V. (2013). Review of encapsulation methods suitable for microbial biological control agents. *Biol. Control* *67*, 380–389.
- Veronese, F.M., and Mero, A. (2008). The impact of PEGylation on biological therapies. *Biodrugs Clin. Immunother. Biopharm. Gene Ther.* *22*, 315–329.
- Videira, M.A., Botelho, M.F., Santos, A.C., Gouveia, L.F., Pedroso de Lima, J.J., and Almeida, A.J. (2002). Lymphatic Uptake of Pulmonary Delivered Radiolabelled Solid Lipid Nanoparticles. *J. Drug Target.* *10*, 607–613.
- Vyas, S.P., Paliwal, R., and Paliwal, S.R. (2011). Chapter 5 - Ocular Delivery of Peptides and Proteins. In *Peptide and Protein Delivery*, C.V.D. Walle, ed. (Boston: Academic Press), pp. 87–103.
- Wacharasindhu, S., Supornsilchai, V., Aroonparkmongkol, S., and Srivuthana, S. (2007). Diagnosis and growth hormone (GH) therapy in children with GH deficiency: experience in King Chulalongkorn Memorial Hospital, Thailand. *J. Med. Assoc. Thai. Chotmaiht Thangphaet* *90*, 2047–2052.
- Walde, P., and Ichikawa, S. (2001). Enzymes inside lipid vesicles: preparation, reactivity and applications. *Biomol. Eng.* *18*, 143–177.
- Walsh, E.E., Schlesinger, J.J., and Brandriss, M.W. (1984). Protection from respiratory syncytial virus infection in cotton rats by passive transfer of monoclonal antibodies. *Infect. Immun.* *43*, 756–758.
- Wan, F., Møller, E.H., Yang, M., and Jørgensen, L. (2012). Formulation technologies to overcome unfavorable properties of peptides and proteins for pulmonary delivery. *Drug Discov. Today Technol.* *9*, e141–e146.
- Wearley, L.L. (1991). Recent progress in protein and peptide delivery by noninvasive routes. *Crit. Rev. Ther. Drug Carrier Syst.* *8*, 331–394.
- Weltzin, R., Hsu, S.A., Mittler, E.S., Georgakopoulos, K., and Monath, T.P. (1994). Intranasal monoclonal immunoglobulin A against respiratory syncytial virus protects against upper and lower respiratory tract infections in mice. *Antimicrob. Agents Chemother.* *38*, 2785–2791.
- Wissing, S.A., and Müller, R.H. (2002). Solid lipid nanoparticles as carrier for sunscreens: in vitro release and in vivo skin penetration. *J. Controlled Release* *81*, 225–233.
- Wissing, S.A., Kayser, O., and Müller, R.H. (2004). Solid lipid nanoparticles for parenteral drug delivery. *Adv. Drug Deliv. Rev.* *56*, 1257–1272.
- Xie, J., and Wang, C.-H. (2007). Encapsulation of proteins in biodegradable polymeric microparticles using electrospray in the Taylor cone-jet mode. *Biotechnol. Bioeng.* *97*, 1278–1290.
- Xie, J., Ng, W.J., Lee, L.Y., and Wang, C.-H. (2008). Encapsulation of protein drugs in biodegradable microparticles by co-axial electrospray. *J. Colloid Interface Sci.* *317*, 469–476.
- Xin Hua Zhou (1994). Overcoming enzymatic and absorption barriers to non-parenterally administered protein and peptide drugs. *J. Controlled Release* *29*, 239–252.
- Xu, Y., and Hanna, M.A. (2006). Electrospray encapsulation of water-soluble protein with polylactide: Effects of formulations on morphology, encapsulation efficiency and release profile of particles. *Int. J. Pharm.* *320*, 30–36.
- Yamamoto, A., and Muranishi, S. (1997). Rectal drug delivery systems Improvement of rectal peptide absorption by absorption enhancers, protease inhibitors and chemical modification. *Adv. Drug Deliv. Rev.* *28*, 275–299.
- Yang, L., and Alexandridis, P. (2000). Physicochemical aspects of drug delivery and release from polymer-based colloids. *Curr. Opin. Colloid Interface Sci.* *5*, 132–143.
- Yang, S.C., Lu, L.F., Cai, Y., Zhu, J.B., Liang, B.W., and Yang, C.Z. (1999). Body distribution in mice of intravenously injected camptothecin solid lipid nanoparticles and targeting effect on brain. *J. Controlled Release* *59*, 299–307.

- Yang, Y.-Y., Chung, T.-S., and Ping Ng, N. (2001). Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. *Biomaterials* 22, 231–241.
- Ye, M., Kim, S., and Park, K. (2010). Issues in long-term protein delivery using biodegradable microparticles. *J. Controlled Release* 146, 241–260.
- Ye, Q., Asherman, J., Stevenson, M., Brownson, E., and Katre, N.V. (2000). DepoFoam™ technology: a vehicle for controlled delivery of protein and peptide drugs. *J. Controlled Release* 64, 155–166.
- Yeo, Y., Baek, N., and Park, K. (2001). Microencapsulation methods for delivery of protein drugs. *Biotechnol. Bioprocess Eng.* 6, 213–230.
- Zhang, N., Ping, Q.N., Huang, G.H., and Xu, W.F. (2005). Investigation of lectin-modified insulin liposomes as carriers for oral administration. *Int. J. Pharm.* 294, 247–259.
- Zhang, N., Ping, Q., Huang, G., Xu, W., Cheng, Y., and Han, X. (2006a). Lectin-modified solid lipid nanoparticles as carriers for oral administration of insulin. *Int. J. Pharm.* 327, 153–159.
- Zhang, Y.Z., Wang, X., Feng, Y., Li, J., Lim, C.T., and Ramakrishna, S. (2006b). Coaxial Electrospinning of (Fluorescein Isothiocyanate-Conjugated Bovine Serum Albumin)-Encapsulated Poly(ϵ -caprolactone) Nanofibers for Sustained Release. *Biomacromolecules* 7, 1049–1057.
- Zheng, C., Guo, Q., Wu, Z., Sun, L., Zhang, Z., Li, C., and Zhang, X. (2013). Amphiphilic glycopolymer nanoparticles as vehicles for nasal delivery of peptides and proteins. *Eur. J. Pharm. Sci.* 49, 474–482.
- Zhou, X.H., and Po, A.L.W. (1991). Peptide and protein drugs: II. Non-parenteral routes of delivery. *Int. J. Pharm.* 75, 117–130.
- Zhou, X.H., and Li Wan Po, A. (1991). Peptide and protein drugs: I. Therapeutic applications, absorption and parenteral administration. *Int. J. Pharm.* 75, 97–115.
- Zuzana Antosova, M.M. (2009). Therapeutic application of peptides and proteins: parenteral forever? *Trends Biotechnol.* 27, 628–635.
- (2002a). Human insulin production and amelioration of diabetes in mice by electrotransfer-enhanced plasmid DNA gene transfer to the skeletal muscle. *Publ. Online* 15 Oct. 2002 Doi101038sjgt3301804 9.
- (2002b). Introduction and overview of peptide and protein pegylation. *Adv. Drug Deliv. Rev.* 54, 453–456.

Etude experimentale

3. Etude expérimentale

3.1. Etude systématique de l'émulsion double

L'objectif de la recherche thérapeutique est de parvenir à élaborer des formulations pharmaceutiques qui ont une efficacité thérapeutique maximale avec le minimum d'effets secondaires indésirables. Pour ce faire, l'encapsulation est une des méthodes les plus utilisées pour améliorer l'effet d'un médicament et augmenter son efficacité et sa biodisponibilité. Elle peut permettre de protéger le principe actif, contrôler le site d'action, la vitesse de sa libération et par ailleurs de masquer le mauvais goût et odeur.

Différentes techniques ont été développées et utilisées pour l'encapsulation de molécules actives. Le choix d'utiliser une de ces techniques (pour encapsuler un principe actif) dépend fortement des caractéristiques physicochimiques du principe actif à encapsuler, et aussi de l'objectif thérapeutique recherché.

Dans cette étude, pour encapsuler le principe actif la méthode d'émulsions multiples (double émulsion / évaporation) a été utilisée car elle a démontré son efficacité pour encapsuler les molécules hydrophile et lipophile sensible à la haute température. Cette technique passe par deux étapes :

1. préparation de la première émulsion (Eau/Huile).
2. préparation de la deuxième émulsion (Eau/Huile/Eau).

Les conditions de préparation jouent un rôle déterminant dans la spécification et dans la qualité des capsules obtenues, tant en termes de forme, de taille et de distribution de taille, stabilité colloïdale et de taux d'encapsulation.

Dans le cadre de notre étude expérimentale, l'encapsulation a été effectuée via la méthode de double émulsion évaporation de solvant. Cette étude réalisée sans principe actif a pour objet de conduire une étude systématique la plus large possible afin d'examiner l'effet de chaque paramètre séparément. Pour ce faire, un seul paramètre a été changé en gardant les autres constants. Les différentes expériences effectuées et les résultats obtenus sont récapitulés dans ce tableau ci-dessous:

Le paramètre étudié	L'effet obtenue sur		
	La taille	La distribution de taille	La forme
La vitesse d'agitation utilisée pour préparer la première émulsion (1ère)	Pas d'effet significatif	Pas d'effet significatif	Pas d'effet significatif
La vitesse d'agitation utilisée pour préparer la deuxième émulsion (2ème)	Plus la vitesse est grande, plus la taille de particules est petite	Les microparticules préparées à 21500 rpm ont une distribution de taille plus étroite que celles préparées à 9500 rpm	Pas d'effet notable sur la morphologie extaïre des particules.
Le temps d'agitation utilisée pour préparer la première émulsion (1ère)	Pas d'effet significatif	Pas d'effet significatif	Pas d'effet significatif
Le temps d'agitation utilisée pour préparer la deuxième émulsion (2ème)	Plus le temps est long, plus la taille de particules est petite	L'homogénéité de la taille des particules augmente quand la seconde émulsion est agitée pendant une longue période de temps	Les particules obtenues après une agitation de 6 min ont une morphologie plus régulière que les particules obtenues après une agitation de 4 min (elles ont une forme de tasse).
La concentration de polymère utilisé (PCL)	Plus la concentration de polymère est grande, plus la taille de particules est grande	Plus la concentration de polymère est grande, plus la distribution de taille est étroite	Plus la concentration de polymère utilisée est grande, plus la forme des particules préparées est régulière
La concentration de stabilisant utilisé (PVA)	Plus la concentration de stabilisant est grande, plus la taille de particules est grande	Pas d'effet notable sur la distribution en taille, mais sa présence assure une très faible sédimentation	Les particules préparées en utilisant le PVA de concentration 0,5% ont une forme arrondie et régulière, tandis que les particules préparées en utilisant le PVA à 2% s'agregent.
Le volume de la phase aqueuse interne	Pas d'effet significatif	Le volume interne est tellement faible par rapport à la phase organique ce qui minimise l'effet de ce volume sur la ditribution en taille	Les particules obtenues en utilisant 1ml de phase aqueuse interne ont des fissures à la surface avec une forme sphérique, tandis que les particules préparées en utilisant 1,5ml ont une forme arrondie et une bonne morphologie.
Le volume de la phase aqueuse externe	Plus le volume de la phase aqueuse extérieure est important, plus la taille de particules est petite.	Une phase aqueuse externe de 150ml a conduit à une distribution de taille plus étroite que pour 50ml.	Les particules obtenues en utilisant 50 ml de la phase aqueuse externe ont une morphologie déformée, tandis que pour 150 ml la forme est arrondie et régulière



Contents lists available at ScienceDirect

Colloids and Surfaces A: Physicochemical and Engineering Aspects

journal homepage: www.elsevier.com/locate/colsurfa

Effects of process parameters on the colloidal properties of polycaprolactone microparticles prepared by double emulsion like process



D. Ibraheem, M. Iqbal, G. Agusti, H. Fessi, A. Elaissari*

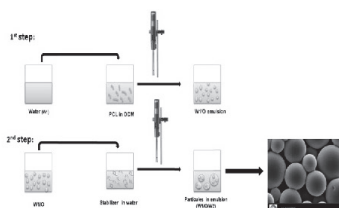
University of Lyon, F-69622, Lyon, University Lyon-1, CNRS, UMR 5007, LAGEP-CPE, 43 bd 11 Novembre 1918, F-69622 Villeurbanne, France

HIGHLIGHTS

- Formulation of polymeric particles through double emulsion like process.
- Effects of process parameters on particles colloidal properties.
- Both agitation time and speed affect the final average particles size.
- The use of high amount of polycaprolactone leads to large particles size.

GRAPHICAL ABSTRACT

In the first step, inner aqueous phase (W_1) was added to dichloromethane (DCM) containing polycaprolactone (PCL) polymer and homogenized to form primary emulsion (W_1/O). In the second step, the primary emulsion was emulsified in the outer aqueous phase (W_2) containing polyvinyl alcohol (PVA) as stabilizer using ultra-turrax to have double emulsion ($W_1/O/W_2$). SEM microgram shows the particles morphology.



ARTICLE INFO

Article history:
Received 19 November 2013
Received in revised form 7 January 2014
Accepted 13 January 2014
Available online 21 January 2014

Keywords:
Double emulsion
Polycaprolactone
Microparticles
Size distribution
Morphology

ABSTRACT

Preparation of polycaprolactone (PCL) based microparticles by double emulsion solvent diffusion like process was studied in this work. The double emulsion was prepared in two steps. In first step, the inner aqueous phase (W_1) was added to dichloromethane (DCM) solution containing PCL and homogenized to form primary emulsion (W_1/O). In the second step, the primary emulsion (W_1/O) was emulsified with the outer aqueous phase (W_2) containing polyvinyl alcohol (PVA) as stabilizer using ultra-turrax at a specific speed and time in order to achieve the double emulsion ($W_1/O/W_2$). Effects of various parameters such as stirring time and speed, polymer amount and the volume fraction of each phase on hydrodynamic particle size, size distribution and zeta potential were investigated.

As a general tendency, zeta potential of all prepared particles was found to be constant irrespective of investigated parameter. Whereas, the increase in polymer amount leads to large particles size and lowest sizes were obtained when high stirring speed was used during the second emulsification step.

© 2014 Published by Elsevier B.V.

* Corresponding author at: University of Lyon, F-69622, Lyon, University Lyon-1, CNRS, UMR 5007, LAGEP-CPE, 43 bd 11 Novembre 1918, F-69622 Villeurbanne, France. Tel.: +33 4 72 43 18 41.

E-mail address: Elaissari@lagep.univ-lyon1.fr (A. Elaissari).

1. Introduction

The key point for pharmaceutical researches is to fabricate pharmaceutical drug delivery system that enhance drug efficiency and diminish the undesirable effects [1,2]. Encapsulation technique is one of the techniques, which are used effectively for achieving this aim. It can be identified as the technique by which the active material is walled or coated by a supported material, that shielding it from the external environment [3–5]. It has found many

applications in different fields like pharmaceuticals [6,7], cosmetics [8,9], foods [10], diagnoses [11] and printing industries [12]. For instance, in pharmaceutical field, drug encapsulation can protect the active ingredient against harsh biological environment; mask the unpleasant taste and smell of the drug. Additionally, it can be used in drug delivery system for targeting the drug to specific site and to control the drug release [13,14].

Encapsulation technique, based on the method of preparation, may lead to the formation of spheres or capsules [15]. Generally, spheres are more stable than capsules. As a result, the drug liberation from the spheres is slower; therefore, spheres can be employed when a prolonged drug release is needed [16]. Encapsulation can be achieved by using various strategies [17]. Among these strategies, biopolymer-based encapsulation techniques are the most appropriate in pharmaceutical domain [18–20], due to the biodegradability and biocompatibility of polymers which is suitable for in vivo applications [21]. Polymer-based encapsulation techniques have been well studied and reviewed by various researcher teams [22–24], including: nanoprecipitation method, emulsion diffusion method, double emulsification method, emulsion-coacervation method, polymer-coating method, and layer-by-layer (LBL) assembly method.

Here we studied particles preparation by double emulsification method (multiple emulsion method); as this technique can be used to encapsulate both hydrophilic and lipophilic substances [25]. In addition, it is suitable for water-soluble fragile drug materials which are very sensitive to the high temperatures, such as nucleic acids and protein [26–28].

Multiple emulsions were described for the first time in 1925 by Seifriz [29], it can be identified as complex [30] polydispersed systems, in which the dispersed phase is itself an emulsion, that has inner phase of the same nature of the double emulsion [32]. In double emulsion two types of emulsion (water in oil and oil in water) are found simultaneously [33] using two types of surfactants (lipophilic and hydrophilic) for stabilizing them. Garti has identified these systems as “emulsions of emulsions” [34]. The presence of two types of emulsion simultaneously in one system, gives it the properties of the two emulsions. The multiple emulsions may be classified into two types: water in oil in water ($W/O/W$) and oil in water in oil ($O/W/O$) [35], the first type ($W/O/W$) is frequently employed for pharmaceutical purposes [36]. The composition of double emulsions and their properties make them promising systems that have potential applications in various fields for example, in pharmaceutical [37], in food industry and in cosmetics [38]. In spite of all these important properties, the applications of multiple emulsions are still limited because of their inherent thermodynamic instability. In recent years, great efforts have been made to improve the multiple emulsions properties, especially, increasing the emulsion stability, decreasing and homogenizing the emulsion droplets size. Many factors can affect the double emulsion stability such as, method of preparation, type of oil phase, type and concentration of emulsifiers and so on [39].

The aim of the present work is to study for the first time the influence of various parameters on colloidal properties (i.e. particles size, zeta potential, size distribution and morphology) of the final particles prepared by double emulsion solvent diffusion like process like. The parameters investigated were: polymer content, concentration of stabilizer (PVA), inner and outer aqueous phase

volumes, time and speed of stirring in 1st and 2nd steps of emulsification process.

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL) ($M_w = 14,000 \text{ g/mol}$), polyvinyl alcohol (PVA) (Mowiol® 4-88, $M_w = 31,000 \text{ g/mol}$), and dichloromethane (DCM) were obtained from Sigma-Aldrich, Germany, distilled water. Ultra-turrax (T-25 basic IKA-WERK), Laser Diffraction Particle Size Analyzer LS 13 320 (Beckman Coulter, USA), Field Emission Scanning Electron Microscope (S-800 Hitachi, Japan). Zetasizer (Nano-ZS, Malvern, UK). Analytical balance (Acculab ALC-110.4) was supplied by Sartorius Group, Germany.

2.2. Preparation of PVA solution

To be used as outer aqueous phase, 0.5% PVA solution was prepared by adding 2.5 g of PVA in 500 ml flask and distilled water was added to make up the volume, and then PVA was dissolved using magnetic stirrer under heating at 60 °C for 40 min to obtain a clear PVA solution.

2.3. Preparation of particle by double emulsion like process

The microparticles were fabricated by double emulsion solvent diffusion method. Two-step emulsification process was used; the primary emulsion (W_1/O) was dispersed as small droplets in the outer aqueous phase (W_2) with the help of ultra-turrax stirrer. PVA was used as emulsion stabilizer in the outer aqueous phase.

2.3.1. Preparation of primary emulsion (1st step)

In the first step, in order to make the primary emulsion (W_1/O); 3 g of polycaprolactone (polymer) was dissolved in 12 ml of DCM and shaken on rolling shaker until form a clear solution. And then 1.5 ml of distilled water was added in PCL solution, this mixture was homogenized properly using ultra-turrax (T-25 basic IKA-WERK) at a specific speed and for a specific time (Table 2) to have the first emulsion (W_1/O).

2.3.2. Preparation of double emulsion (2nd step)

In the second step, the primary emulsion (W_1/O) was added in the outer aqueous phase (W_2) containing 0.5% PVA as stabilizer. This mixture was homogenized by using ultra-turrax (T-25 basic IKA-WERK) at specific speed for specific time (Table 2), to achieve the double emulsion ($W_1/O/W_2$). Finally, the diffusion of organic solvent from dispersed primary emulsion droplets to outer aqueous phase (W_2), resulted into the formation of solidified suspended polycaprolactone (PCL) particles. In 2nd step we used excess of outer aqueous phase (W_2) in order to facilitate the diffusion of organic solvent from PCL particle to outer aqueous phase. Fig. 1 represents the two steps process for microparticles preparation by double emulsion solvent diffusion like process.

2.4. Reference emulsion composition

The value of parameters indicated in Table 1 was used to prepared reference emulsion. A set of experiments were performed,

Table 1
Composition of reference parameters of double emulsion.

Primary emulsion's parameters (1st step)				Double emulsion's parameters (2nd step)			
Amount of PCL (g)	Volume of inner phase w_1 (ml)	Stirrer speed (rpm)	Stirrer time (min)	PVA (% w/v)	Stirrer speed (rpm)	Stirrer time (min)	Volume of outer phase w_2 (ml)
3	1.5	17,500	5	0.5%	21,500	5	150

Table 2

Parameters (changed and fixed) in different set of experiments. The "bold" values show the parameters, which were changed in their respective recipes.

Studied parameters	Primary emulsion's parameters (1st step)				Double emulsion's parameters (2nd step)				Average particle size (µm)
	PCL(g)	Inner phase W ₁ (ml)	Stirring time (min)	Stirring speed (rpm)	PVA (%)	Outer phase W ₂ (ml)	Stirring time (min)	Stirring speed (rpm)	
Stirring speed for 1st emulsion	3	1.5	5	6500	0.5	150	5	21,500	6.5
	3	1.5	5	9500	0.5	150	5	21,500	6.5
	3	1.5	5	13,500	0.5	150	5	21,500	6.8
	3	1.5	5	17,500	0.5	150	5	21,500	6.7
Stirring speed for 2nd emulsion	3	1.5	5	17,500	0.5	150	5	6500	37.9
	3	1.5	5	17,500	0.5	150	5	9500	21.8
	3	1.5	5	17,500	0.5	150	5	13,500	13.7
	3	1.5	5	17,500	0.5	150	5	21,500	7
Stirring time for 1st emulsion	3	1.5	2	17,500	0.5	150	5	21,500	7.7
	3	1.5	4	17,500	0.5	150	5	21,500	7
	3	1.5	6	17,500	0.5	150	5	21,500	7.8
	3	1.5	8	17,500	0.5	150	5	21,500	7.3
Stirring time used for 2nd emulsion	3	1.5	5	17,500	0.5	150	2	21,500	9.9
	3	1.5	5	17,500	0.5	150	4	21,500	8.3
	3	1.5	5	17,500	0.5	150	6	21,500	7.7
	3	1.5	5	17,500	0.5	150	8	21,500	6.6
Concentration of stabilizer (PVA)	3	1.5	5	17,500	0.05	150	5	21,500	12.7
	3	1.5	5	17,500	0.1	150	5	21,500	8.65
	3	1.5	5	17,500	0.2	150	5	21,500	8.5
	3	1.5	5	17,500	0.5	150	5	21,500	10.6
	3	1.5	5	17,500	1	150	5	21,500	9.1
	3	1.5	5	17,500	2	150	5	21,500	10.6
Amount of polymer used (PCL)	1	1.5	5	17,500	0.5	150	5	21,500	4.2
	2	1.5	5	17,500	0.5	150	5	21,500	6
	3	1.5	5	17,500	0.5	150	5	21,500	8.5
	4	1.5	5	17,500	0.5	150	5	21,500	11.6
Volume of inner aqueous phase (W ₁)	3	1	5	17,500	0.5	150	5	21,500	8.7
	3	1.2	5	17,500	0.5	150	5	21,500	9.6
	3	1.5	5	17,500	0.5	150	5	21,500	9.2
	3	2	5	17,500	0.5	150	5	21,500	8.6
Volume of outer aqueous phase (W ₂)	3	1.5	5	17,500	0.5	50	5	21,500	10.8
	3	1.5	5	17,500	0.5	100	5	21,500	7.9
	3	1.5	5	17,500	0.5	150	5	21,500	9.8
	3	1.5	5	17,500	0.5	200	5	21,500	8.9

to study the effect of different parameters on the characteristics of the particles prepared via double emulsification by changing only one parameter at a time and keeping all other parameters fixed. For example, to study the parameter "polymer amount" four samples were prepared with different amount of PCL i.e. 1 g, 2 g, 3 g and 4 g while keeping all other conditions (stirring time, stirring speed, phase volume, etc.) constant (Table 1); and particles size were measured for all samples.

2.5. Particles size measurement

The particles size and size distribution were studied using Beckman Coulter LS 13 320 Laser Diffraction Particles Size Analyses. The samples were added drop by drop into the sample cell comprising continuous phase (deionized water), the pump speed was adjusted to 20% for appropriate mixing of sample. When the obscuration reached 8–9% then sample analysis was started.

2.6. Zeta potential

Zeta potential was measured at different pH values at 25 °C, for this purpose solution of 1 mM (milli Molar) concentration of NaCl was prepared and its pH was adjusted to different values like 3, 5, 7, 9 and 11. After that each sample's zeta potential was measure at these pH values with help of Zetasizer (Nano-ZS, Malvern).

2.7. SEM observation

Scanning Electron Microscopy, SEM, was performed with a Hitachi S800 FEG microscope at the "Centre Technologique des Microstructures" (CTµ) at the University of Lyon (Villeurbanne, France). A drop of diluted aqueous suspension of microparticles was deposited on a flat steel holder and dried at room temperature. The sample was finally coated under vacuum by cathodic sputtering with platinum. The samples were observed by SEM under an accelerating voltage of 15 kV.

3. Results and discussion

The purpose of this work was to study the effects of different parameters such as polymer amount, stabilizer concentration, stirring time and stirring speed used, on the size of particles prepared by double emulsion like process. Double emulsion W₁/O/W₂ was prepared by a two-step emulsification process using PVA as stabilizer in the second step.

3.1. Effects of different parameters on particle size and size distribution

3.1.1. Effect of stirring speed

Since the emulsion is a mixture of two or more immiscible liquids so, in order to make it uniformly dispersed, it is necessary

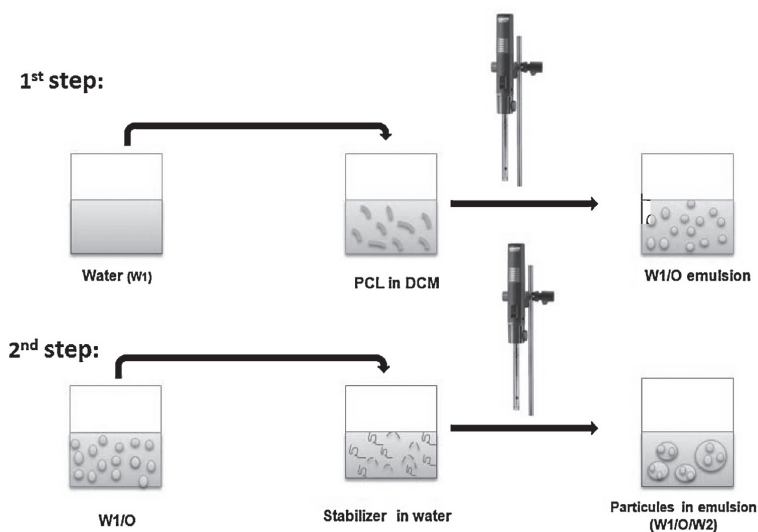


Fig. 1. Schematic illustration of a two-step process in formation microparticles via double emulsion solvent diffusion like process.

to provide this system with the adequate energy [40], either by high stirring or by ultrasound system [34]. However, the agitation used to provide the needed energy should not be very severe, especially during the second step of emulsion preparation, because it may rupture the droplets obtained from the first step [34,36]. Yang et al. have found that the stirring speed is a governing factor in size determining of the particles prepared via double emulsification technique [40]. The work of Yang's team demonstrates that, high stirring speed produces very small size particles because of the dismantling of second emulsion into smaller droplets. However, the final particles yield is low, due to the breaking down of the resultant microspheres [40]. In this work the necessary energy was supplied to the system via high stirring using ultra-turrax and the influence of stirring speed on the final particles size was studied in both steps of emulsification. In first step, the stirring speed used to prepare the first emulsion was changed only, while stirring speed of second emulsion and other conditions were kept constant (Table 2). In the second step, contrary to first step was done, it means that stirring speed of first emulsion with all other parameters were kept constant and only the stirring speed for preparation

of second emulsion was changed. The particles size obtained were measured by using *Laser Diffraction Particle Size Analyzer*, the final particles size was in micro range as shown in Fig. 2.

It is obvious from Fig. 2 that the change in stirring speed from 6500 rpm to 9500 rpm during first emulsion preparation has no influence on the size of final particles. And the particles size changed slightly when the stirring speed was further increased i.e. from 9500 rpm to 13,500 rpm, at this stirring speed the resultant particles size was 6.8 μm . By increasing starting speed further to 17,500 rpm, the particle size decreased to 6.7 μm . Fig. 2 shows that, the stirring speed used to prepare the first emulsion has no significant effect on the size of the resulting particles.

In second step of double emulsion, by increasing the stirring speed of ultra-turrax and keeping all other parameters constant, has led to a proportional reduction in the obtained particles size as shown in Fig. 2. Here, the size of particles was 37.6 μm at stirring speed of 6500, and particle size gradually decreased to 21.8 μm , 13.7 μm and 7.0 μm at stirring speed of 9500 rpm, 13,500 rpm and 21,500 rpm respectively. The findings of this study proved that the stirring speed in the second step of double emulsions preparation is a significant factor in particle size determination, i.e. by increasing the stirring speed in 2nd step of emulsion preparation, the size

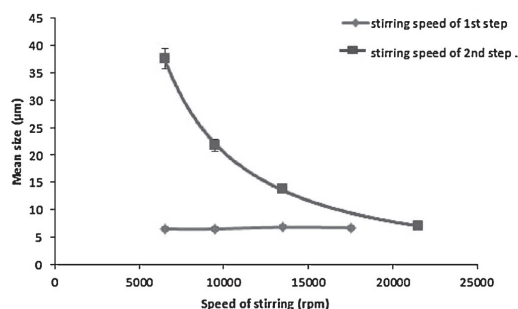


Fig. 2. Effect of stirring speed in 1st step and 2nd step of emulsification on the mean particle size prepared by double emulsion.

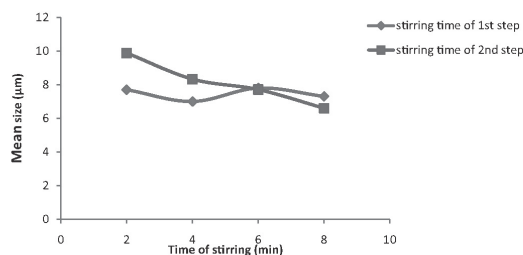


Fig. 3. Effect of stirring time of 1st step and 2nd step of emulsification on the mean particle size.

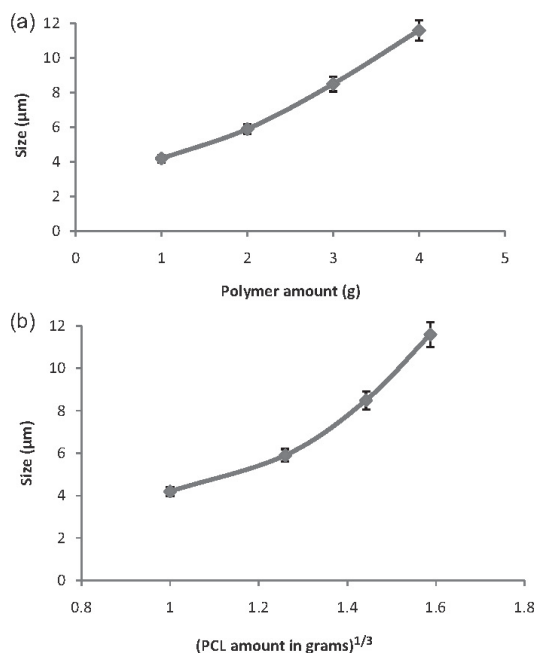


Fig. 4. (a) Effect of polymer amount on the mean particle size. (b). Average hydrodynamic size versus (polymer amount)^{1/3}.

of obtained particles decreased proportionally, Which is in accordance with the results reported by Yang [40].

3.1.2. Effect of stirring time

The time duration of providing energy is quite important because short time of stirring grants insufficient energy to the system, while long time of stirring may lead to break down of the emulsion, which in turn will lead to low entrapment efficiency. In this experiment, the stirring time of the first emulsion was changed only, while keeping all other parameters of the recipe constant (Table 2). Four recipes were prepared with different stirring time (2 min, 4 min, 6 min and 8 min) and size of particles achieved were 7.7 μm, 7 μm, 7.8 μm, 7.3 μm respectively (Fig. 3). These results demonstrated that the time duration of stirring for the first emulsion (W_1/O) has no significant effect on the particle size prepared

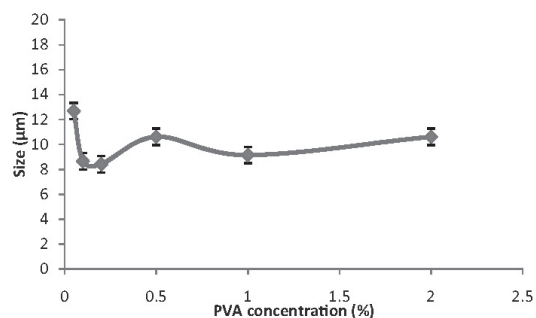


Fig. 5. Effect of stabilizer (PVA) versus mean particle size.

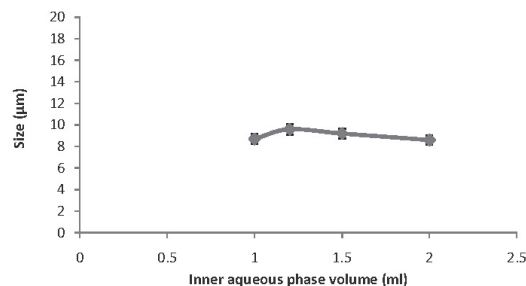


Fig. 6. Inner aqueous phase (W_1) versus PCL mean particles size.

via multiple emulsions solvent diffusion like process as shown in Fig. 3.

The stirring time of the second emulsion was investigated too, by changing the stirring time for the second emulsion and keeping all other conditions unchanged. It was found that the stirring time is an important and influential factor in determining the final particles size in double emulsion. Adequate agitation time is required to obtain small, mono-dispersed particles and to avoid aggregation of the formed particles. However, the stirring time must not exceed a certain limit in order to avoid emulsion spoilage. Here, different stirring time i.e. 2 min, 4 min, 6 min and 8 min were used in 2nd step emulsification, which resulted into particulate dispersion with mean size 9.88 μm, 8.33 μm, 7.7 μm and 6.6 μm, respectively. Fig. 3 shows that there is a gradual decrease in particles size as stirring time increase, this reduction in particle size may be due to supply of input power for longer period of time.

3.1.3. Effect of polymer amount

The polymer amount may be an important factor influencing the particle's characteristics like encapsulation efficacy and particle size. In this work, polycaprolactone was used as polymer and its effects were investigated at different amount of polymer (1, 2, 3 and 4 g) by keeping all other conditions constant. The results obtained (Fig. 4a) implies that at small amount (1 g) of polymer small particles were obtained (4.2 μm) but as the amount of polymer was increased further to 2 g, 3 g and 4 g, the particle size increased significantly which were 5.9 μm, 8.5 μm and 11.6 μm, respectively. These results revealed that by increasing the amount of polymer, the particle size also increases and similar results were reported by Lamprecht et al. [41].

In order to point out the real effect of polymer, we deduced the mathematic relationship between the average particle size (R) and the polymer amount (M), which is; $R = (3/4\pi d N_p)^{1/3} \times (M)^{1/3}$ where (d) is the density of polymer, N_p is the number of particles.

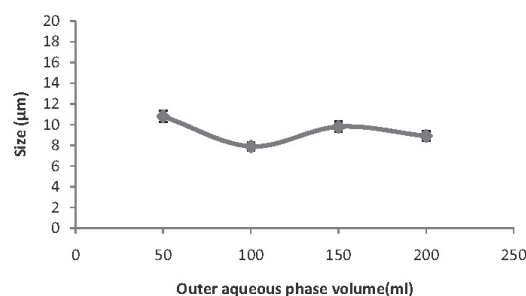


Fig. 7. Effect of outer aqueous phase volume (W_1).

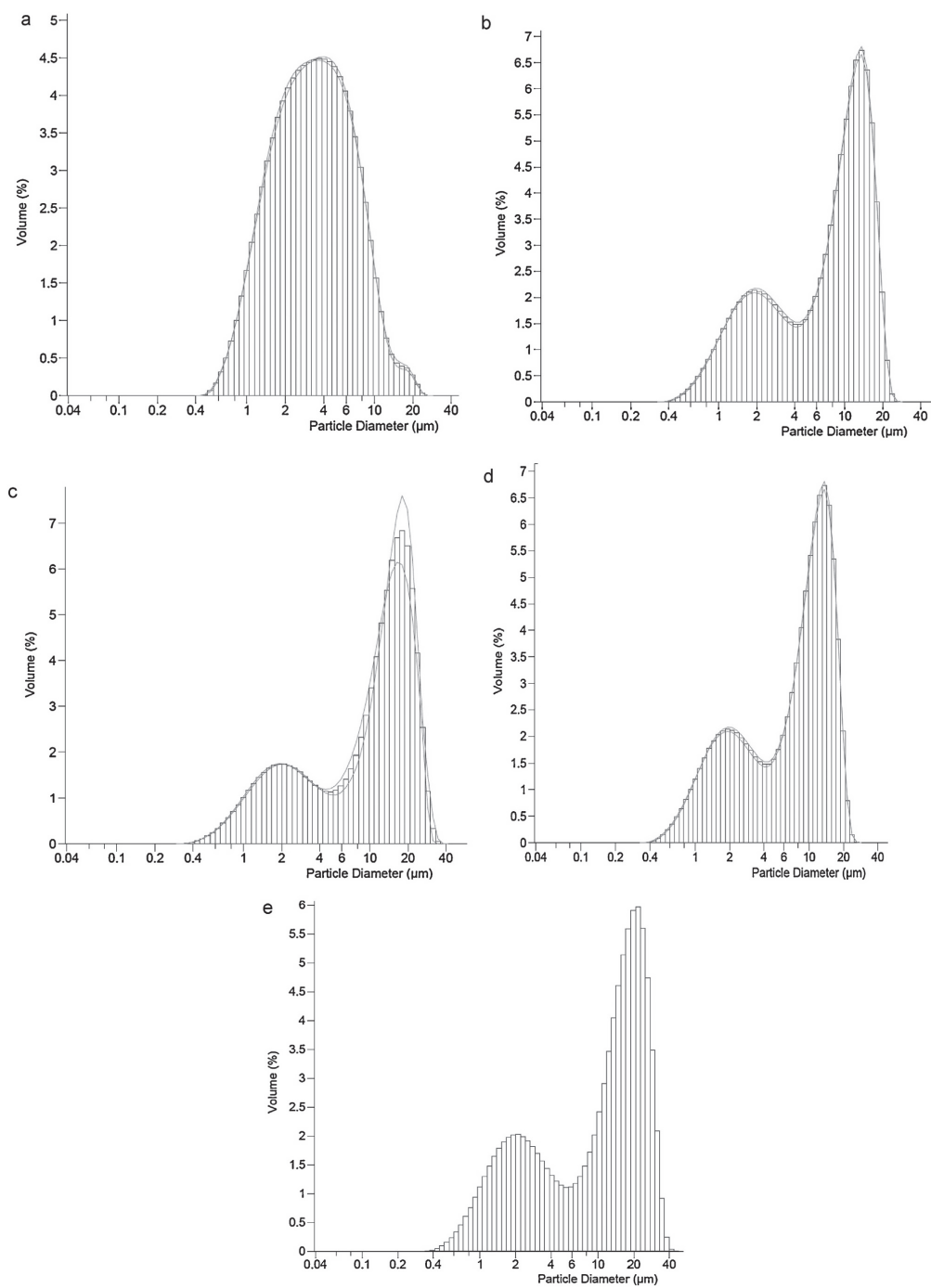


Fig. 8. (a) Particle size distribution of sample prepared using 1g of PCL. (b) Particle size distribution of sample prepared using 3g of PCL. (c) Particle size distribution of sample prepared using 4g of PCL. (d) Particle size distribution of sample prepared using 0.5% g PVA. (e) Particle size distribution of sample prepared using 0.05% g PVA.

Table 3
Zeta potential values (in mV) of different recipes at different pH.

Parameters		Zeta potential (mV) as a function of pH				
		pH=3	pH=5	pH=7	pH=8	pH=10
Stirring time in 1st step of emulsion	2 min	-0.70	-2.42	-2.41	-3.21	-1.58
	4 min	-1.04	-2.41	-2.71	-3.38	-2.01
	6 min	-1.16	-2.33	-2.64	-2.83	-1.70
	8 min	-0.993	-2.14	-1.69	-3.01	-1.44
Stirring time in 2nd step of emulsion	2 min	1.72	-2.11	-0.65	-2.91	-1.81
	4 min	-1.22	-2.46	-2.32	-3.28	-2.02
	6 min	1.96	-1.38	-0.63	-3.22	-1.33
	8 min	-1.18	-1.87	-2.13	-3.22	-1.62
PCL content	1 g	-1.88	-2.55	-1.65	-3.55	-1.72
	2 g	-0.89	-1.82	-2.44	-2.72	-2.10
	3 g	-1.12	-2.51	-2.37	-3.12	-2.01
	4 g	-0.62	-3.92	-1.83	-3.25	-1.76
PVA (wt./v)	0.2%	0.37	-3.59	-2.85	-3.45	-2.03
	0.5%	-0.98	-2.12	-1.66	-2.23	-2.15
	1%	-0.88	-3.40	-2.33	-3.26	-1.92
	2%	-1.05	-2.53	-1.42	-2.83	-1.70
Stirring speed in 1st step of emulsion	6500 rpm	-0.90	-1.85	-1.52	-1.83	-1.49
	9500 rpm	-0.89	-2.12	-1.28	-2.40	-0.94
	13,500 rpm	-1.14	-1.32	-1.59	-2.48	-1.46
	17,500 rpm	-0.59	-1.79	-1.19	-2.39	-1.86
Stirring speed in 2nd step of emulsion	6500 rpm	-1.56	-1.20	-3.14	-2.11	-1.93
	9500 rpm	-0.82	-1.51	-1.65	-1.35	-0.92
	13,500 rpm	-0.91	-1.73	-2.25	-2.00	-1.44
	21,500 rpm	-0.721	-2.79	-1.87	-3.19	-1.30
Inner (W ₁) aqueous phase volume	1 ml	-0.96	-3.38	-1.23	-3.95	-1.77
	1.2 ml	-1.66	-2.75	-2.66	-2.73	-2.25
	1.5 ml	-0.97	-2.52	-2.29	-3.59	-2.00
	2 ml	-1.11	-3.21	-1.59	-3.48	-1.73
Outer (W ₂) aqueous phase volume	50 ml	-1.24	-4.67	-3.74	-4.78	-3.32
	100 ml	-1.67	-4.12	-3.16	-3.90	-2.43
	150 ml	-1.02	-3.52	-1.56	-3.57	-2.09
	200 ml	1.00	-3.88	-2.98	-3.97	-2.30

In this relationship, if the number of particles is constant independent of the used polymer amount, the slope that represents the relationship between the particle size and $(M)^{1/3}$ will be straight. But in our case it seems that the slope increases with the increasing of polymer amount in the formulation, as illustrated in Fig. 4b. Such increase in the slope exhibits that using more polymer amount leads to increase the number of obtained particles in the formation. Such increase in the number and in the size of particles can be attributed to the aggregation of unstable particles that can be enhanced by increasing the solid content (polymer amount) and also by low stabilizing efficiency of PVA.

3.1.4. Effects of stabilizer concentration

The addition of suitable stabilizer plays a key role in liquid–liquid dispersion [42]. The concentration and type of stabilizer affect the stability and formulation of emulsion. The stability of emulsion is very important because during the evaporation of solvent, the volume of emulsion can be decreases, which in turn increase its viscosity. This may affect the final size of the droplet and may results in the coalescence and aggregation of the droplets during solvent evaporation [43].

The stabilizer stays at oil/water interface during solvent evaporation. In recent times polyvinyl alcohol (PVA) is frequently used as an emulsion stabilizer [44] and its concentration in the external water phase is considered to be vital factor to influence the size of microparticles [40]. Since PVA is a high molecular weight polymer, the presence of PVA in the outermost water phase (W₂) may increase the viscosity of the dispersion phase, resulting in an increased difficulty to reduce the emulsion particles to smaller size [40,45].

In this study, six samples were prepared with different concentration of PVA in the outer aqueous phase (W₂), it was found that by increasing PVA concentration (0.05%, 0.1%, and 0.2%) has led to marked decrease in particle size and minimum size was achieved at 0.2% PVA which was 8.4 μm (Fig. 5). By increasing further the PVA concentration to 0.5%, 1% and 2% the particle size were slightly increased with some variations, which was 10.6 μm, 9.1 μm and

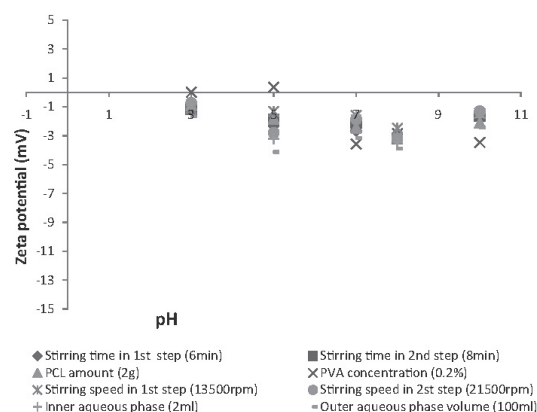


Fig. 9. Zeta potential of PCL microparticles as a function of pH, at different conditions.

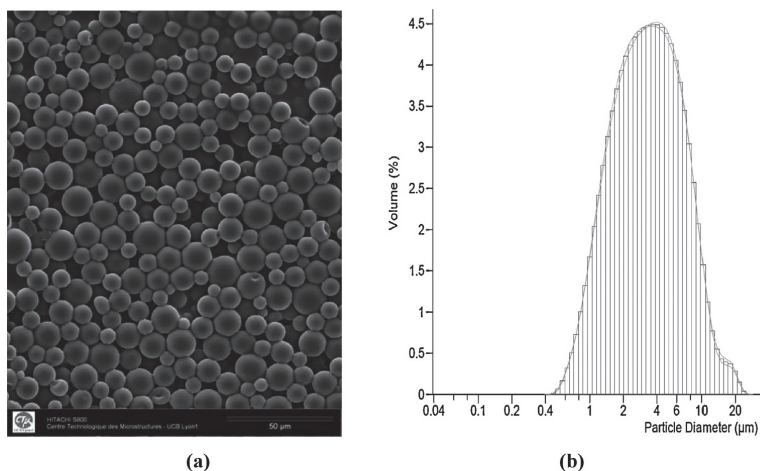


Fig. 10. (a) SEM image of PCL particles prepared by double emulsion like method. The scale bar represents 50 μm . (b) Particle size distribution diameter from Laser Diffraction analysis.

10.6 μm , respectively. Additionally one sample was prepared without PVA in outer aqueous phase (W_2) but in this case no particles were formed and the emulsion was absolutely unstable. The results obtained (Fig. 5) showed that 0.2% PVA was more appropriate in achieving smaller size microparticles in this method.

3.1.5. Effect of inner aqueous phase volume

In this work, we used different volumes of internal aqueous phase (W_1) including 1 ml, 1.2 ml, 1.5 ml and 2 ml of distilled water to determine its effects on the final PCL particle prepared by double emulsion. These volumes were emulsified in 25% solution of polycaprolactone in DCM. It was found that the volume of the internal aqueous phase has no significant effect on the size of the final particle in double emulsion. By increasing the volume of inner phase there was insignificant decrease in size of microparticles with some variations (Fig. 6). Smallest particle size was achieved at 2 ml of

inner aqueous phase. The smaller particle size at high volume of inner aqueous phase may be due to the factor that; the high polymer amount solution coagulates faster during the second emulsion and results in tighter structure due to chain entanglement [40]. So at high volume ratio of inner aqueous phase (W_1), polymer may form thin layer over water droplet and thus a low probability to coagulation into large particle.

3.1.6. Effect of outer aqueous phase volume

Four samples were prepared with different volumes of outer aqueous phase (Table 2) and its effect was evaluated on the final PCL particles prepared by double emulsion. It was found that by increasing the volume of outer aqueous phase (W_2) the size of the microparticles decreased slightly with some variations (Fig. 7). Larger particles were obtained at 50 ml and smallest particles were achieved at 100 ml volume of the outer aqueous phase in this study.

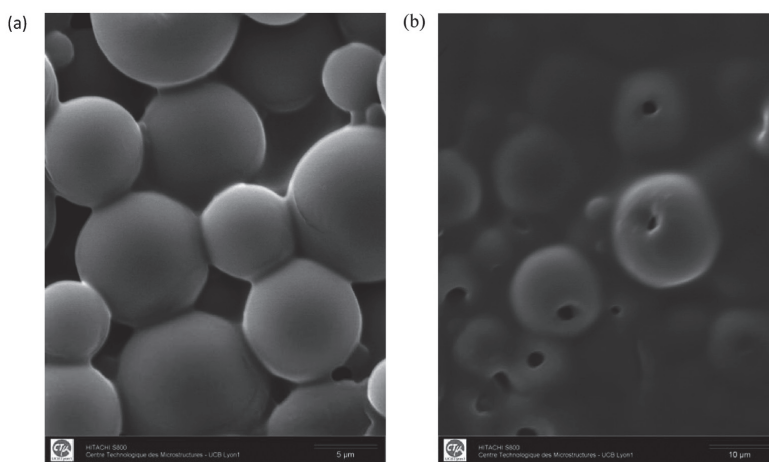


Fig. 11. (a) SEM image of PCL particles prepared by double emulsion like method. The scale bar represents 5 μm . (b) SEM image of PCL particles prepared by double emulsion like method. The scale bar represents 10 μm .

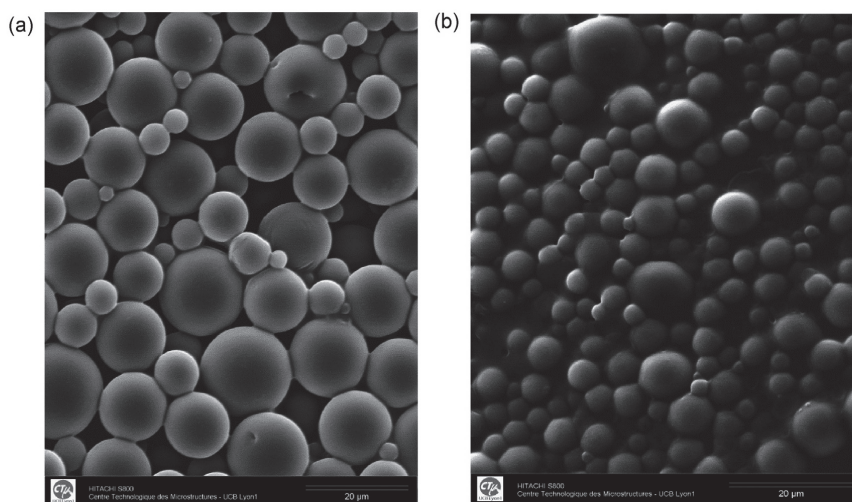


Fig. 12. (a) SEM image of PCL microparticles prepared using 3 g of PCL. (b) SEM image of PCL microparticles prepared using 1 g of PCL.

This may be due to decrease in the viscosity of the emulsion at high volume of outer phase, so there is efficient shearing force to reduce the particle size.

3.1.7. Effect on particle size distribution

Particle size distribution for different recipes was investigated using laser diffraction. Size distribution was found to be large and ranging from 0.4 to 40 μm revealing incontestably the polydispersity of the prepared dispersions. But for low polymer amount (i.e. 1 g) one single size distribution peak (0.4–20 μm) was observed (Fig. 8a). Whereas, above 1 g of PCL two peaks were obtained as shown in Fig. 8b and c.

In case of PVA concentration, particle size distribution ranges from (0.4 to 40 μm) irrespective of PVA amount with the presence of two distributions as shown in (Fig. 8d and e).

3.2. Zeta potential

Actually, the value of zeta potential reflects the charge of particles surfaces and this value depends on three factors, the chemical nature of the polymer, the surfactant, and medium pH values [22].

In this study, zeta potential was measured for all samples at different pH i.e. pH 3, 5, 7, 9 and 11 (Table 3), and it was found that there was no significant change in the values of zeta potential of all samples (Fig. 9), which means that changing of PCL microparticles preparation conditions such as changes in polymer concentration, stabilizer amount, stirring time and speed, phase volume for 1st and 2nd emulsion have no significant effect on the zeta potential. In fact, the zeta potential was found in between +1 and –4 mV which can be considered in zero range reflecting the non charge character of the particles as already reported in the literature [46]. This low zeta potential can be attributed to non-charge character of PCL.

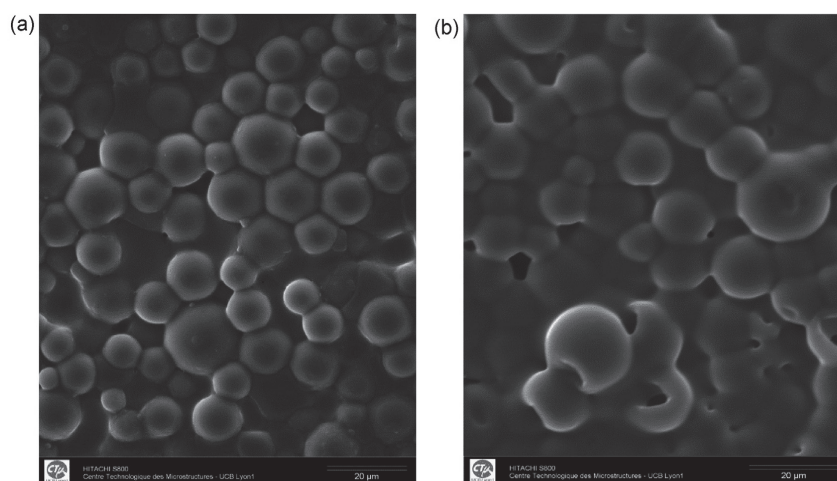


Fig. 13. (a) SEM image of PCL microparticles prepared in 0.5% PVA concentration. (b) SEM image of PCL microparticles prepared in 2% PVA concentration.

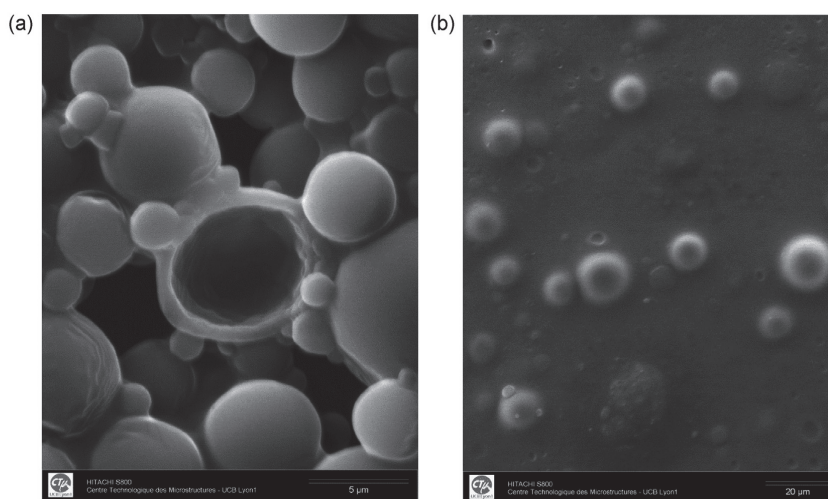


Fig. 14. (a) SEM image of PCL microparticles prepared under 4 min stirring. The scale bar represents 5 μm . (b) SEM image of PCL microparticles prepared under 6 min stirring.

3.3. Effects of different parameters on the morphology of the particles

The morphology of the PCL microparticles prepared by double emulsion technique was observed with help of Field Emission Scanning Electron Microscope (S-800 SEM Hitachi, Japan). Fig. 10a shows that the shape of the obtained microparticles can be assumed to be spherical with smooth surface and unimodal size distribution Fig. 11(b). Compare to the results obtained from Laser Diffraction Particle Size Analyzer, the particles measured with SEM were slightly smaller; this may be due to contraction induced by drying during evaporation of solvent. SEM images (Fig. 11a) show that some particles are observed to be connected with each other, which may be due to surface tension of water acting on

microparticles during drying which is in accordance with the results observed by Wu et al. [47]. These images show that all the particles are rounded shape. Some particles have pores on their surface, which may be due to evaporation of solvent during drying (Fig. 11b).

3.3.1. Polymer amount

The polymer amount is a significant factor that affects the characteristic of microparticles prepared by double emulsion method [40].

In this work, we observed two samples prepared with polymer amounts of 1 g and 3 g. From SEM image observations it was found that the particles obtained with 3 g polymer (Fig. 12a) having

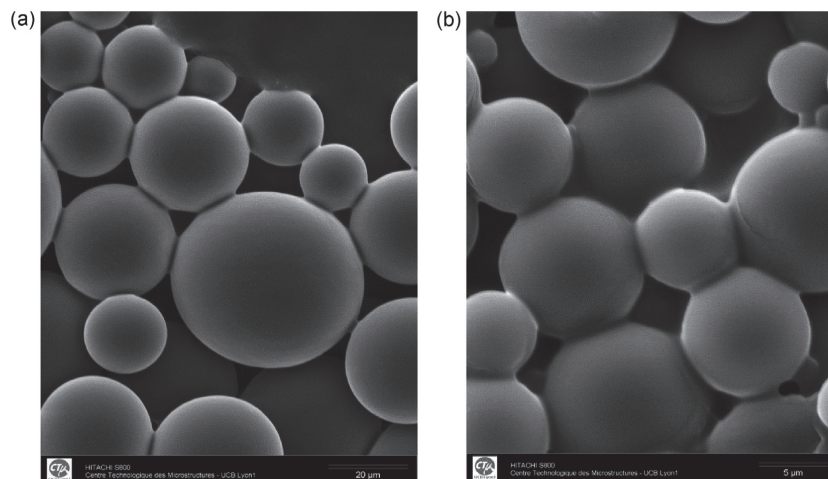


Fig. 15. (a) SEM image of PCL microparticles prepared under a stirring speed of 9500 rpm for the second emulsion. (b) SEM image of PCL microparticles prepared under a stirring speed of 21,500 rpm for the second emulsion. The scale bar represents 5 μm .

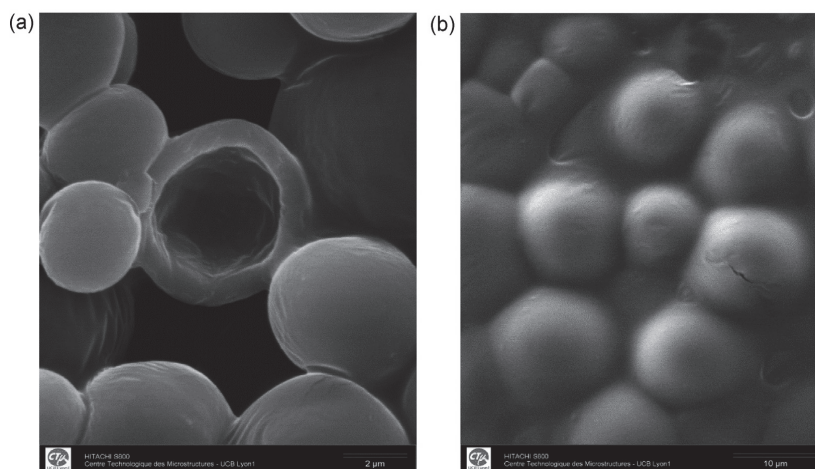


Fig. 16. (a) SEM image of PCL microparticles prepared with 50 ml of outer aqueous phase. The scale bar represents 2 µm. (b) SEM image of PCL microparticles prepared with 150 ml of outer aqueous phase. The scale bar represents 10 µm.

narrow size distribution and more regular shape as compare to particles obtained from 1 g polymer amount as shown in Fig. 12b.

3.3.2. The influence of PVA concentration in the external water phase

The significant influence of the concentration of PVA in the external water phase on the size of resultant particles is reported in many studies [48–50]. In this study, two samples were prepared with PVA concentration of 0.5% and 2% in the external water phase and its influence on the particle morphology was studied using SEM. From the SEM images it is evident that the particles prepared with 0.5% PVA has shown good morphology with regular rounded shape (Fig. 13a) as compare to particles prepared with 2% PVA, which were clumped together as show in Fig. 13b. This clumping

may be due excessive PVA, which results in sticking of particles during drying.

3.3.3. The influence of time of agitation

The influence of agitation time for both first and second emulsion was studied. For 1st emulsion, particles were prepared with agitation time of 4 min and 6 min. From the SEM images, it was found that agitation time of first emulsion has negligible effect on the size and morphology of particles.

In second emulsion the particles were prepared with the same agitation time i.e. 4 min and 6 min. When the samples of second emulsion were observed with SEM, it was found that the particles prepared with 6 min agitation time has regular morphology (Fig. 14b) on the other hand the particles with 4 min agitation time have not well encapsulated the inner water phase and some

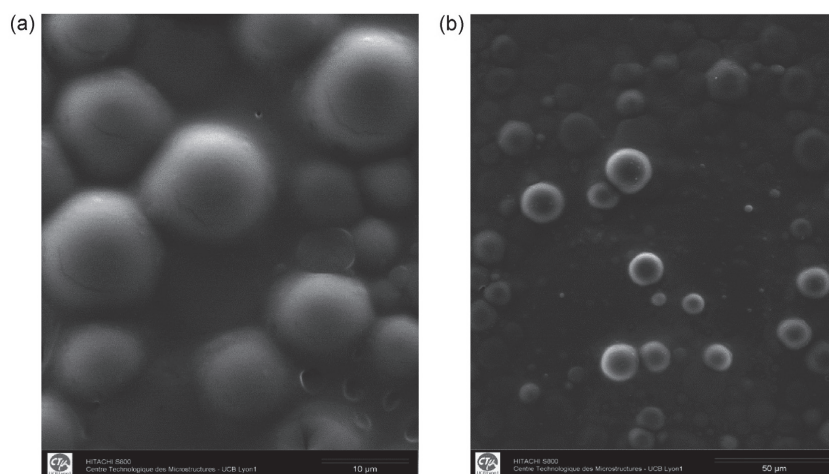


Fig. 17. (a) SEM image of PCL microparticles prepared using 1000 µl inner aqueous phase volume. The scale bar represents 10 µm. (b) SEM image of PCL microparticles prepared using 1500 µl inner aqueous phase volume. The scale bar represents 50 µm.

particles have cup shape morphology as shown in Fig. 14a. The homogeneity of particles size increased when the second emulsion was agitated for long period of time, these results in accordance with the results reported by Ayoub et al. [2].

3.3.4. The influence of stirring speed

The stirring speed is the vital parameter affecting the particle size because it supplies the necessary energy to disperse the dispersed phase into continuous phase, some researchers reported that, the second emulsion is preferred to prepare by avoiding sever mixing because it may destroy the first emulsion W_1/O prepared in the first step [34]. From SEM images, it was found that stirring speed of first emulsion has no significant influence on the particle shape and size. In case of second emulsion, SEM study established that the microparticles prepared with 21,500 rpm have narrow size distribution (Fig. 15b) as compare to particles prepared with 9500 rpm (Fig. 15a).

3.3.5. Effect of outer water-phase volume

The volume of external water phase is an important factor that plays vital role in determining the size, shape and size distribution of resultant particles.

From SEM observation, it was found that the particles prepared by using external water phase 50 ml, some particles obtained have cup shape morphology as shown in Fig. 16a. On the other hand the particles obtained with 150 ml external aqueous phase has regular rounded shape and narrow size distribution (Fig. 16b).

3.3.6. Influence of inner water phase (W_1) volume

The effect of this parameter was studied by comparing the SEM photographs of two samples, in first one the internal aqueous phase was 1000 μl and in the second it was 1500 μl .

From the SEM images observation, it was found that the particle obtained with 1000 μl internal aqueous phase have cracks on their surfaces with spherical shape Fig. 17a, while the other particles which are prepared by using 1500 μl as internal aqueous phase have rounded shape and good morphology.

4. Conclusion

Multiple emulsions are important systems; consisting of two immiscible phases, this system is thermodynamically unstable so suitable emulsifying agent is added in order to stabilize it.

In this study the conditions and parameters which influencing properties especially particle size and morphology of double emulsion were well studied. Microparticles were prepared by using two-step emulsification solvent diffusion process. The effect of different parameters such as Stirring time (of 1st and 2nd step), stirring speed (of 1st and 2nd step), polymer amount, stabilizer concentration, phase volume of aqueous passes (inner and outer) on the partial size was investigated.

The main finding of this study was that:

There was no significant effect of stirring speed in double emulsion preparation during step first, but during step 2nd with an increase in stirring speed the particle size of emulsion decreased proportionally.

The time duration of stirring in first step of double emulsion has no significant impact on particle size while during 2nd step longer stirring time has led to smaller size particles.

The concentration of polycaprolactone (selected polymer for encapsulating) has a significant influence on the particles size i.e. particles size proportionately increased when the amount of PCL were increased. In case of stabilizer concentration it was concluded

that at low concentration of PVA (stabilizer) smaller size particle can be achieved than at higher concentration.

The relative volumes of aqueous phases are also an important factor in double emulsion particle size evaluation; from this study it is established, that the phase volume of inner aqueous phase (W_1) has no significant effect on the particle size of double emulsion while on the other hand volume of outer aqueous phase (W_2) has a prominent effect on the final particle size.

From SEM images observation, it was found that the surface of obtained microparticles can be assumed to be spherical with smooth surface, having narrow size distribution. Compare to the results from Laser Diffraction Particles Size Analyses, the particles measure with SEM were slightly smaller in size this may be due to contraction induced by drying during evaporation of solvent.

In this work we study the effects of different parameters affecting the particle size of double emulsion, further evaluation study have to be performed in order to determine suitable amount of active ingredient such as proteins to be loaded in the inner aqueous phase (W_1) and also to study the release of active medicaments from these microparticles.

References

- [1] E.V.R. Campos, N.F.S. de Melo, E. de Paula, A.H. Rosa, L.F. Fraceto, Screening of conditions for the preparation of poly(ϵ -caprolactone) nanocapsules containing the local anesthetic articaine, *J. Colloids Sci. Biotechnol.* 2 (June (2)) (2013) 106–111.
- [2] M. Ayoub, N. Ahmed, N. Kalaji, C. Charcosset, A. Magdy, H. Fessi, A. Elaissari, Study of the effect of formulation parameters/variables to control the nanoencapsulation of hydrophilic drug via double emulsion technique, *J. Biomed. Nanotechnol.* 7 (April (2)) (2011) 255–262.
- [3] V. Nedovic, A. Kalusevic, V. Manojlovic, S. Levic, B. Bugarski, An overview of encapsulation technologies for food applications, *Proc. Food Sci.* 1 (2011) 1806–1815.
- [4] Z. Fang, B. Bhandari, Encapsulation of polyphenols – a review, *Trends Food Sci. Technol.* 21 (October (10)) (2010) 510–523.
- [5] R.M. Nitika Agnihotri, Microencapsulation – a novel approach in drug delivery: a review, *Indo Glob. J. Pharm. Sci.* 2 (2012) 1–20.
- [6] R. Langer, Drug delivery and targeting, *Nature* 392 (April (6679 Suppl.)) (1998) 5–10.
- [7] M.L. Hans, M.A. Lowman, Biodegradable nanoparticles for drug delivery and targeting, *Curr. Opin. Solid State Mater. Sci.* 6 (August (4)) (2002) 319–327.
- [8] M.J. Cocero, A. Martín, F. Mattea, S. Varona, Encapsulation and co-precipitation processes with supercritical fluids: fundamentals and applications, *J. Supercrit. Fluids* 47 (January (3)) (2009) 546–555.
- [9] F.S. Poletto, R.C.R. Beck, S.S. Guterres, A.R. Pohlmann, Polymeric nanocapsules: concepts and applications, in: R. Beck, S. Guterres, A. Pohlmann (Eds.), *Nanocosmetics and Nanomedicines*, Springer, Berlin, Heidelberg, 2011, pp. 49–68.
- [10] Y.P. Neo, S. Ray, J. Jin, M. Gizdavic-Nikolaidis, M.K. Nieuwoudt, D. Liu, S.Y. Quek, Encapsulation of food grade antioxidant in natural biopolymer by electrospinning technique: a physicochemical study based on zein–gallic acid system, *Food Chem.* 136 (January (2)) (2013) 1013–1021.
- [11] M. Ahmad, K. Rashid, M. Nadeem, K. Masood, S. Ali, M. Nafees, N. Gull, Mumtaz-ul-Haq, N. Ibrahim, A. Saeed, A. Qureshy, F. Aleem, H. Naseer, S. Mehmood, S.W. Hyder, A simple method to prepare aqueous dispersion of iron oxide nanoparticles and their biodistribution study, *J. Colloids Sci. Biotechnol.* 1 (December (2)) (2012) 201–209.
- [12] D. Palomino, C. Yamunake, P.L. Coustumer, S. Stoll, Stability of TiO_2 nanoparticles in presence of fulvic acids. Importance of pH, *J. Colloids Sci. Biotechnol.* 2 (March (1)) (2013) 62–69.
- [13] A. Doustgani, E.V. Farahani, M. Imani, A.H. Doulabi, Dexamethasone sodium phosphate release from chitosan nanoparticles prepared by ionic gelation method, *J. Colloids Sci. Biotechnol.* 1 (June (1)) (2012) 42–50.
- [14] N.V.N. Jyothi, P.M. Prasanna, S.N. Sakarkar, K.S. Prabha, P.S. Ramaiah, G.Y. Srawan, Microencapsulation techniques, factors influencing encapsulation efficiency, *J. Microencapsul.* 27 (May (3)) (2010) 187–197.
- [15] R. Arshady, Albumin microspheres and microcapsules: methodology of manufacturing techniques, *J. Control. Release* 14 (October (2)) (1990) 111–131.
- [16] R. Herrero-Vanrell, M.F. Refojo, Biodegradable microspheres for vitreoretinal drug delivery, *Adv. Drug Deliv. Rev.* 52 (October (1)) (2001) 5–16.
- [17] J. Sara, Risch, Encapsulation: overview of uses and techniques Encapsulation and Controlled Release of Food Ingredients, vol. 590, American Chemical Society, 1995, pp. 2–7.
- [18] G.M. Barratt, Therapeutic applications of colloidal drug carriers, *Pharm. Sci. Technol. Today* 3 (May (5)) (2000) 163–171.
- [19] M.V. Chaubal, Application of formulation technologies in lead candidate selection and optimization, *Drug Discov. Today* 9 (July (14)) (2004) 603–609.

- [20] P. Legrand, G. Barratt, V. Mosqueira, H. Fessi, J.P. Devissaguet, Polymeric nanocapsules as drug delivery systems, *S.T.P. Pharm. Sci.* 9 (1999) 411–418.
- [21] M. Li, O. Rouaud, D. Poncelet, Microencapsulation by solvent evaporation: state of the art for process engineering approaches, *Int. J. Pharm.* 363 (November (1–2)) (2008) 26–39.
- [22] C.E. Mora-Huertas, H. Fessi, A. Elaissari, Polymer-based nanocapsules for drug delivery, *Int. J. Pharm.* 385 (January (1–2)) (2010) 113–142.
- [23] X. Rong, Y. Xie, X. Hao, T. Chen, Y. Wang, Y. Liu, Applications of polymeric nanocapsules in field of drug delivery systems, *Curr. Drug Discov. Technol.* 8 (September (3)) (2011) 173–187.
- [24] K.S. Soppimath, T.M. Aminabhavi, A.R. Kulkarni, W.E. Rudzinski, Biodegradable polymeric nanoparticles as drug delivery devices, *J. Control. Release* 70 (January (1–2)) (2001) 1–20.
- [25] A. Yaqoob Khan, S. Talegaonkar, Z. Iqbal, F. Jalees Ahmed, R. Krishan Khar, Multiple emulsions: an overview, *Curr. Drug Deliv.* 3 (October (4)) (2006) 429–443.
- [26] H.K. Makadia, S.J. Siegel, Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier, *Polymers* 3 (August (4)) (2011) 1377–1397.
- [27] P. Couvreur, M.J. Blanco-Prieto, F. Puisieux, B. Roques, E. Fattal, Multiple emulsion technology for the design of microspheres containing peptides and oligopeptides, *Adv. Drug Deliv. Rev.* 28 (October (1)) (1997) 85–96.
- [28] R. Alex, R. Bodmeier, Encapsulation of water-soluble drugs by a modified solvent evaporation method. I. Effect of process and formulation variables on drug entrapment, *J. Microencapsul.* 7 (September (3)) (1990) 347–355.
- [29] W. Seifriz, *Studies in Emulsions*, III–V, 2002.
- [30] T. Schmidts, D. Dobler, A.C. Guldán, N. Paulus, F. Runkel, Multiple W/O/W emulsions—using the required HLB for emulsifier evaluation, *Colloids Surf. Physicochem. Eng. Asp.* 372 (December (1–3)) (2010) 48–54.
- [31] J. Jiao, D.G. Rhodes, D.J. Burgess, Multiple emulsion stability: pressure balance and interfacial film strength, *J. Colloids Interface Sci.* 250 (June (2)) (2002) 444–450.
- [32] N. Akhtar, Y. Yazan, Formulation and characterization of a cosmetic multiple emulsion system containing macadamia nut oil and two antiaging agents, *Turkish J. Pharm. Sci.* (2005) 173–185.
- [33] N. Garti, Double emulsions—scope, limitations and new achievements, *Colloids Surf. Physicochem. Eng. Asp.* 123–124 (May) (1997) 233–246.
- [34] D.D. Vasiljevic, J.V. Parojcic, M.M. Primorac, Rheological and droplet size analysis of W/O/W multiple emulsions containing low concentrations of polymeric emulsifiers, *J. Serb. Chem. Soc. March* (2009) 801–816.
- [35] R. Kumar, M. Senthil Kumar, N. Mahadevan, Multiple emulsions: a review, *Int. J. Recent Adv. Pharm. Res.* (2012) 9–19.
- [36] L. Sapei, M.A. Naqvi, D. Rousseau, Stability and release properties of double emulsions for food applications, *Food Hydrocolloids* 27 (June (2)) (2012) 316–323.
- [37] I. Kobayashi, M. Nakajima, S. Mukataka, Preparation characteristics of oil-in-water emulsions using differently charged surfactants in straight-through microchannel emulsification, *Colloids Surf. Physicochem. Eng. Asp.* 229 (November (1–3)) (2003) 33–41.
- [38] D. Vasiljevic, J. Parojcic, M. Primorac, G. Vuleta, An investigation into the characteristics and drug release properties of multiple W/O/W emulsion systems containing low concentration of lipophilic polymeric emulsifier, *Int. J. Pharm.* 309 (February (1–2)) (2006) 171–177.
- [39] Y.Y. Yang, T.S. Chung, N.P. Ng, Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method, *Biomaterials* 22 (February (3)) (2001) 231–241.
- [40] A. Lamprecht, N. Ubrich, M. Hombreiro Pérez, C. Lehr, M. Hoffman, P. Maincent, Biodegradable monodispersed nanoparticles prepared by pressure homogenization–emulsification, *Int. J. Pharm.* 184 (July (1)) (1999) 97–105.
- [41] L. Sánchez-Silva, J.F. Rodríguez, P. Sánchez, Influence of different suspension stabilizers on the preparation of Rubitherm RT31 microcapsules, *Colloids Surf. Physicochem. Eng. Asp.* 390 (October (1–3)) (2011) 62–66.
- [42] R. Jalil, J.R. Nixon, Biodegradable poly(lactic acid) and poly(lactide-co-glycolide) microcapsules: problems associated with preparative techniques and release properties, *J. Microencapsul.* 7 (September (3)) (1990) 297–325.
- [43] S.C. Lee, J.T. Oh, M.H. Jang, S.I. Chung, Quantitative analysis of polyvinyl alcohol on the surface of poly(D,L-lactide-co-glycolide) microparticles prepared by solvent evaporation method: effect of particle size and PVA concentration, *J. Control. Release Off. J. Control. Release Soc.* 59 (May (2)) (1999) 123–132.
- [44] A.U. Khan, N.M. Ahmad, N. Mahmood, Rheological studies on stabilised zirconia aqueous suspensions, *J. Colloids Sci. Biotechnol.* 1 (December (2)) (2012) 175–184.
- [45] C.E. Mora-Huertas, H. Fessi, A. Elaissari, Influence of process and formulation parameters on the formation of submicron particles by solvent displacement and emulsification–diffusion methods: critical comparison, *Adv. Colloids Interface Sci.* 163 (April (2)) (2011) 90–122.
- [46] Y. Wu, R.L. Clark, Controllable porous polymer particles generated by electro-spraying, *J. Colloids Interface Sci.* 310 (June (2)) (2007) 529–535.
- [47] N. Celebi, N. Erden, A. Türkyilmaz, The preparation and evaluation of salbutamol sulphate containing poly(lactic acid-co-glycolic acid) microspheres with factorial design-based studies, *Int. J. Pharm.* 136 (June (1–2)) (1996) 89–100.
- [48] H. Jeffery, S.S. Davis, D.T. O'Hagan, The preparation and characterization of poly(lactide-co-glycolide) microparticles. II. The entrapment of a model protein using a (water-in-oil)-in-water emulsion solvent evaporation technique, *Pharm. Res.* 10 (March (3)) (1993) 362–368.
- [49] M.K. Yeh, A.G.A. Coombes, P.G. Jenkins, S.S. Davis, A novel emulsification-solvent extraction technique for production of protein loaded biodegradable microparticles for vaccine and drug delivery, *J. Control. Release* 33 (March (3)) (1995) 437–445.

3.2. Encapsulation des acides nucléiques

La thérapie génique est une méthode prometteuse pour traiter un grand nombre de maladies graves. Son application avec réussite chez l'homme est directement associée à la découverte de systèmes de transfert du gène d'intérêt, efficacement et sans risque au noyau de la cellule ciblée. Des systèmes divers et variés sont proposés pour atteindre cet objectif ; au départ en utilisant des virus, en passant par l'utilisation de systèmes physiques, et pour arriver à encapsuler le gène par l'utilisation de différentes techniques et vecteurs comme les liposomes, adsorption sur particules cationiques, encapsulation dans des couches de polymères etc... . Dans ce contexte, notre laboratoire a développé un grand nombre de méthode d'encapsulations de molécules actives et également des biomolécules. Une des méthodes pour encapsuler l'ADN dans les nanoparticules biodégradables est basée sur l'utilisation de la technique émulsion multiple et l'évaporation de solvant. Ceci permet d'assurer la protection et la délivrance du gène encapsulé.

Notre étude présente l'application de la technique d'émulsion multiple et évaporation de solvant, afin d'encapsuler l'ADN dans un matrice de polymère biodégradable. Le polycaprolactone (PCL) est choisi pour encapsuler l'ADN grâce à sa biocompatibilité et sa biodégradabilité. Le (PCL) est dissous dans le dichlorométhane (DCM) comme solvant organique. Cette solution est la phase huileuse de la première émulsion (E/H). Alors que la phase aqueuse de cette même émulsion est une solution d'ADN à concentration donnée. Pour la préparation de cette émulsion, la phase aqueuse est ajoutée à la phase huileuse sous agitation forte favoriser la fragmentation des gouttes aqueuses dans la solution de polymère et par conséquent la formation de la première émulsion eau dans huile (E/H). Cette émulsion (E/H) est ajoutée à une solution d'alcool polyvinylique (PVA) dans l'eau distillée sous agitation contrôlée pour avoir une émulsion double eau dans huile dans eau (E/H/E). La dernière étape est l'élimination du DCM par évaporation au rotavapor. L'ADN hydrophile est localisé dans les cavités hydrophiles dispersées dans la matrice polymère. Cette étude d'encapsulation est complétée en caractérisant les dispersions finales en terme de taille, la distribution de taille, morphologie, stabilité colloïdale et propriétés électrocinétiques. La taille hydrodynamique est de l'ordre de 260 nm et une distribution en taille relativement étroite. Le potentiel zêta des particules étudié en fonction du pH montre que les particules sont faiblement chargées et négativement. La morphologie des particules examinée par microscopie électronique à transmission montre que les particules obtenues ont une forme

sphérique et homogène. Finalement, l'efficacité d'encapsulation de l'ADN est quantifiée par spectrophotomètre UV, la quantité encapsulée étant de l'ordre de 95%.

En conclusion, la technique d'émulsion multiple et l'évaporation de solvant a été étudiée et appliquée pour encapsuler l'ADN afin d'être utilisée efficacement en thérapie génique. On a trouvé qu'il est possible d'encapsuler l'ADN dans un réservoir polymère stable. Par conséquent, la technique d'émulsion multiple et l'évaporation de solvant peut être prometteuse comme méthode pour encapsuler l'ADN pour les applications in vivo.



DNA Encapsulation via Double Emulsion Like Process

D. Ibraheem, H. Fessi*, and A. Elaissari

University of Lyon, F-69622, Lyon, France; University Lyon-1, Villeurbanne; CNRS, UMR 5007,
LAGEP- CPE; 43 bd 11 Novembre 1918, F-69622 Villeurbanne, France

Gene therapy is one of the advanced methods used for treatment of incurable diseases such as cancer, acquired immunodeficiency syndrome, cardiovascular and infectious diseases. However, the efficiency of gene therapy is directly related to the access of nucleic acid (DNA) to the nucleus avoiding its degradation via enzymatic effect. So, the challenge is the access of non-degradable gene into the target cell-nucleus. This goal can be achieved by encapsulating the selected gene in a polymer matrix in order to be protected from degradation. Accordingly, this work aims to encapsulate DNA model in the biodegradable polycaprolactone (PCL) particles using water in oil in water (W/O/W) double emulsion evaporation technique. Via this method, DNA solution was mixed with dichloromethane (DCM) solution containing PCL in order to obtain the first water in oil emulsion (W₁/O). Then, the obtained W₁/O emulsion was dispersed in the aqueous phase containing poly(vinyl alcohol) (PVA) as a stabilizing agent in order to obtain the final water in oil in water emulsion (W₁/O/W₂) after DCM evaporation. In this study a special attention was dedicated to study the effect of DNA concentration on the colloidal properties of the final particles, including hydrodynamic particles size, size distribution, and zeta potential, in addition to its encapsulation efficiency by the biodegradable PCL particles. The obtained results proved that the w/o/w double emulsion–evaporation technique is highly efficient for encapsulation of DNA in submicron (260 nm) PCL colloidal particles with high encapsulation efficiency.

Keywords: Encapsulation, DNA, Biodegradable Polymer, Nanoparticles, Double Emulsion–Evaporation.

1. INTRODUCTION

Over the past two decades, gene therapy has attracted the attention of scientists as a promising approach that can be employed effectively to cure a wide range of serious heritable and non heritable diseases such as cancer, acquired immunodeficiency syndrome, cardiovascular and infectious diseases.¹ Unlike the traditional treatment, gene therapy works at the malfunction sources instead of addressing the malfunction results. This can be achieved by inserting a functional gene,² which plays the role of active molecule, into the targeted cell in order to treat the disease or to repair a dysfunction caused by a genetic defect.

Theoretically, gene therapy is considered as a simple therapeutic method and depends on replacing the faulty gene with a healthy one that will express the required protein.³ Practically, it is a complex process due to several obstacles which have to be overcome by transgene to achieve the targeted nucleus of the human cell where

it should give its proper expression.⁴ Accordingly, the efficiency of gene therapy is directly related to the access of nucleic acid (DNA) to the nucleus avoiding its degradation by enzymatic effect.^{5,6} DNA is a hydrophilic negatively charged biomacromolecule and it is threatened to degradation by nucleases enzymes.^{6,7} So the exiting challenge in gene therapy approach is the access of non-degradable gene into the target cell-nucleus.⁸

For a long time, virus based carriers were worthily employed in order to avoid DNA degradation by the enzymes located in the biological medium, and to carry DNA molecule to the target cell nucleus passing through the cell membrane. Such virus based carriers approach of nucleic acid transportation is called *viral vectors*.⁹ However, the drastic reduction in immunological response, which may be raised by these viruses when used *in vivo* therapy, in addition to the high cost for preparation of such carriers have restricted their use.¹⁰ This consequently stimulated scientists to develop new methods leading to more efficient and safe therapy, and can be easily used on the industrial scale with low cost, as compared with viral vectors.⁶

*Author to whom correspondence should be addressed.

In this regards, another interesting approach (*non-viral vectors*) based on the electrostatic interaction of the negatively charged DNA and positively charged well selected polymers (polyplex)¹¹ or lipid (lipoplex)¹² has been investigated and used in gene therapy. However, this more safe approach can be used as a promising alternative for viral vectors, but with less efficiency. In order to enhance the efficacy of *non-viral vectors*, DNA-encapsulation appeared to be of great interest, which may be performed via various processes such as water-in-oil emulsion, liposomes, or polyelectrolyte capsules.⁸ Among the emulsification techniques, double emulsion evaporation method (DEE) is considered as one of the most interesting approaches for encapsulating nucleic acids, leading to fabrication of biodegradable polymer nanoparticles containing DNA. In addition, DEE method is more suitable for encapsulating both hydrophobic or hydrophilic drugs in submicron and micro-particles.¹³

As can be deduced from its name, double emulsion process consists in the emulsification of two emulsions; the first emulsion plays the role of the internal phase, which contains the active hydrophilic molecules or biomolecules (e.g., protein)¹⁴ or drug, while the continuous phase of the second emulsion contains the final external phase containing the stabilizing agent.¹⁵ Generally, there are two types of double emulsion; water-in-oil-in water (w/o/w) and oil-in-water-in-oil (o/w/o).¹⁶

Although the hydrodynamic size of the resulting particles using such method is micrometric, nanoparticles have been also obtained after optimization of the operating conditions^{17,18} by using high speed shear mixers e.g., ultraturrax or by using ultra-sonication technique. Additional factors can affect significantly the hydrodynamic particles size, including time of stirring, stirring speed and concentration of the stabilizing surfactant.

Different methods have been explored to ensure DNA encapsulation. Double emulsion like process has been described as one of the most appropriate method leading to encapsulation of hydrophilic drugs and proteins.^{19,20} Double emulsion-solvent evaporation using polycaprolactone (PCL) was generally selected leading to biodegradable polymer matrix, which is highly favourable for therapeutic applications.^{21,22}

Then, this work aims to prepare polycaprolactone (PCL) nanoparticles loaded with DNA in order to be used in therapeutic purposes; especially in gene therapy. The encapsulation process was carried out using double emulsion evaporation method, which is proved to be the most appropriate method to encapsulate hydrophilic molecules such as DNA and proteins within micro or nanoparticles as reported by various authors.^{19,20,23} The most characteristic features of the obtained particles were investigated including size and size-distribution, morphology, and zeta potential as a function of pH of the final dispersion medium. In addition, the encapsulation efficiency of DNA was also estimated via depletion method.

2. MATERIALS AND METHODS

2.1. Materials

Polycaprolactone (PCL) (Mw = 14.000 g/mol), polyvinyl alcohol (PVA) (Mw = 31.000 g/mol), dichloromethane (DCM) and NaCl were purchased from Sigma-Aldrich, Germany and were used as received. Sheared Salmon sperm DNA (Amboion 1 ml of 10 mg DNA/ml) was from Invitrogen, U.S.A. Water of Milli-Q grade was used in all experiments.

2.2. Methods

2.2.1. Preparation of Nanoparticles

A series of DNA containing polycaprolactone (PCL) nanocapsules were prepared using double emulsion-evaporation method (DEE) at different DNA concentrations. This approach is usually performed in two stages; the first one is the preparation of the first emulsion (W_1/O), which will be the internal phase in the final emulsion. The second stage is the preparation of (W_1/O)/ W_2 emulsion by mixing the first (W_1/O) emulsion with water phase containing the suitable stabilizing agent (in this case, PVA was used).

At first, PCL polymer was dissolved in dichloromethane (DCM).²⁴ The total volume of the first water phase containing DNA of the first emulsion was fixed at 1.5 ml. Then, DNA solution (at a given concentration) was added to the organic (12 ml DCM) phase containing 3 g of polymer (PCL), followed by high shear mixing by using ultraturrax at a speed of 17500 rpm during 15 minutes forming the first W_1/O emulsion.

The prepared W_1/O emulsion was then mixed with 150 ml of 0.5 wt% PVA aqueous solution using the ultraturrax at 21500 rpm shearing speed during 15 minutes in order to obtain the final $W_1/O/W_2$ double emulsion. The DCM organic solvent was evaporated from $W_1/O/W_2$ double emulsion under reduced pressure to obtain the final stable dispersion of PCL-encapsulated DNA stabilized with PVA polymer.

Here, it is worthy to mention that all double emulsion preparation parameters in this research were chosen according to previously done experimental works.^{13,23,25,26} These optimized parameters include the concentration of PCL and PVA, the stirring speed and time used for preparing each phase of the double emulsion. According to these research attempts, the best hydrophilic drug-loaded nanoparticles are obtained via double emulsion prepared at these parameters.

A schematic illustration for preparation of DNA encapsulated with PCL polymer particles using double emulsion evaporation process is shown in Figure 1.

2.3. Characterization

2.3.1. Particle Size and Size Distribution Measurements

The average hydrodynamic size (D_h) of the prepared DNA containing PCL-particles was measured via dynamic light

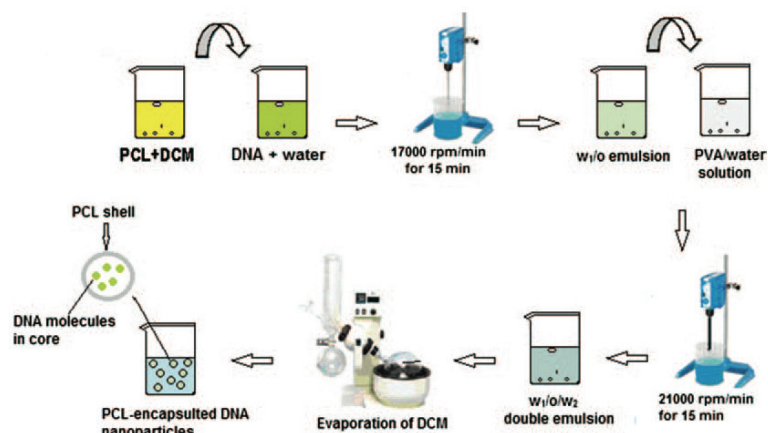


Fig. 1. Double emulsion–evaporation method for encapsulation of DNA.

scattering technique in 10^{-3} M NaCl solution at 25 °C using Zetasizer NanoZS2000 (Malvern instrument). Each recorded value was the average of 30 measurements.

2.3.2. Transmission Electron Microscopy (TEM)

The morphology and microstructure of the PCL-encapsulated DNA colloidal particles were investigated by transmission electron microscopy using Phillips CM120 microscope, CMEABG, University of Claude Bernard Lyon I. Briefly, a drop of water-diluted sample was deposited onto a carbon-coated copper grid and then it was left to dry at room temperature overnight before TEM imaging.

2.3.3. Zeta Potential Measurements

Zeta potential of the prepared colloidal particles was measured using Malvern Zetasizer (NanoZS2000) instrument. The particles (after solvent evaporation) were first diluted in 1 mM NaCl solution, and then the electrophoretic mobility was measured at 25 °C. The measured electrophoretic mobility was then converted to zeta potential (ζ) using Smoluchowski equation $\zeta = \mu / \epsilon \eta$,²⁷ where η and ϵ are the viscosity and the dielectric constant of the dispersion medium, respectively. The reported zeta potential values are the average of five measurements.

2.3.4. DNA Encapsulation Efficiency Measurements

The encapsulation efficiency of DNA in the resulting nanoparticles was calculated using the depletion method. In this method, the concentration of free DNA (non-encapsulated) in the final aqueous phase of the dispersion was determined. The supernatant was collected and the amount of DNA was measured using UV spectrophotometer at 260 nm wavelength.

3. RESULTS AND DISCUSSION

The formulation used in this study was first examined and then optimized via systematic study by investigating all external parameters, chemical composition of the recipe and also the nature and the concentration of the used stabilizing agent in the aqueous phase.

3.1. Particle Size and Size Distribution Measurements

As previously mentioned, a series of PCL particles encapsulated DNA were prepared via double emulsion–evaporation technique and using various DNA concentrations ranging from 1.33 to 3.33 mg/ml. The size of the obtained PCL-DNA particles was found to be submicron and ranging from 250 to 270 nm irrespective of DNA concentration in the formulation recipe as shown in Table I. In addition, the prepared PCL-DNA particles exhibited narrow size distribution, as shown in Figure 2.

Here, it is interesting to notice that the particle size and size distribution are almost the same before and after solvent evaporation and the effect of DNA amount can be considered as negligible which explains the robustness of such optimized process. As expect, since the volume of the internal water phase wasn't changed, the final particles sizes are then not affect.

Table I. Particle size of PCL-encapsulated DNA particles as a function of DNA concentration.

DNA concentration (mg/ml)	Hydrodynamic particle size (nm)
0.00	265
1.33	273
2.33	260
2.66	253
3.33	258

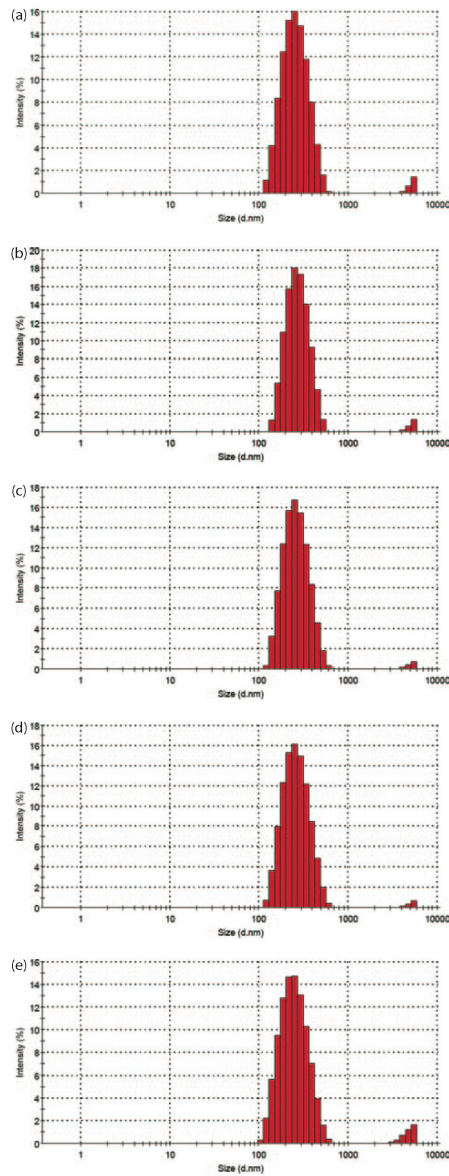


Fig. 2. Size distribution of PCL-encapsulated DNA colloidal particles at different DNA concentrations; (a) DNA free particles, (b) 1.33 mg/ml DNA, (c) 2.33 mg/ml DNA, (d) 2.66 mg/ml DNA, (e) 3.33 mg/ml DNA.

4

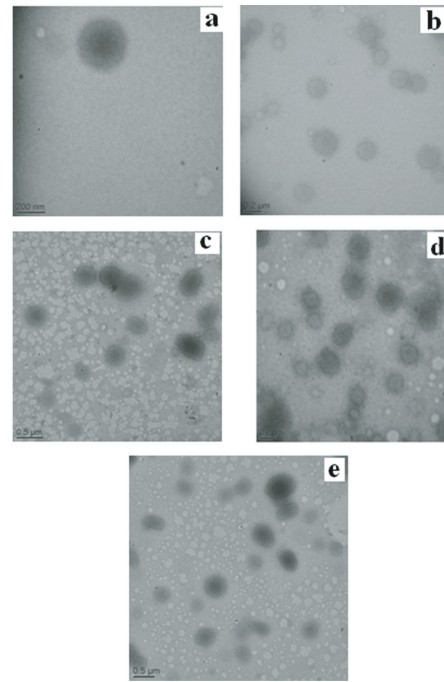


Fig. 3. TEM micrographs of PCL-encapsulated DNA colloidal particles at different DNA concentrations; (a) DNA free particles, (b) 1.33 mg/ml DNA, (c) 2.33 mg/ml DNA, (d) 2.66 mg/ml DNA, (e) 3.33 mg/ml DNA.

3.2. Transmission Electron Microscopy Analysis

The morphology of the prepared particles was examined using transmission electron microscopy (TEM). The first analysis shows the spherical shape of the obtained particles and also the slight polydispersity of all dispersions irrespective of DNA concentration as shown in Figure 3. Secondly, the location of DNA can't be evidenced from these images and this can be attributed to the low contrast difference between the used polymer and the DNA location part.

3.3. Zeta Potential Measurements

Since the access to surface charge density of such biodegradable system is impossible, the surface charge density was deduced from the measured zeta potential. Zeta potential (ζ) measurements were carried out in order to study the effect of pH and DNA encapsulated amount on the surface charge of the formed particles. As shown in Figure 4, the zeta potential of DNA free particles was found to be negative irrespective of pH of the dispersion medium. This can be attributed to the presence of carboxylic groups at the end of PCL chains.

J. Colloid Sci. Biotechnol. 2, 1–6, 2014

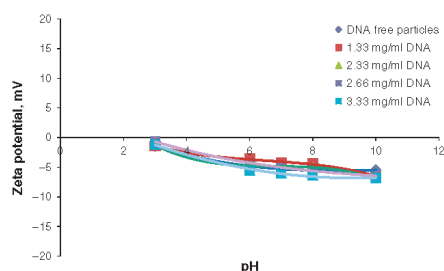


Fig. 4. Zeta potential of PCL-encapsulated DNA colloidal particles as a function of pH at different DNA concentrations; (●) DNA free particles, (■) 1.33 mg/ml DNA, (▲) 2.33 mg/ml DNA, (×) 2.66 mg/ml DNA, (∗) 3.33 mg/ml DNA.

Regarding the PCL particles containing encapsulated DNA, the determined zeta potential was found to be both pH and DNA concentration independent. In addition, the zeta potential profiles for DNA free and DNA containing PCL particles are the same irrespective of pH. Such behaviour can be attributed to the encapsulation of DNA rather than adsorption on the particles surface. In fact, if the DNA was only adsorbed on the PCL particles, the zeta potential should be higher than for DNA free particles. For more comparison, the surface charge density of particles dispersion was directly estimated from the measured electrophoretic mobility and calculated using the following approximated equation valid for low surface charge density related to both Helmholtz's and Smoluchowski's formula:²⁷

$$\mu = \frac{\sigma}{\eta\kappa} \quad (1)$$

Where, μ is the electrophoretic mobility, σ is the surface charge density, η is the viscosity and κ is the Debye length. The deduced surface charge densities are reported in Table II.

As clearly seen in Table II, DNA free and DNA containing PCL colloidal particles exhibit low negative zeta potential and consequently low surface charge density in the investigated pH range and conditions. In fact, the estimated average surface charge density was found to be in between 0.2 to 1.5 $\mu\text{C}/\text{cm}^2$.

It's interesting to notice that the zeta potential of all particles slightly decreases by increasing pH of the aqueous

Table III. Encapsulation efficiency of DNA as a function of DNA concentration.

Initial DNA concentration in aqueous phase (mg/ml)	Initial DNA concentration in PCL (mg/g of PCL)	Encapsulated DNA (mg/g PCL)	Encapsulation efficiency (%)
1.33	0.66	0.5983	95.34
2.33	1.16	1.1075	97.77
2.66	1.50	1.4553	98.31
3.33	1.66	1.5764	47.33

medium. This can be attributed to the negative surface charge density enhancement arising from the possible hydrolysis of the PCL polymer as recently discussed by Mora-Huertas et al.²⁸

3.4. Encapsulation Efficiency of DNA

The encapsulation efficiency of DNA in the PCL particles was quantified using depletion-based method. The concentration of free DNA (non-encapsulated) in the centrifuged supernatant was first determined using UV at 260 nm wavelength. The concentration of encapsulated DNA is determined by calculating the difference between the initial concentration and the residual DNA in the supernatant. The concentration of initial and encapsulated DNA was expressed in mg per gram of PCL polymer used in the recipe, as reported in Table III.

As can be deduced from the encapsulation efficiency, the maximum encapsulation amount was found to be directly related to the initial DNA amount used in the internal aqueous phase. In fact, until 1.5 mg DNA/g PCL polymer, the encapsulated amount can be considered as constant and the encapsulation efficiency is above 90%. This can be attributed to high hydrophilic character of DNA which majority found in the first emulsion (i.e., nanodroplets surrounded by DCM and polymer. Then, the probability to reach the external aqueous phase during the shearing process is low and consequently, the total DNA amount was found to be encapsulated in polymer matrix, which leads to good encapsulation efficiency.

In comparison to what has been reported in the literature using biodegradable polymers, the encapsulated DNA amount is mainly related to the nature of the used polymers and encapsulation process. In fact, the encapsulated DNA amount was found to be in between 0.6 to 1.5 mg/g

Table II. Zeta potential (mV)/surface charge density ($\mu\text{C}/\text{cm}^2$) of PCL-DNA colloidal particles as a function of pH at different DNA concentrations.

DNA concentration (mg/ml)	Zeta potential (mV)/ σ ($\mu\text{C}/\text{cm}^2$) at different pH values				
	3.5	6.3	7.0	8.3	10
0	-1.4/-0.31	-4.7/-1.04	-6.3/-1.36	-5.4/-1.20	-4.4/-0.96
1.33	-1.4/-0.31	-3.6/-0.80	-4.2/-0.88	-4.4/-0.96	-5.6/-1.20
2.33	-1.2/-0.26	-4.6/-0.96	-5.1/-1.12	-4.8/-1.04	-7.0/-1.52
2.66	-0.6/-0.12	-4.3/-0.96	-4.8/-1.04	-5.7/-1.20	-6.4/-1.36
3.33	-1.1/-0.24	-5.5/-1.20	-6.0/-1.28	-6.3/-1.36	-6.8/-1.44

of poly(D,L-lactic-co-glycolic acid) based microspheres⁷ and 5 mg/g in the case of PLA-PEG prepared via water in oil in water emulsion evaporation process.²⁹ The use of organic phase self-emulsification DNA encapsulated amount in poly (D,L-lactic-co-glycolic acid) (PLGA) microspheres was found to be around 3.3 mg/g.³⁰ ~~The encapsulated DNA amount (in between 0.5 to 1.5 mg/g) in the case of our study was found to be in agreement with the reported results.~~

4. CONCLUSIONS

The encapsulation of active molecules is the best method to protect and to deliver in proper way in well-targeted *in-vivo*-site. Various approaches have been used to encapsulate such attractive molecules starting from chemistry base process to capsules formulation using preformed polymers. In case of the encapsulation of biomolecules, the use of chemistry-based process was not well appropriate since it may induce chemical modification and denaturation of biomolecules principally in the case of proteins. Consequently, the encapsulation of biomacromolecules is mainly performed using a preformed polymer and non-denaturing solvents and conditions.

Submicron PCL-particles are prepared using double emulsion followed by solvent evaporation under optimized conditions. The second emulsification step was found to be the key parameter leading to final particles control. Unfortunately, the high polydispersity of the prepared systems cannot be controlled or solved using such process, which needs special attention in the near future.

Regarding the DNA encapsulation, the optimized formulation and conditions are used in order to examine the effect of DNA concentration. As expected, the average particles size was found to be constant irrespective of DNA concentration. This can be attributed to marginal effect of chemical composition of the internal aqueous phase (at a constant volume). In addition, the determined zeta potential versus pH was also found to be constant irrespective of DNA concentration. In fact, the negative zeta potential profiles were found to be the same and DNA concentration independent. Regarding the surface charge densities, the deduced values are low to maintain good colloidal stability, which need the use of stabilizing agent. As a consequence, the encapsulation of DNA using double emulsion solvent evaporation method could be a promising alternative to the conventional methods, which are chemistry-based approaches such as polymerization and polyplex assembly for gene therapy applications.

Acknowledgments: The authors thank and appreciate the research grant from Syrian government. The authors

also thank Dr. M. M. Eissa from Polymer and Pigments Department, National Research Centre, Dokki, Giza, Egypt for his help, suggestions and discussion.

References and Notes

1. X. Guo and L. Huang, *Acc. Chem. Res.* 45, 971 (2012).
2. F. D. Ledley, *Human Gene Therapy* 6, 1129 (1995).
3. D. S. Latchman, *Gene* 264, 1 (2001).
4. F. D. Ledley, *Human Gene Therapy* 2, 77 (1991).
5. L. Brannon-Peppas, B. Ghosn, K. Roy, and K. Cometta, *Pharm. Res.* 24, 618 (2007).
6. H. Y. Nam, J. H. Park, K. Kim, I. C. Kwon, and S. Y. Jeong, *Archives of Pharmacol Research* 32, 639 (2009).
7. D. Wang, D. R. Robinson, G. S. Kwon, and J. Samuel, *J. Control. Release* 57, 9 (1999).
8. A. N. Zelikin, A. L. Becker, A. P. R. Johnston, K. L. Wark, F. Tuutti, and F. Caruso, *ACS Nano* 1, 63 (2007).
9. G. R. Whittaker, *Adv. Drug Deliv. Rev.* 55, 733 (2003).
10. N. S. Templeton, *Biosci. Rep.* 22, 283 (2002).
11. B. Ma, S. Zhang, H. Jiang, B. Zhao, and H. Lv, *J. Control. Release* 123, 184 (2007).
12. P. L. Felgner, Y. Barenholz, J. P. Behr, S. H. Cheng, P. Cullis, L. Huang, J. A. Jessee, L. Seymour, F. Szoka, A. R. Thierry, E. Wanger, and G. Wu, *Human Gene Therapy* 8, 511 (1997).
13. M. Ayoub, N. Ahmed, N. Kalaji, C. Charcosset, A. Magdy, H. Fessi, and A. Elaissari, *J. Biomed. Nanotechnol.* 7, 255 (2011).
14. N. Kalaji, N. Sheibat-Othman, H. Saadaoui, A. Elaissari, and H. Fessi, *E. Polymers* 10, 1 (2009).
15. N. Garti, *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 123–124, 233 (1997).
16. A. Benichou, A. Aserin, and N. Garti, *Advances in Colloid and Interface Science* 108–109, 29 (2004).
17. M. Blanco and M. Alonso, *European Journal of Pharmaceutics and Biopharmaceutics* 43, 287 (1997).
18. M. Tobío, R. Gref, A. Sánchez, R. Langer, and M. J. Alonso, *Pharm. Res.* 15, 270 (1998).
19. S. Cohen, T. Yoshioka, M. Lucarelli, L. H. Hwang, and R. Langer, *Pharm. Res.* 8, 713 (1991).
20. R. Alex and R. Bodmeier, *J. Microencapsul.* 7, 347 (1990).
21. V. R. Sinha, K. Bansal, R. Kaushik, R. Kumria, and A. Trehan, *Int. J. Pharm.* 278, 1 (2004).
22. M.-A. Benoit, B. Baras, and J. Gillard, *Int. J. Pharm.* 184, 73 (1999).
23. M. F. Zambaux, F. Bonneaux, R. Gref, P. Maincent, E. Dellacherie, M. J. Alonso, P. Labrude, and C. Vigneron, *J. Control. Release* 50, 31 (1998).
24. M. Li, O. Rouaud, and D. Poncelet, *Int. J. Pharm.* 363, 26 (2008).
25. U. Bilati, E. Allémann, and E. Doelker, *AAPS PharmSciTech.* 6, E594 (2005).
26. A. Deloge, N. Kalaji, N. Sheibat-Othman, V. S. Lin, P. Farge, and H. Fessi, *J. Nanosci. Nanotech.* 8, 467 (2009).
27. R. J. Hunter, *Zeta Potential in Colloid Science: Principles and Applications*, Academic Press, London, New York (1981).
28. C. Mora-Huertas, F. Couenne, H. Fessi, and A. Elaissari, *J. Nanoparticle Research* 14, 1 (2012).
29. C. Perez, A. Sanchez, D. Putnam, D. Ting, R. Langer, and M. Alonso, *J. Control. Release* 75, 211 (2001).
30. F. F. Zhuang, R. Liang, C. T. Zou, H. Ma, C. X. Zheng, and M. X. Duan, *J. Biochem. Biophys. Methods* 52, 169 (2002).

Received: 25 October 2013. Accepted: 5 November 2013.

3.3. Encapsulations des protéines

Dans cette étude, le procédé double émulsion évaporation de solvant a été utilisé pour encapsuler la protéine d'albumine humaine (HSA, utilisée comme modèle) à l'intérieur des particules de polycaprolactone. L'utilisation de ce polymère biodégradable est dans l'objectif d'une application immunothérapeutique *in vivo*. Comme préalablement décrit plus haut, le procédé d'encapsulation utilisé dans le cadre de cette étude est basé sur l'utilisation d'une solution organique de polycaprolactone dans le dichlorométhane (DCM) et une solution aqueuse d'alcool polyvinylique (PVA) comme stabilisant. Les particules de polycaprolactone élaborées contenant de l'albumine encapsulée ont été caractérisées en déterminant la taille, la distribution de taille, la stabilité colloïdale (en examinant judicieusement le potentiel zêta des particules en fonction du pH), et la morphologie des particules. Les résultats obtenus ont montré que les particules de polycaprolactone chargées en albumine humaine sont de taille submicronique (avec une taille moyenne proche de 340 nm) et une distribution de taille large montrant la polydispersité des particules. Les particules élaborées sont bien sphériques avec une surface relativement lisse et présentent une bonne stabilité colloïdale en présence de l'agent stabilisant le PVA. Mais ces particules présentent une très faible densité de charge négative à la surface comme mesurée par le potentiel zêta trouvé compris entre 0 et -5 mV. En ce qui concerne l'efficacité d'encapsulation de la protéine modèle (HSA), un pourcentage élevé (jusqu'à 95 %) de la protéine utilisée a été encapsulée à l'intérieur des particules de polycaprolactone, comme analysé par microscopie confocale et évalué par le dosage de Bradford spécifique aux protéines.

Preparation and characterization of albumin –loaded polycaprolactone nanoparticles for *in vivo* applications

D. Ibraheem¹, G. Agusti¹, Mohamed M. Eissa², A. Elaissari¹, H. Fessi^{1*}

¹ University of Lyon, F-69622, Lyon, France; Université Lyon-1, Villeurbanne; CNRS, UMR 5007, LAGEP - CPE; 43 bd 11 Novembre 1918, F-69622 Villeurbanne, France

² Polymers and Pigments Department, National Research Centre, Dokki, Giza 12622, Egypt.

Correspondence to: Fessi@lagep.univ-lyon1.fr

Abstract

In this research, double emulsion-solvent evaporation method (DEE) was used to encapsulate the human albumin protein within the biodegradable polycaprolactone (PCL) polymer in order to be used in drug delivery applications. The encapsulation process was carried out with the aid of dichloromethane (DCM) as the organic solvent and polyvinyl alcohol (PVA) as colloidal stabilizer. The obtained human albumin–loaded polycaprolactone particles were characterized in terms of their size, size distribution, colloidal stability (using zeta potential measurements), and particles morphology. The obtained results showed that the human albumin–loaded polycaprolactone particles are submicron in size (≈ 340 nm) with broad size distribution. In addition, they have core-shell structure with spherical morphology, and have good colloidal stability in presence of PVA stabilizer but with low negative surface charge density. More interestingly, a high percentage (up to 95%) of the used human albumin protein was encapsulated within PCL particles, as evidenced by the confocal microscopic images and Bradford assay of the prepared particles.

Key words: Encapsulation, Human albumin protein, Polycaprolactone, Double emulsion/solvent evaporation, Therapeutic applications.

1. Introduction:

In the last decades, biotechnologists have focused on the encapsulation of drugs because it offers many advantages such as protecting the drug against degradation, facilitating its intracellular penetration, increasing the accuracy of drug delivery to the infected site, and controlling the rate of drug release ¹. This consequently leads to achievement of maximal therapeutic action with minimal side effects ². In addition, this technology is becoming increasingly important especially when the drug is hydrophilic and fragile like protein. Nowadays, the use of protein and its derivatives for preventing and/or treating the diseases has received a great interest ^{3,4}. In fact, the understanding and knowledge of the chemical composition and structure of proteins will lead to development in the treatment of many incurable diseases, which in turn enhances the progress in the field of biotechnology ^{4,5,6,7}. Unfortunately, many obstacles are still limiting the *in vivo* using of protein such as its instability ⁷, inability to achieve its intracellular target ⁸ and its short half-life in the blood circulation ^{5,9,10,11}. In this regard, scientists have concentrated their efforts to resolve these problems.

The incorporation or encapsulation of protein drug inside a biodegradable polymeric particle is one of the preferable techniques that are used for protecting, localizing and controlling the release of therapeutic protein ^{5,10,12,13,14}. Various technologies have been used for encapsulating protein such as emulsion/solvent evaporation or extraction, coacervation, and spray-drying ¹⁵. Among these techniques, the water-in-oil-in-water (w/o/w) emulsion/ solvent extraction technique is considered one of the most suitable methods for proteins encapsulation within biodegradable polymeric particles ^{16,17,18}. Many biodegradable polymers like poly(DL-lactide-co-glycolide)(PLGA) ^{19,20,21}, polylactic acid or polylactide (PLA) ^{20,22,23} and polycaprolactone (PCL)^{20,24,25} have been used to encapsulate several types of proteins. Actually, the nature of selected polymer ensures the achievement of good protein release profile ²⁵.

In this work, PCL is chosen to encapsulate human albumin protein via double emulsion-solvent evaporation technique. The characteristics of the prepared particles have been attentively studied, including size, size distribution, zeta potential, and morphology. In addition, the encapsulation efficiency of human protein was also investigated by Bradford's assay, which is now the typical method used for estimation of proteins quantitatively. Furthermore, scanning electron microscopy was used to observe the structure and morphology of the protein-loaded

particles. The protein distribution within the prepared particles was also investigated by using confocal laser scanning microscopy.

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL) ($M_w = 14.000$ g/mol), polyvinyl alcohol (PVA) ($M_w = 31.000$ g/mol), dichloromethane (DCM), Bradford's Reagent, NaCl, Human Albumin (HA) and Human serum albumin- Fluorescein isothiocyanate conjugate (FITC-HSA) (60 kDa) were purchased from Sigma-Aldrich, Germany and used as such. Deionized water of Milli-Q grade was used in all experiments.

2.2. Methods

2.2.1. Preparation of particles

The human albumin protein (HA)-loaded polycaprolactone (PCL) nanoparticles were prepared according to a modified w/o/w double emulsion/ solvent evaporation like method. This technique is considered one of the most suitable techniques for encapsulating hydrophilic fragile materials such as proteins^{5,26}. By using this method, five samples of w/o/w emulsion containing HA were prepared as follow: Protein solution was first emulsified in DCM solution containing PCL polymer. The obtained w/o emulsion was then introduced in water solution containing PVA as the stabilizing agent in order to perform w/o/w emulsion, as schematically described in Figure 1. In this study, the concentration of albumin was varied in order to investigate the limitation of such process. After solvent evaporation, the final dispersions were characterized by examining the average particles size, size distribution, morphology and the proteins distribution in the PCL polymer matrix.

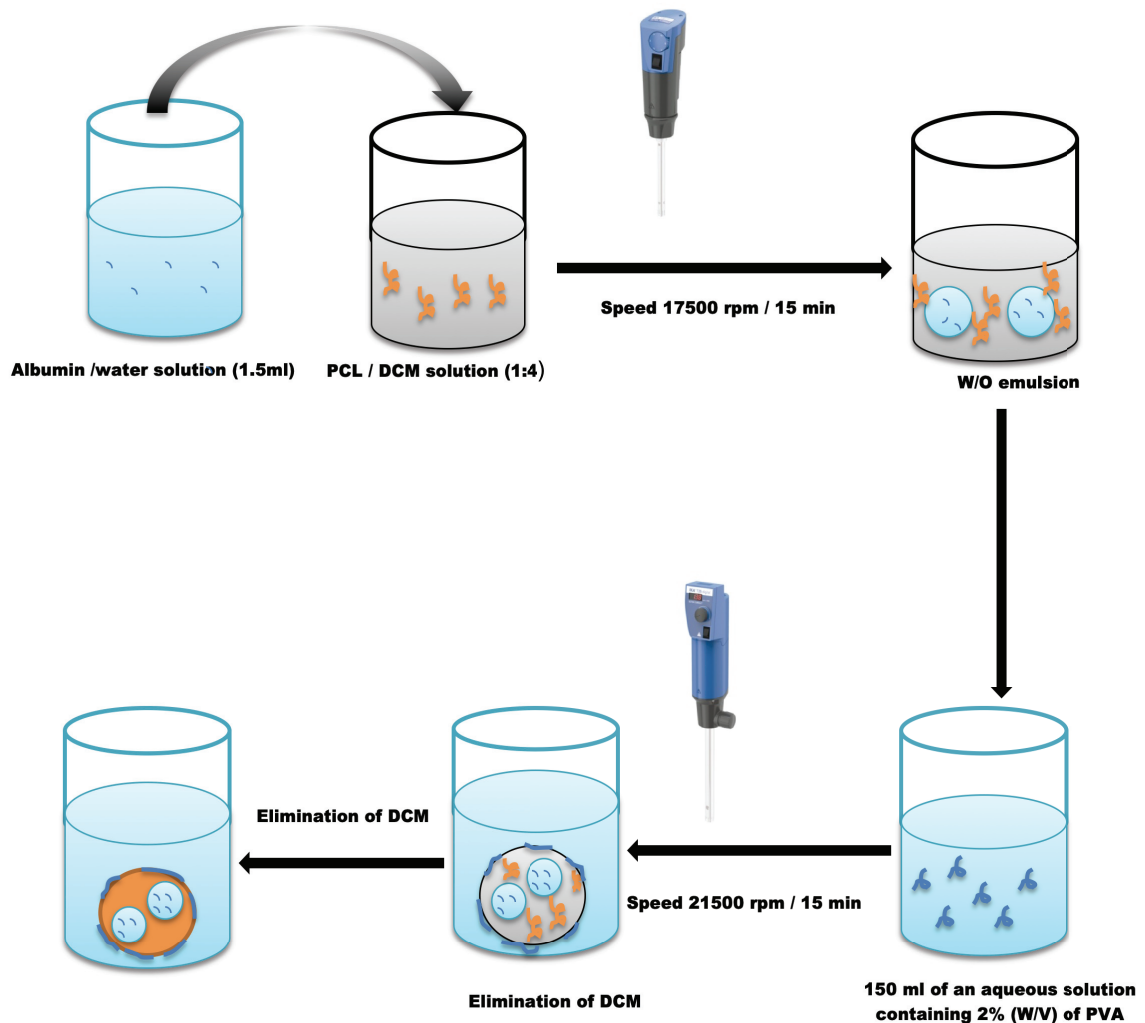


Figure 1. Schematic illustration of protein encapsulation using double emulsion/solvent evaporation like technique.

3. Characterization

3.1. Particle size analysis

Hydrodynamic particle size and size distribution of the prepared dispersions were determined and examined using Zetasizer 3000 HSA (Malvern Instruments, UK). Each prepared sample was highly diluted with 1mM NaCl solution, and then the size was analyzed by performing at least five measurements for each sample.

3.2. Scanning electron microscopy

The morphology and the surface texture of nanoparticles were observed by scanning electron microscopy (SEM) (FEG Hitachi S 800). The fabricated nanoparticles were mounted onto metal stubs using double-sided adhesive tape. After being vacuum-coated with a thin layer of gold (100-150 Å), the obtained particles were examined by SEM at 15 kV or 10 kV.

3.3. Confocal laser scanning microscopy

The distribution of the albumin inside the samples was examined by using the confocal laser scanning microscopy (Leica Microsystems TCS SP2/Germany). Two samples of albumin fluorescent-loaded PCL particles are prepared via double emulsion solvent evaporation method. Thanks to the presence of FITC-HSA in the prepared particles. One drop of each sample was spread on a cover slip. Then, the fluorescein-labeled particles were excited by a 488 nm argon laser.

3.4. Zeta potential

Colloidal stability is a critical factor for using the encapsulated protein particles *in vivo* therapeutic applications. Colloidal stability of the prepared Albumin-loaded PCL particles was investigated in term of zeta potential (i.e. surface charge density) measurement as a function of pH of the dispersion medium. Zeta potential of the prepared particles, which deduced from their electrophoretic mobility, was estimated at different pH values (3, 5, 7, 9, and 11). Measurements were carried out at 25 °C using Zetasizer Nano series (Malvern Instruments, UK) after diluting samples in 1mM NaCl solution.

3.5. Encapsulation efficiency

The protein encapsulation efficiency using such double emulsion like process was evaluated by examining the amount of free soluble protein in the supernatant after particles removal by centrifugation. The protein concentration was determined using Bradford's reagent, which is highly specific to protein. At first, a calibration standard curve was drawn depending on the UV absorbance of Bradford-protein complex solutions at different protein concentrations, as shown in Figure 2. Then, the concentration of non-encapsulated albumin was determined as follow: Five different samples of the emulsion (at different albumin concentrations) were centrifuged and the supernatant was collected and diluted two times in Milli-Q deionized water. Finally, 3 ml of Bradford solution and 0.1ml of diluted protein solution sample were mixed in UV cell. The absorbance of each sample was measured at 595 nm and the protein concentration was deduced from the calibration curve. Then, the encapsulation efficiency was determined from the initial and the residual protein concentrations.

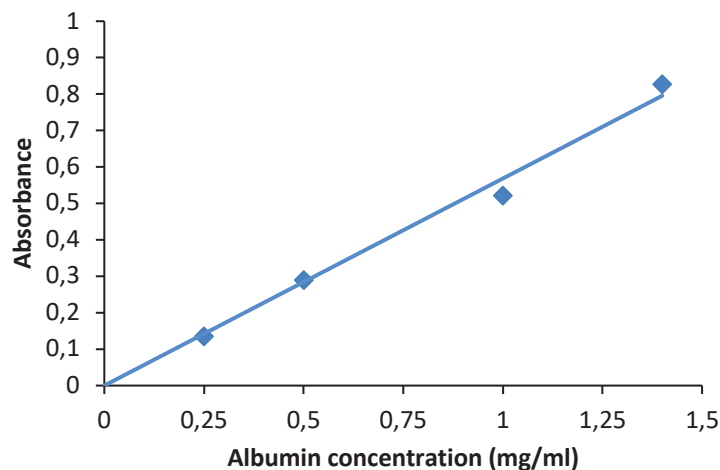


Figure 2. Standard calibration curve shows the UV absorbance as a function of protein concentration (mg/ml) in the water phase.

4. Results and discussion

A modified water-in-oil-in-water (w/o/w) double-emulsion solvent/evaporation method was used to prepare protein-loaded polycaprolactone particles. In fact, this process is suitable method compared to authors due to the possibility of encapsulating both hydrophilic and hydrophobic molecules²⁷. However, the encapsulation of biomacromolecules was not widely investigated. To target such objective, various biodegradable and biocompatible polymers such as poly(lactide) (PLA)²⁸ and poly(lactide-co-glycolide) (PLGA)^{29,30,31,32} have been used to formulate and to prepare biodegradable polymer based colloidal particles containing protein for *in vivo* therapeutic applications. In spite of the advantages offered by these polymers, they suffer from one major drawback, which represented in the strong acidic environment (pH 2-3) created during their degradation, causing denaturation of the encapsulated protein^{33,34}. In order to avoid this problem, PCL was chosen to encapsulate proteins. In addition, PCL offers other various advantages suitable for controlling drug delivery²⁸ such as; slow degradation, long term drug release and high intercellular permeability.

In this study, dichloromethane (DCM) was selected as good solvent of PCL and also due to its favorable physicochemical characteristics such as rapid evaporation, appreciable immiscibility with water³⁵. Experimentally, the first emulsion was prepared by adding the protein solution to PCL/DCM solution and the mixture was then homogenized during 15 minutes at a speed 17500 rpm. As it is mentioned in the literatures, it is important to use surfactant or stabilizer to prepare stable particles or dispersions^{36,37,38}. In this work, PVA (0.5 % wt/v in water) was used as stabilizer during the preparation of the second dispersion. This second dispersion was achieved by pouring the first emulsion (w/o) into 150 ml of PVA/water solution and then homogenized at 21500 rpm for 15 min. Here, it is worth to mention that the preparation

conditions were chosen according to previously done studies ³⁹. After eliminating the organic solvent (DCM) via evaporation at 30°C under reduced pressure, a water dispersible protein-loaded colloidal particles were obtained.

4.1. Particles size and size distribution

Size of colloidal particles is an important and critical parameter in the biomedical applications, especially in drug delivery domain ^{40, 41}. The nano or submicron particles exhibit many benefits such as longer half-life as compared with the larger ones ^{42,43,44}, better ability to cross epithelial cell membranes due to their small size ⁴⁵, more targeting and controlled drug release which ensure more efficacy with minimal side effects ^{46,47} and larger reactive area ^{41,43}. These advantages have more importance when biomolecules, like proteins and nucleic acids, are the encapsulated materials ^{3,48}.

In this work, human albumin-loaded polycaprolactone particles were prepared. The average hydrodynamic size of the prepared particles was investigated. The obtained results are shown in Figure 3 in which the particles size was found to be constant, irrespective of the protein amount used in the formulation recipe. This can be attributed to the constant internal water volume (1.5 ml) used in the formulation of the first emulsion.

In addition, it is clear that the albumin-loaded PCL particles are submicron in size and the average diameter of the particles lies between 320 and 350 nm. Regarding these results, it could be observed that there is no significant change in the size of the prepared colloidal particles whatever the concentration of Human Albumin.

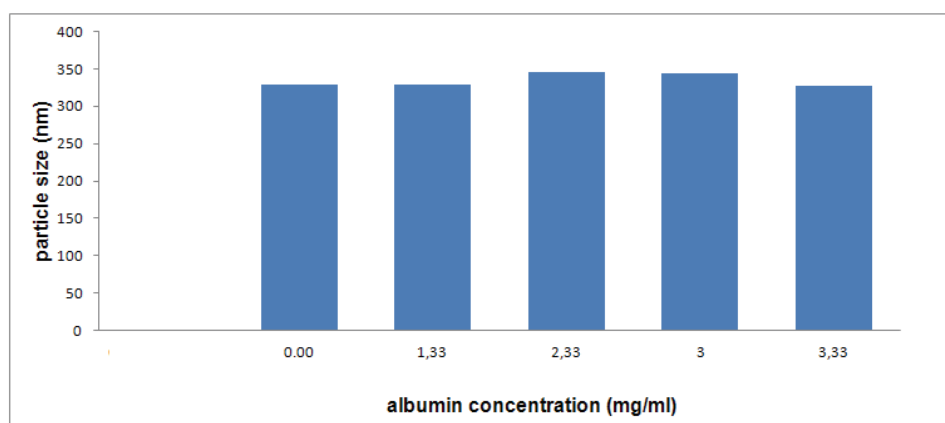


Figure 3. Particle size of human albumin-loaded polycaprolactone colloidal particles as a function of albumin protein concentration.

Regarding size distribution of the prepared particles, which reflects the polydispersity of the dispersion, it was examined and as expected, it was found to be broad, revealing the high

polydispersity of the prepared particles. The results of size distribution measurements are illustrated in Figure 4 for two samples; (a) proteins free and (b) protein-loaded PCL particles which prepared using 3.33 mg/ml of proteins. In fact, the standard deviation calculated from monomodal analysis was found to be in between 30 to 50%. This result is in agreement with those reported in the literature showing comparable broad size distribution of the particles prepared via double emulsion solvent evaporation process for particles above 2 μm size^{20, 25, 49}.

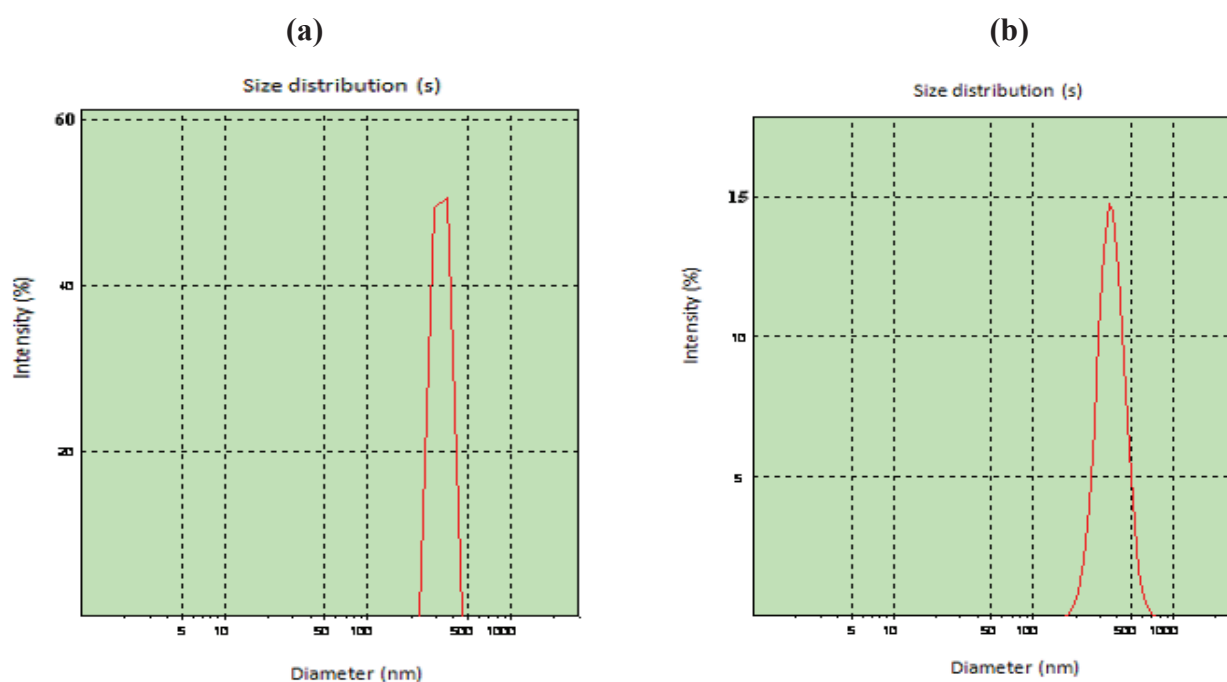


Figure 4. Size distribution for (a) protein-free PCL particles and for (b) protein containing PCL particles prepared using 3.33 mg/ml.

4.2. Zeta potential

Zeta-potential of particles is an important factor in colloidal science. Zeta Potential deduced from electrophoretic mobility value reflects the surface charge density and consequently, the colloidal stability domain of a given dispersion⁵⁰. In fact, the zeta potential for low charged particles can be assumed proportional to the surface charge density when considering hard and smooth spherical particles^{51,52}. In this work, zeta potential was investigated as a function of pH at fixed salinity and temperature. The determined zeta potential values show that there is no significant effect of pH on the surface charge of performed polycaprolactone particles with or without albumin (Figure 5). The low zeta potential values of the prepared particles can be attributed to the low surface charge of polycaprolactone (i.e. few end-carboxylic groups per PCL chain from partial hydrolysis) and also to the screening effect of PVA colloidal stabilizer. As generally observed for charged particles, the use of non-charged stabilizer leads to the surface charge screening effect and to the shift in the slipping plan position, which induce

decreases in the absolute value of the measured zeta potential. Then, the observed low zeta potential can be attributed to the combination of both effects: low hydrolysis of polycaprolactone and shift of the sleeping plan position far from the particles surface.

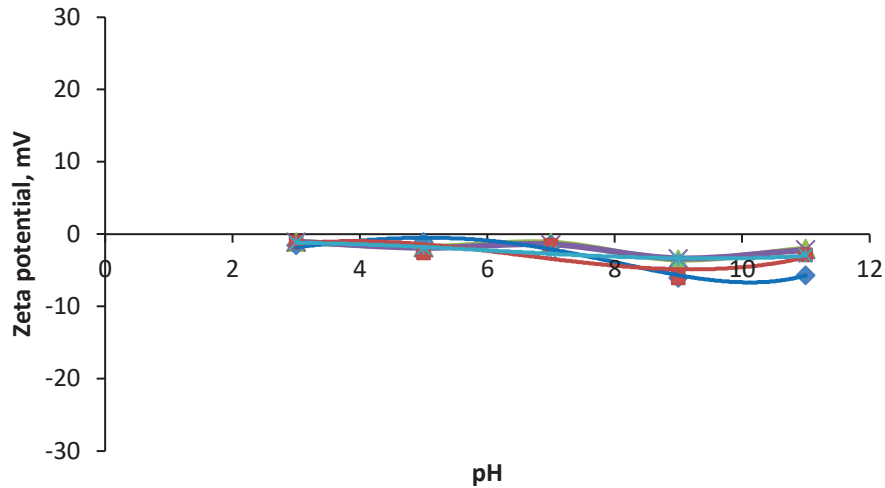


Figure 5. Zeta potential of PCL-encapsulated HA colloidal particles as a function of pH of dispersion medium; (◆) HA free PCL particles, (■) 1.33 mg/ml HA, (▲) 2.33 mg/ml HA, (×) 3 mg/ml HA, (*) 3.33 mg/ml HA.

4.3. Encapsulation efficiency of HA protein

As above mentioned, the encapsulation efficiency of human albumin (HA) protein was investigated and quantified using classical Bradford's titration method^{53, 54, 55}. The Bradford assay depends on specific interactions between basic amino acids residues of the protein and Coomassie blue⁵⁶. Consequently, when the Bradford's reagent is added to the sample containing free protein, the color of the sample changed to blue instantaneously, as shown in Figure 6 (sample 2). After centrifugation, the concentration of free human albumin in the collected supernatant was analyzed and quantified. A reference sample (Figure 6, sample 1) containing water and Bradford solution was prepared for comparison. The rapid macroscopic analysis shows that the analyzed supernatant of the albumin-encapsulated-PCL particles after centrifugation (samples 3) exhibits the same color (i.e. green) as of the reference sample. These observations show that all protein molecules were encapsulated with PCL particles, and there is no free soluble protein in the supernatant.

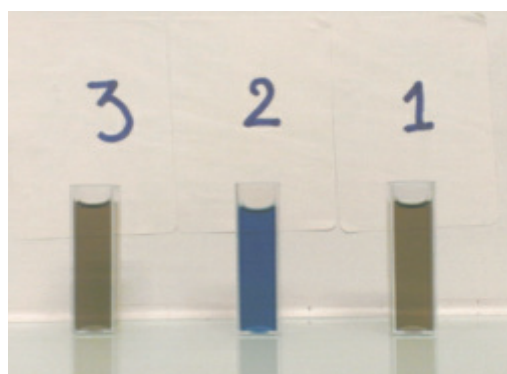


Figure 6. Macroscopic color examination of Bradford's reagent containing solutions, (1) reference sample prepared by addition of 0.1 ml of distilled water to 3 ml of Bradford's reagent solution, (2) sample containing 0.1 ml of albumin solution in water added to 3 ml of Bradford's reagent solution, and (3) sample obtained by addition of 0.1 ml of the supernatant of albumin-encapsulated-PCL particles added to 3 ml of Bradford's reagent solution.

Regarding the quantitative aspect, the UV absorbance for each sample was measured and the obtained values are reported in Table 1. The concentration of free HA protein in the supernatant was deduced using the calibration curve (Figure 2). The encapsulated protein amount was then calculated using depletion method. Surprisingly, the encapsulation efficiency was found to be above 80%, revealing the high encapsulation of protein molecules using double emulsion solvent evaporation like process. This high encapsulation efficiency can be attributed to the efficient formation of water in oil nanodroplets during the preparation of the first emulsion. The formed nanodroplets are surrounded by PCL in DCM solvent leading to low probability to have protein solution (internal phase) in contact with the external aqueous phase during the second emulsification process. In addition, this can be attributed to the local and rapid PCL precipitation during the second emulsification step promoted by the moderate solubility of DCM in water.

Table 1. Protein encapsulation efficiency as a function of initial protein concentration.

Initial protein concentration in water phase (mg/ml)	Free protein concentration in supernatant (mg/ml)	Encapsulated protein in PCL (mg/g)	Encapsulation efficiency (%)
1.33	0.18	1.15	86.5
2.33	0.2	2.13	91.4
3.00	0.14	2.86	95.3
3.33	0.18	3.15	94.6

4.4. Morphology study

In order to study the shape and the morphology of the prepared PCL particles, scanning electron microscopy (SEM) was used, and the obtained images are shown in Figure 7. As can be seen from images, the obtained particles have spherical morphology and exhibit smooth surface. Regarding size distribution, it's clearly evidenced from the SEM images the heterogeneous size distribution, which is in agreement with size distribution measurements.

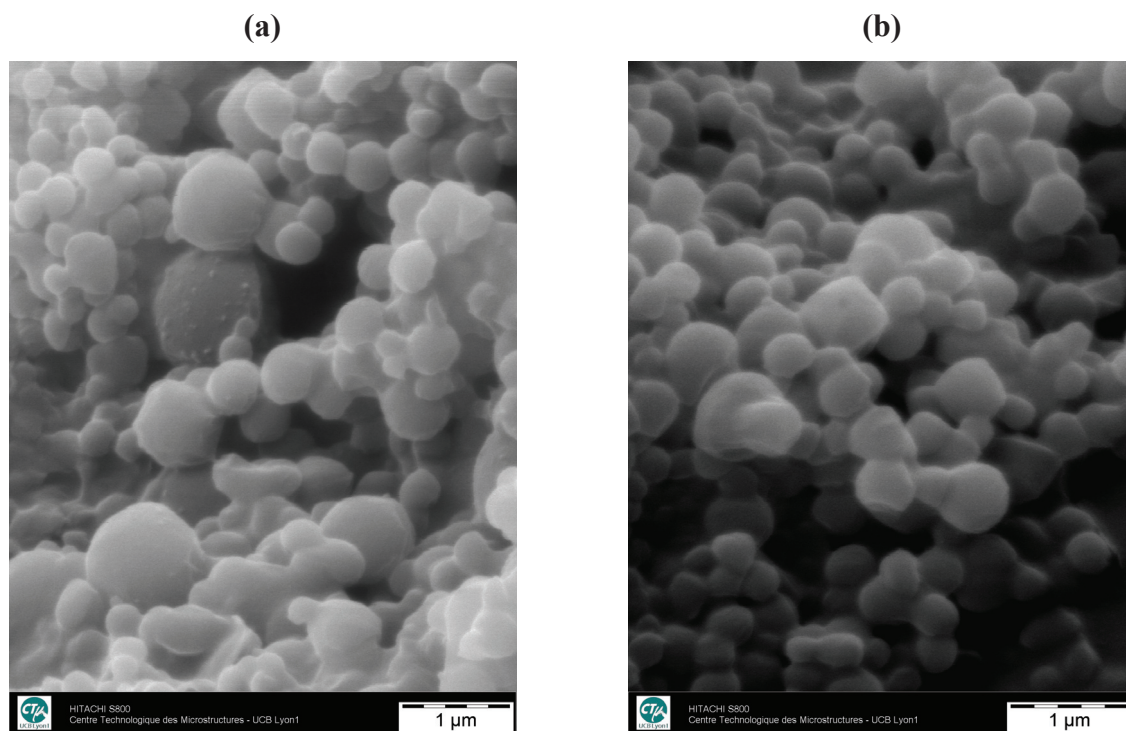


Figure 7. Scanning electron micrographs of (a) protein-free PCL particles and (b) protein-loaded PCL particles prepared using 3.33 mg/ml of protein.

4.5. Protein distribution analysis

Confocal laser scanning microscopy was used to observe the protein molecules distribution in the polycaprolactone matrix. To target such objective, FITC-HSA was used and the obtained images are shown in Figure 8. For better analysis, the images taken for confocal laser scanning microscopy were performed under white light and under laser light of 488 nm wave length, which corresponding to the excitation wave length of FITC molecule.

As clearly seen from Figure 8, the FITC-albumin is exclusively present inside the polycaprolactone-based particles since no marked fluorescence was found in the aqueous medium as background noise which explains the high encapsulation efficiency of the human albumin protein, as above reported. In addition, the comparison between image (Figure 8-a) performed under white light and that obtained under light of 488 nm wavelength for the same sample (Figure 8-b) shows that all particles are labeled indicating the presence of proteins in all

prepared particles. These results are in agreement with those obtained from protein encapsulation efficiency measurements.

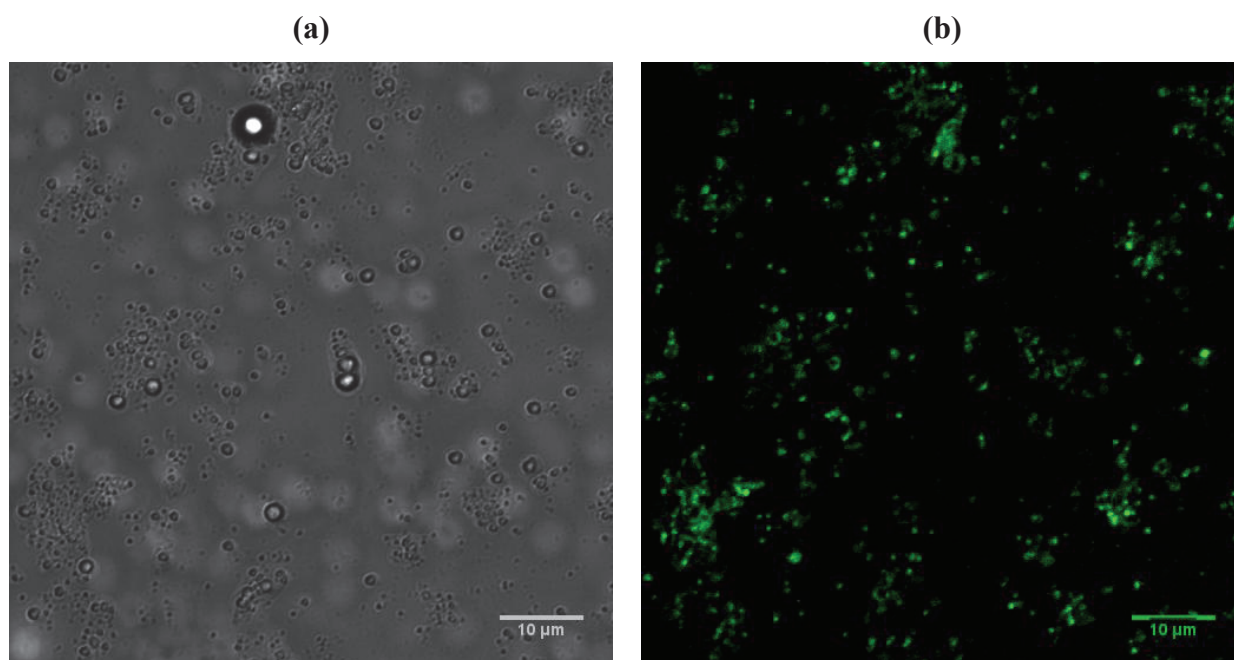


Figure 8. Confocal laser scanning microscope images of human albumin protein-loaded polycaprolactone particles (a) performed under white light and (b) under light of 488 nm wavelength.

Conclusion

Proteins therapy (i.e. immuno-therapy) is attractive and promising area for various chronic and dangerous diseases treatments. The major problems related to such application are the instability and short half-life of such biomacromolecules in the blood circulation, which limit their *in vivo* use. In order to overcome these drawbacks, many processes and approaches related to protein protection have been developed. Encapsulation approach is one of the processes, which has successfully used to improve the properties of active molecules and proteins and to make them more suitable and stable for *in vivo* applications. In this direction, double emulsion evaporation technique is an easy process that has been widely employed to encapsulate different types (hydrophobic and hydrophilic) of biomolecules.

In this work, human albumin protein used as protein model was encapsulated by double emulsion solvent evaporation method using the biodegradable polycaprolactone (PCL). The obtained polycaprolactone colloidal particles containing proteins are stable, submicron in size, and have spherical morphology with low negative surface charge density. As it was expected, such elaboration process leads to heterogeneous size distribution since the process is mainly

shearing based technology. In addition, the confocal SEM images showed that all particles are labeled (i.e. containing proteins), and proteins are dispersed in polymer matrix with high encapsulation efficiency (>80%).

These positive results indicate that double emulsion/solvent evaporation like process is a promising and efficient technique for encapsulating the water-soluble biomolecules with high encapsulation efficiency.

References

1. M. N. Singh, K. S. Y. Hemant, M. Ram and H. G. Shivakumar, *Res. Pharm. Sci.* **5**, 65–77 (2010).
2. K. Kita and C. Dittrich, *Expert Opin. Drug Deliv.* **8**, 329–342 (2011).
3. M. Blanco and M. Alonso, *Eur. J. Pharm. Biopharm.* **43**, 287–294 (1997).
4. D. J. Cahill, *J. Immunol. Methods* **250**, 81–91 (2001).
5. J. Xie, W. J. Ng, L. Y. Lee and C.-H. Wang, *J. Colloid Interface Sci.* **317**, 469–476 (2008).
6. S. D. Putney, *Curr. Opin. Chem. Biol.* **2**, 548–552 (1998).
7. H. Lilie, *EMBO Rep.* **4**, 346–351 (2003).
8. C. S. Russell and L. A. Clarke, *Clin. Genet.* **55**, 389–394 (1999).
9. S. D. Putney and P. A. Burke, *Nat. Biotechnol.* **16**, 153–157 (1998).
10. T. G. Park, W. Lu and G. Crotts, *J. Controlled Release* **33**, 211–222 (1995).
11. D. Blanco and M. J. Alonso, *Eur. J. Pharm. Biopharm.* **45**, 285–294 (1998).
12. T. G. Park, H. Yong Lee and Y. A. Sung Nam, *J. Controlled Release* **55**, 181–191 (1998).
13. H. V. Maulding, *J. Controlled Release* **6**, 167–176 (1987).
14. J. H. Eldridge *et al.*, *Mol. Immunol.* **28**, 287–294 (1991).
15. M. X. L. Tan and M. K. Danquah, *Chem. Eng. Technol.* **35**, 618–626 (2012).
16. H. Jeffery, S. S. Davis and D. T. O'Hagan, *Int. J. Pharm.* **77**, 169–175 (1991).
17. H. Jeffery, S. S. Davis and D. T. O'Hagan, *Pharm. Res.* **10**, 362–368 (1993).
18. R. C. Mehta, B. C. Thanoo and P. P. Deluca, *J. Controlled Release* **41**, 249–257 (1996).
19. G. Crotts and T. G. Park, *J. Microencapsul.* **15**, 699–713 (1998).
20. Y.-Y. Yang, T.-S. Chung and N. Ping Ng, *Biomaterials* **22**, 231–241 (2001).
21. H. K. Makadia and S. J. Siegel, *Polymers (Basel)* **3**, 1377–1397 (2011).
22. Y. Xia, Q. Xu, C. Wang and D. W. Pack, *J. Pharm. Sci.* **102**, 1601–1609 (2013).
23. F.-H. Xu and Q. Zhang, *Yao Xue Xue Bao* **42**, 1–7 (2007).
24. S. R. Jameela, N. Suma and A. Jayakrishnan, *J. Biomater. Sci. Polym. Ed.* **8**, 457–466 (1997).

25. Y.-Y. Yang, T.-S. Chung, X.-L. Bai and W.-K. Chan, *Chem. Eng. Sci.* **55**, 2223–2236 (2000).
26. S. Cohen, T. Yoshioka, M. Lucarelli, L. H. Hwang and R. Langer, *Pharm. Res.* **8**, 713–720 (1991).
27. A. Deloge *et al.*, *J. Nanosci. Nanotechnol.* **8**, 467–474 (2009).
28. V. R. Sinha, K. Bansal, R. Kaushik, R. Kumria and A. Trehan, *Int. J. Pharm.* **278**, 1–23 (2004).
29. T. Feczko, J. Tóth, G. Dósa and J. Gyenis, *Chem. Eng. Process. Process Intensif.* **50**, 757–765 (2011).
30. S. Nazarian, S. L. M. Gargari, I. Rasooli, S. Hasannia and N. Pirooznia, *Res.* doi:10.1016/j.micres.2013.06.005
31. M. Morales-Cruz *et al.*, *Results Pharma Sci.* **2**, 79–85 (2012).
32. M. A. Holgado *et al.*, *Int. J. Pharm.* **380**, 147–154 (2009).
33. M.-A. Benoit, B. Baras and J. Gillard, *Int. J. Pharm.* **184**, 73–84 (1999).
34. S. P. Schwendeman *et al.*, *Dev. Biol. Stand.* **87**, 293–306 (1996).
35. M. Li, O. Rouaud and D. Poncelet, *Int. J. Pharm.* **363**, 26–39 (2008).
36. A. Y. Khan, S. Talegaonkar, Z. Iqbal, F. J. Ahmed and R. K. Khar, *Curr. Drug Deliv.* **3**, 429–443 (2006).
37. A. T. Florence and D. Whitehill, *Int. J. Pharm.* **11**, 277–308 (1982).
38. N. Garti and A. Aserin, *Adv. Colloid Interface Sci.* **65**, 37–69 (1996).
39. M. Ayoub *et al.*, *J. Biomed. Nanotechnol.* **7**, 255–262 (2011).
40. S. Y. Kim, I. G. Shin and Y. M. Lee, *J. Control. Release Off. J. Control. Release Soc.* **56**, 197–208 (1998).
41. A. Z. Wilczewska, K. Niemirowicz, K. H. Markiewicz and H. Car, *Pharmacol. Reports Pr* **64**, 1020–1037 (2012).
42. Y. Malam, M. Loizidou and A. M. Seifalian, *Trends Pharmacol. Sci.* **30**, 592–599 (2009).
43. W. H. De Jong and P. J. Borm, *Int. J. Nanomedicine* **3**, 133–149 (2008).
44. S. D. Perrault, C. Walkey, T. Jennings, H. C. Fischer and W. C. W. Chan, *Nano Lett.* **9**, 1909–1915 (2009).
45. M. Wacker, *Int. J. Pharm.* **457**, 50–62 (2013).
46. K. S. Soppimath, T. M. Aminabhavi, A. R. Kulkarni and W. E. Rudzinski, *J. Controlled Release* **70**, 1–20 (2001).
47. N. Gulati and H. Gupta, *Recent Patents Drug Deliv. Formul.* **5**, 133–145 (2011).
48. S. S. Suri, H. Fenniri and B. Singh, *J. Occup. Med. Toxicol. Lond. Engl.* **2**, 16 (2007).

49. N. Kalaji, N. Sheibat-Othman, H. Saadaoui, A. Elaissari and H. Fessi, *E-Polym.* **10**, 1–12 (2009).
50. M. Kosmulski, P. Próchniak and J. B. Rosenholm, *J. Colloid Interface Sci.* **342**, 110–113 (2010).
51. K. Ohsawa, M. Murata and H. Ohshima, *Colloid Polym. Sci.* **264**, 1005–1009 (1986).
52. C. E. Mora-Huertas, H. Fessi and A. Elaissari, *Int. J. Pharm.* **385**, 113–142 (2010).
53. N. J. Kruger, in *Basic Protein Pept. Protoc.* (Walker, J. M.) 9–15 (Humana Press, 1994). at <http://link.springer.com/protocol/10.1385/0-89603-268-X%3A9>
54. M. M. Bradford, *Anal. Biochem.* **72**, 248–254 (1976).
55. H. Mohammedi *et al.* , *Arch. Inst. Pasteur Algérie Inst. Pasteur Algérie* **57**, 151–162 (1989).
56. H.-K. Ku *et al.* , *Anal. Biochem.* **434**, 178–180 (2013).

4. Conclusion générale

La thérapie biomoléculaire (exemple : immunothérapie et la thérapie génique) utilise des molécules biologiques ou des modificateurs de la réponse biologique pour le traitement de différentes maladies telles que le cancer, le syndrome d'immunodéficience acquise, le diabète, les troubles auto-immunes, les troubles de la croissance et de nombreuses autres maladies dégénératives et dangereuses. Il existe plusieurs classes de médicaments "macromoléculaires" qui sont utilisés en thérapie : les protéines, les peptides, l'ADN de plasmide (ADNp), les oligodésoxy nucléotides anti-sens (ASODN) et les RNA silencing. Dans cette thèse, nous avons étudié deux types importants de biomolécules thérapeutiques : l'acide désoxyribonucléique (ADN) (utilisé en thérapie génique) et les protéines comme modèle de l'immunothérapie.

La thérapie génique est basée sur l'introduction du matériel génétique (acide désoxyribonucléique) dans les cellules d'un individu dans le but d'apporter un bénéfice thérapeutique aux patients. Il s'agit principalement de traiter les troubles génétiques en remplaçant le gène défectueux par un gène réparateur, un gène sain normal ou en complétant du gène déficient.

On distingue deux sortes de thérapie génique : la thérapie génique somatique et la thérapie génique germinale. La thérapie génique somatique permet de remplacer le gène défectueux dans les cellules somatiques et n'a d'incidence que sur la personne traitée. En revanche, la thérapie génique germinale, modifie le patrimoine génétique des ovules et des spermatozoïdes. La modification génétique aurait lieu non seulement dans toutes les cellules somatiques mais aussi dans les cellules germinales et se transmettrait alors à toute la descendance. Cette thérapie génique germinale est interdite pour l'être humain. En effet, il subsiste beaucoup de questions éthiques, sociales et commerciales qui limitent l'utilisation de la thérapie génique.

Le problème essentiel qui limite l'application de cette stratégie *in vivo* est la difficulté de livraison de gène jusqu'au noyau de la cellule ciblée. Pour avoir un résultat thérapeutique adéquat, il est important que le gène arrive sans dégradation à son site avec efficacité. Cependant les propriétés physico-chimiques de l'ADN (sa nature polyélectrolytique, anionique, hydrophile et macromoléculaire) et aussi ses propriétés biologiques (sa dégradation rapide) ne lui permettent pas d'être utilisé seul comme un agent thérapeutique et rendent son utilisation dépendante des propriétés du vecteur qui l'acheminera à la cible cellulaire en compactant, en masquant sa charge négative et en le protégeant des dégradations possibles.

De nombreux vecteurs ont été utilisés pour atteindre cet objectif. Tout d'abord, quelques virus ont été modifiés et utilisés comme vecteurs viraux qui se sont avérés d'une bonne efficacité. Mais de nombreux problèmes posés par l'emploi de tels vecteurs (production, immunogénicité, sûreté, coût) ont conduit à imaginer des systèmes synthétiques capables d'assurer les mêmes fonctions que les virus, mais sans leurs inconvénients.

Les vecteurs synthétiques représentent une alternative prometteuse aux vecteurs viraux. Ils sont simples à élaborer, à utiliser et ils sont peu immunogènes. Ces vecteurs sont généralement des dérivés polyélectrolytes cationiques, de nature lipidique ou polymérique, ils sont capables d'interagir par interactions électrostatiques attractives avec les phosphodiester anioniques de l'ADN et de le compacter sous forme particulières appelées lipoplexes ou polyplexes. Le complexe obtenu est globalement positivement chargé, ce qui favorise son accès au noyau.

Dans le but d'améliorer les systèmes de transport du gène, des techniques d'encapsulation variées sont développées et utilisées tels que les liposomes, la couche après couche et la double émulsion/ évaporation du solvant. Cette dernière a fait l'objet de la partie expérimentale de cette thèse.

En ce qui concerne l'immunothérapie, l'utilisation de protéines a été largement examinée. La deuxième biomolécule qui a été étudiée dans cette thèse est une protéine. Grâce à leur forte spécificité et grande diversité, les protéines sont des agents thérapeutiques potentiels pour traiter plusieurs maladies graves. Malheureusement, l'immunogénicité, le mauvais ciblage et la dégradation rapide dans le milieu biologique ont rendu la plupart des protéines inefficaces et insuffisantes comme médicaments. Comme l'ADN, l'utilisation efficace de protéines comme médicaments est liée à trouver des stratégies appropriées pour remédier à ces inconvénients (l'instabilité de la protéine *in vivo*, l'absorption mauvaise, et la demi-vie courte) qui limitent leur utilisation *in vivo*.

De nombreuses stratégies ont été développées afin d'améliorer l'efficacité de la protéine comme agent thérapeutique, telles que l'augmentation de la perméabilité, l'inhibition des enzymes et la modification de la structure de la protéine ou la protection de la protéine. L'une des stratégies la plus utilisée est l'encapsulation dans une matrice liquide ou polymérique. Dans cette direction, plusieurs techniques d'encapsulations ont été utilisées, mais une seule a été marginalisée ou très peu étudiée et il s'agit du procédé de la double émulsion-évaporation de solvant.

La technique de la double émulsion-évaporation de solvant a été choisie comme procédé d'encapsulation de biomolécules en raison de manque d'études systématiques de cette technique et son utilisation pour encapsuler des biomacromolécules hydrophiles thermosensibles tels que les protéines et l'ADN.

La partie expérimentale de ce travail a débuté par la réalisation d'une étude systématique la plus large possible afin de mieux comprendre les paramètres affectant les propriétés colloïdales des particules finales et également d'optimiser les conditions opératoires utilisées (double émulsion-évaporation de solvant). Le polymère utilisé est le polycaprolactone sélectionné pour sa biodégradabilité et pour sa bonne biocompatibilité. Le dichlorométhane est le solvant organique choisi en raison de sa température d'ébullition basse et de sa non-miscibilité totale avec l'eau. L'alcool polyvinylique (PVA) est utilisé comme agent stabilisant.

Dans la première partie expérimentale dédiée à l'étude systématique, différents paramètres qui peuvent affecter les propriétés colloïdales (la taille, distribution en taille, charge de surface etc...) des particules obtenues ont été étudiés. Ces paramètres sont ; la vitesse et le temps d'agitation pour les deux étapes d'émulsification, la concentration du polymère utilisé, la concentration d'agent stabilisant et le volume des phases aqueuses (interne et externe). Cette étude a été réalisée en changeant uniquement un seul paramètre à la fois.

Il ressort de cette étude que la vitesse et la durée d'agitation utilisée pour préparer la première émulsion n'ont pas d'effet notable sur la taille et la distribution en taille des particules obtenues. En revanche, la vitesse et la durée d'agitation utilisées pour préparer la seconde émulsion affectent considérablement la taille des particules qui diminue avec l'augmentation de l'énergie fournie au système.

En ce qui concerne la concentration en polymère principal, une influence importante sur la taille des particules a été mise en évidence. En effet, plus la concentration du polymère est augmentée dans la formulation, plus la taille des particules est importante. En revanche, la distribution en taille et les propriétés électrocinétiques restent inchangées. D'autre part, il a été constaté que la concentration d'agent stabilisant affecte également la taille des particules, mais une forte concentration induit la formation de mousse.

Le changement du volume de la phase aqueuse interne n'a pas d'influence sur la taille des particules. En revanche, le changement du volume de la phase aqueuse externe a un effet marquant sur la taille des particules obtenues.

Le procédé de double émulsion-évaporation de solvant a été utilisé dans les conditions appropriées pour encapsuler les biomolécules (l'ADN ou de la protéine HSA). Après avoir bien maîtrisé le procédé, une étude basée sur l'encapsulation de l'ADN ou de la protéine modèle a été réalisée. Il ressort de cette étude que la présence de l'ADN ou de la protéine dans la phase aqueuse interne n'affecte pas les propriétés colloïdales des particules. Les propriétés électrocinétiques (versus pH du milieu) des particules sont inchangées quelque soit la

concentration en biomolécules (ADN ou protéines). Il est à noter également que la morphologie et la stabilité colloïdale ne sont pas affectées ce qui traduit la robustesse de ce procédé.

En ce qui concerne l'efficacité d'encapsulation, la quantification par déplétion et par spectrophotométrie montre que l'encapsulation de l'ADN et la protéine (HSA) est très élevée, car des taux d'encapsulations supérieurs à 90% ont été obtenus. En ce qui concerne la répartition des protéines dans la matrice polymère, elle a été examinée en utilisant une protéine fluorescente (marquée à la fluorescéine) montrant que les particules sont bien fluorescentes et sans présence de fluorescence libre dans la phase continue. Ceci traduit la bonne encapsulation de la protéine.

Malheureusement, le manque de temps ne nous a pas permis d'effectuer l'étude des profils de libération des particules préparées et d'examiner également l'activité des protéines et la dénaturation ou non de l'ADN après encapsulation.