Élaboration d'un système de libération contrôlée des facteurs de croissance FGF-2 et TGF-β1 en vue de leur utilisation en odontologie conservatrice et endodontie

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Elaboration d'un système de libération contrôlée des facteurs de croissance FGF-2 et TGF-β1 en vue de leur utilisation en odontologie conservatrice et endodontie

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À ma femme Hiba

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Abréviations

AFM	Atomic force microscopy
aFGF	Acidic FGF
bFGF	Basic FGF
BMP	Bone morphogenetic proteins
BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine
$Ca(OH)_2$	Calcium hydroxide
CTGF	Connective tissue growth factors
DCM	Dichloromethane
EGF	Epidermal growth factor
ELISA	Enzyme-Linked ImmunoSorbent Assay
FDA	Food and Drug Administration
IGF	Insulin-like growth factors
MEB	Microscopie électronique à balayage
MTA	Mineral trioxide aggregate
MTT test	Méthode de numération des cellules vivantes
NGF	Nerve growth factor
PCL	Polycaprolactone
PDGF	Platelet-derived growth factors
PEG	Poly(ethylene glycol)
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Poly(lactide-co-glycolide)
PVA	Poly vinyl alcohol
TGF-β	Transforming growth factor β
VEGF	Vascular endothelial growth factors
W/O/W	Water-in-oil-in-water

Introduction générale

Introduction générale

Lors d'une lésion ou d'une effraction pulpaire d'origine bactérienne, physique ou chimique, l'utilisation de matériaux de coiffage pulpaire tel que l'hydroxyde de calcium ou le MTA (Minéral trioxide aggregate) est souvent le traitement proposé pour encourager la régénération dentinaire. La formation de nouveaux tissus aurait lieu grâce à des facteurs de croissance tels que VEGF, PDGF, FGF-2 et TGF-β1 sécrétés naturellement dans la dentine lors des lésions carieuses. Pour accélérer la formation de nouveaux tissus, l'ingénierie tissulaire permet d'apporter des facteurs de croissance exogènes.

Un certain nombre de molécules bioactives (telle les facteurs de croissance FGF-2 et TGF-ß1) sont séquestrées dans la dentine et peuvent être libérées lors des lésions carieuses. Il a été démontré récemment qu'elles peuvent également être libérées par les fibroblastes pulpaires lésés, ce qui suggère leur rôle dans les événements de réparation dentinaire après un traumatisme. L'utilisation exogène de ces facteurs de croissance sur la pulpe exposée *in vitro* ou *in vivo* a démontré leur potentiel dans la signalisation de réparation dentinaire. Chacune de ces molécules constitue donc un agent thérapeutique potentiel.

L'intérêt majeur de la libération contrôlée d'une molécule, comme les facteurs de croissance, se présente dans la modulation possible des fonctions cellulaires et de la formation tissulaire des sites affectés. Cependant, vue la fragilité des facteurs de croissance, un système de libération (véhicule) est indispensable pour assurer l'efficacité clinique et la régénération tissulaire au site d'application.

La microencapsulation est une des méthodes proposées pour protéger la protéine dans une matrice de polymère. Les microparticules sont capables de protéger les protéines contre la dégradation et de contrôler leur libération. Parmi les différentes méthodes de microencapsulation, celle basée sur l'émulsion multiple W/O/W par extraction de solvant a été choisie dans cette thèse à cause de la nature hydrophile des protéines.

L'idée est de développer un système de libération contrôlée pour une utilisation locale sur une effraction pulpaire qui permettra d'assurer la protection et la délivrance des facteurs de croissance, cela assurera ainsi un effet optimal au niveau de la régénération et la réparation dentinaire.

Le but de ce travail est donc d'étudier l'effet du FGF-2 ou du TGF-β1 sur les étapes précoces de la régénération dentinaire en utilisant deux approches originales:

- L'élaboration des microparticules chargées en FGF-2 et du TGF-β1 pour assurer leur protection et leur libération prolongée tout en gardant les activités biologiques de ces facteurs en contact avec les cellules de la pulpe dentaire.

- L'évaluation de l'effet des microparticules sur la régénération dentinaire en utilisant un modèle de culture de dents entières *ex vivo*.

Cette thèse est subdivisée en deux parties et huit chapitres. La première partie constitue une revue de la bibliographie en trois chapitres et présente d'abord : un rappel du concept de l'ingénierie tissulaire de la pulpe dentaire et le rôle des facteurs de croissance FGF-2 et TGF-β1 dans ce concept. Ensuite les systèmes de délivrance colloïdaux biocompatibles utilisés pour l'ingénierie tissulaire de la pulpe dentaire sont abordés. Enfin la microencapsulation des protéines type facteur de croissance FGF-2 et TGF-β1 est détaillée avec la méthode de préparation des microparticules chargées en FGF-2 et TGF-β1, les caractérisations de ces microparticules, la modélisation mathématique de la libération à travers ces microparticules et les méthodes biologiques pour évaluer l'effet de ces microparticules en contact avec la pulpe dentaire

La deuxième partie aborde le travail personnel et comprend cinq chapitres. Dans les deux premiers chapitres, une série d'expériences va permettre d'optimiser le procédé d'encapsulation et de valider une forme galénique finale : les microparticules. Cette forme sera adaptée à l'utilisation finale en termes de caractéristiques physicochimiques et profil de libération. Pour cela une étude d'optimisation de procédés de préparation des microparticules libérant un principe actif biologique type facteur de croissance sera effectuée avec une étude des effets des paramètres de formulation sur les caractéristiques des microparticules. La stabilité colloïdale et physicochimique des microparticules avant et après leur séchage et redispersion dans l'eau sera également étudiée. Suite à la validation et l'optimisation de procédés de fabrication, le troisième chapitre sera consacré à la préparation, la caractérisation et la modélisation de libération des microparticules chargées en FGF-2 et TGF-ß1. Dans ce chapitre, les microparticules subiront une caractérisation profonde, en même temps une étude de modélisation mathématique de la cinétique de libération sera effectuée. Ensuite, dans le quatrième chapitre, une fois la partie technologique finalisée, les microparticules subiront des tests biologiques *in vitro* qui commenceront par étudier la toxicité des microparticules. Ensuite, des tests de prolifération des fibroblastes pulpaires humains permettent de vérifier la

conservation de l'activité biologique des facteurs de croissance encapsulés. Enfin, le cinquième chapitre traitera de l'utilisation des microparticules sur une effraction pulpaire afin de confirmer leurs activités biologiques *ex-vivo*. Pour cela nous utiliserons un modèle de culture de dent entière humaine récemment validé par l'équipe de professeur About de la faculté d'odontologie de Marseille.

Partie bibliographique

Partie bibliographique

Chapitre 1

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1. La dent

La dent se compose de trois parties :

- la couronne, située hors de l'os alvéolaire.
- le collet par lequel la couronne s'unit avec la racine.
- la racine, incluse dans l'os alvéolaire.

Chaque dent présente une cavité centrale ou endodonte. L'endodonte communique avec le reste de l'organisme par un orifice situé à l'apex de la racine : le foramen apical.

L'endodonte abrite la partie vivante de la dent : la pulpe dentaire composée d'un tissu conjonctif rougeâtre et de vaisseaux et nerfs correspondants. La cavité endodontique est entourée d'une substance dure, la dentine. La dentine coronaire est elle-même recouverte d'un tissu translucide très dur, l'émail. La dentine radiculaire est recouverte d'un tissu résistant, jaune, opaque : le cément.

1.1 La structure de la dent

1.1.1 L'émail :

L'émail recouvre la couronne dentaire jusqu'au collet. C'est la structure la plus dure et la plus minéralisée de l'organisme. Les sels minéraux présentent environ 95 % de son poids. Sa partie minérale est essentiellement phosphocalcique. L'émail est un tissu translucide. Radiologiquement, il est le plus opaque des tissus dentaires. Il a une structure prismatique. Les prismes d'émail sont orientés perpendiculairement à la jonction amélo-dentinaire. L'émail est un tissu inerte, non innervé. La carie de l'émail est indolore.

1.1.2 Le cément :

Le cément est un tissu minéralisé qui recouvre toute la surface externe de la dentine radiculaire. Le cément est un tissu calcifié analogue à l'os. Il a une teneur en sels minéraux réduites (45 % environ). Sous l'effet de stimulations fonctionnelles, il peut, comme l'os, subir des remaniements (résorption, apposition). Le cément permet l'attache du ligament parodontal, de l'os alvéolaire à la racine de la dent. Les fibres desmodontales sont fixées au cément et à l'os alvéolaire. Le cément assure donc l'attache et la fixation de la dent.

1.1.3 Le complexe pulpo-dentinaire (Piette et Goldberg 2001) :

La dentine et la pulpe dentaire forment une unité anatomo-fonctionnelle appelée le complexe pulpo-dentinaire. Ce complexe est recouvert par l'émail au niveau de la couronne et par le cément au niveau de la racine, il comprend :

1.1.3.1 La dentine :

La dentine est un tissu minéralisé formé à partir d'une matrice extracellulaire déposée par les odontoblastes présents à la périphérie pulpaire La dentine est moins minéralisé que l'émail. Elle contient 70 % de sels minéraux (avec principalement des carbonates de calcium), et 30 % de trame organique constituée essentiellement par des fibres de collagènes

1.1.3.2 La pulpe :

La pulpe est un tissu conjonctif lâche qui possède une composition et une structure semblable à celles des autres tissus conjonctifs de l'organisme (fibres de collagènes, vaisseaux sanguins, vaisseaux lymphatiques et fibres nerveuses). A la périphérie de la pulpe, en contact avec la dentine, on trouve les odontoblastes, cellules sécrétrices de la matrice organique dentinaire.

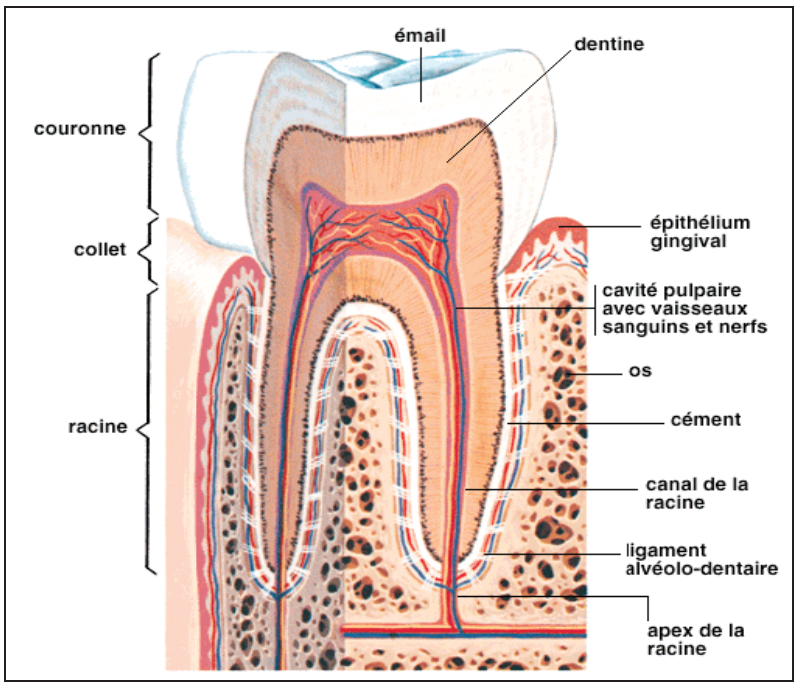

Figure 1.1 : la structure de la dent

1.2 Pourquoi conserver la vitalité pulpaire (maintenir l'intégrité du complexe pulpo-dentinaire) ?

La préservation de la vitalité pulpaire est une préoccupation essentielle du clinicien, car c'est un des principaux facteurs du maintien des dents sur l'arcade, dans un contexte d'allongement de la durée de vie. En effet une dent dépulpée est plus fragile du fait des modifications structurales, biologiques et mécaniques induites par la disparition de la pulpe et

la perte des fonctions assurées par le complexe dentino-pulpaire (Goldberg, 2008). Ces modifications sont :

- La suppression de la pulpe provoque la perte des fonctions neurosensorielles et proprioceptives ainsi que la régulation des pressions intrapulpaires.
- La vascularisation pulpaire assure l'hydratation de la dentine. Cette hydratation permet à la dentine de constituer une masse résiliente sous l'émail, capable d'absorber les chocs, limitant ainsi le risque de fissure et de fracture. La perte de la vascularisation rend donc la dent dépulpée plus susceptible à la fracture.
- La suppression de la pulpe entraine la perte des fonctions de défense de la dent, qui s'opposent à la pénétration bactérienne et à la diffusion des infections dans l'organisme, dont les répercussions sur la santé générale peuvent être graves.
- Enfin la suppression de la pulpe vivante entraine la perte des réactions pulpo-dentinaires protectrices et réparatrices en réponse aux agressions bactériennes ou traumatiques. La dentine tertiaire, réparatrice ou réactionnelle ainsi produite selon l'intensité de l'agression est une barrière naturelle limitant le risque de nécrose pulpaire.

La compréhension de toutes ces fonctions pulpaires et ses modifications induites par la depulpation accompagné par l'importance d'éviter un traitement endodontique souvent difficile à mise en ouvre justifie l'utilisation systématique d'une démarche conservatrice visant à la préservation de la vitalité pulpaire dans toutes les situations cliniques appréhendées

1.3 Comment conserver la vitalité pulpaire (maintenir l'intégrité du complexe pulpo-dentinaire) ?

Selon l'académie canadienne d'endodontie, trois procédures sont cliniquement utilisées pour maintenir la vitalité de la pulpe dentaire selon le volume de la lésion carieuse ou l'étendue du tissu dentaire perdu :

A. Le pansement de protection :

Un matériau de remplissage protecteur est placé au fond d'une cavité profonde pour former une barrière protectrice afin de réduire les risques de tout dommage additionnel à la pulpe et favoriser sa guérison.

B. Le coiffage pulpaire indirect

Un pansement ou un ciment protecteur est placé au-dessus d'une couche de dentine déminéralisée proche de la pulpe et laissé en place pour agir comme barrière protectrice, afin

de réduire tout dommage additionnel à la pulpe et permettre la guérison, ainsi que les recouvrements possibles de la pulpe des dents déciduales ou permanents. Ce ciment permet la reminéralisation, il sera enlever au bout de 15 à 21 jours pour vérifier la formation du tissu minéralisé. Ensuite la restauration finale sera appliquée.

C. Le coiffage pulpaire direct

Un agent de coiffage pulpaire est placé en contact avec le tissu pulpaire vivant exposé accidentellement pendant la suppression de la carie ou par traumatisme. L'objectif est de sceller de manière étanche et d'isoler la pulpe contre l'infiltration bactérienne et provoquer le développement d'un pont dentinaire.

1.4 Matériaux de coiffages pulpo-dentinaires

Actuellement, l'hydroxyde de calcium est considéré comme le matériau de choix pour la réalisation de coiffage pulpo-dentinaire. C'est un produit alcalin (pH=12) qui nécrose la pulpe superficielle et permet la formation d'un tissu de cicatrisation. Sous ce dernier, des cellules pulpaires se différencient en odontoblastes qui synthétisent ensuite un tissu d'aspect dentinaire (le pont dentinaire) qui augmente progressivement d'épaisseur et se minéralise de façon plus ou moins homogène. Mais, l'hydroxyde de calcium présente plusieurs inconvénients tel que : la dégradation avec le temps, le manque d'homogénéité dans le pont dentinaire, des propriétés de scellement inappropriées et la possibilité d'avoir des effets toxiques et d'aboutir à une destruction du tissu pulpaire.

Le MTA (Minéral trioxide aggregate) a été proposé en 1995 comme un matériau de coiffage direct (Abedi et Ingle, 1995). Le MTA présente une meilleure biocompatibilité, de meilleures propriétés de scellement et un potentiel cytotoxique plus faible comparé à l'hydroxyde de calcium (Hasheminia et al, 2008). Toutefois, le MTA a également plusieurs inconvénients qui limitent son utilisation : retard de temps de durcissement, difficulté de manipulation, couleur grise (ou pas esthétique) et prix très élevé.

A coté des problèmes pratiques, d'autres inconvénients du MTA et $CA(OH)_2$ peuvent être mentionnés. Ils concernent le type et le délai de réponse de la pulpe (destruction d'une partie des cellules pulpaires, délai de plusieurs semaines généralement nécessaire avant la formation du pont dentinaire) et surtout leur efficacité qui apparait corrélée à l'importance de l'atteinte dentinaire.

Afin de palier ces inconvénients, l'utilisation de molécules d'origine biologique permettant la stimulation directe des cellules odontoblastiques ou l'induction de la

différenciation odontoblastique à partir de cellules souches ou cellules progénitrices présent dans la pulpe a été proposé. Cette approche est appelée thérapie biologique ou ingénierie tissulaire de la pulpe dentaire (Tziafas et al 2000, Goldberg et al 2006).

2. L'ingénierie tissulaire de la pulpe dentaire

2.1 Le principe de l'ingénierie tissulaire

L'ingénierie tissulaire est l'ensemble des techniques dont le but est de régénérer des tissus autologues, viables et fonctionnels. Il s'agit d'un secteur multidisciplinaire qui applique les principes de l'ingénierie et de la biologie. Ces techniques peuvent être divisées en deux groupes :

- Les méthodes dans lesquelles des cultures cellulaires autologues sont faites *in vitro* puis autotransplantées, soit sous forme de tissus solides et structurés sur une matrice, soit sous forme de suspensions cellulaires (thérapie cellulaire).

- Les méthodes d'implantation de matrices *in vivo* guidant la régénération des tissus de l'hôte. Ces deux groupes de techniques sont souvent associés (Zini et coll., 2004).

Le principe fondamental de l'ingénierie tissulaire repose sur le développement de substitut biologique pour la réparation et la régénération d'un organe ou d'une fonction tissulaire. Il résulte d'une combinaison de trois éléments (figure 1.2) :

1. matrice (échafaudage) :
2. molécule-signal (facteurs de croissance) :
3. cellules

Le concept choisi est simple : remplacer un tissu endommagé par le même tissu naturel et sain.

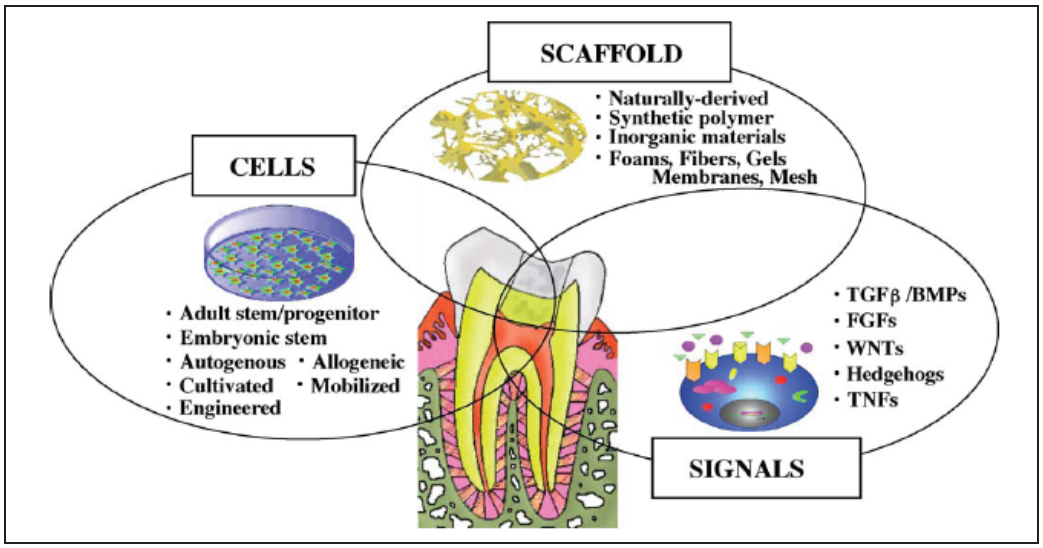

Figure 1.2 : Les éléments essentiels de l'ingénierie tissulaire et de la régénération dentinaire. Le trio contient les cellules souches, la matrice d'échafaudage et les molécules bioactifs de signalisation (les facteurs de croissance). D'après (Nakashima, 2005)

Différentes stratégies sont envisageables en ingénierie tissulaire :

- La conduction, ou approche conductrice, utilise les biomatériaux de façon passive pour faciliter la croissance et la capacité de régénération des tissus existants.
- L'induction, ou approche inductrice, utilise les polymères biodégradables comme véhicules pour délivrer des facteurs de croissance ou des gènes sur le site hôte.
- La transplantation cellulaire utilise un véhicule similaire pour transplanter des cellules ou des parties de tissu dans le site hôte.

C'est le principe de la technique d'induction qui nous intéresse principalement, puisque nous cherchons à encapsuler des facteurs de croissance, le TGF-β1 et le FGF-2, en préservant ses propriétés au contact des tissus, afin de le libérer au contact du tissu hôte : le tissu pulpaire dentaire.

2.2 Généralités sur l'ingénierie tissulaire en odontologie (thérapeutiques biologiques).

La chirurgie dentaire fut longtemps considérée comme un art alliant thérapeutique symptomatique et artisanat de précision. Aujourd'hui l'odontologie est une science biomédicale.

Dans ce contexte, en basant sur la découverte et l'utilisation de molécules impliquées dans la différenciation cellulaire et dans la formation initiale de tissus minéralisés et la compréhension du rôle important de cellules souches, l'ingénierie tissulaire de la pulpe dentaire constituera un outil privilégié dans la pratique odontologique.
Au cours de ces dernières années, dans le cadre de la recherche odontologique, les travaux de recherche ont été dirigés vers l'ingénierie tissulaire. Ils ont deux ambitions :
1- Générer des dents, et ainsi on peut imaginer remplacer les organes dentaires accompagnés de leurs tissus environnants (os cément, ligament alvéolo-dentaire).
2- Régénérer des tissus pulpaires et leur faire produire de la dentine réparatrice, refermant les plaies pulpaires, ou générer des matériaux de remplacement de type dentinaire à partir de ces cellules progénitrices. On peut alors envisager de traiter les effets de caries et lésions pulpaires par ces nouveaux biomatériaux.

Dans ce travail l'ingénierie tissulaire est appliquée pour provoquer la dentinogénèse avec l'intervention des molécules bioactives (facteurs de croissance type FGF-2 et TGF-β1) impliquées dans la formation des tissus minéralisés type dentine réparatrice.
Avant d'aborder ces facteurs de croissance et leur rôle dans la réparation dentinaire, il est utile de rappeler le mécanisme de la dentinogénèse permettant la réparation du complexe pulpodentinaire.

3. Mécanismes cellulaires de la dentinogénèse

Les odontoblastes, cellules pulpaires organisées en palissade à la surface dentinaire, ont une activité de biosynthèse de la matrice organique, qui se minéralise secondairement. Cette apposition est initialement de l'ordre de 4µm par jour. Elle ralentit, mais ne cesse jamais totalement (ce potentiel pouvant être réactivé en cas d'agression). Cette dentinogénèse s'effectue de façon centrifuge, au détriment de l'espace initialement occupé par la pulpe.

En cas d'altération par effraction accidentelle ou à la suite d'une progression carieuse rapide, il semble que des cellules pulpaires puissent se différencier en odontoblastes dits « de seconde génération », si la couche odontoblastique originelle est définitivement altérée.

Toutefois, ces cellules de remplacement n'expriment pas les molécules caractéristiques du phénotype odontoblastique, et il est probable qu'il s'agit de cellules acquérant un phénotype proche du type ostéoblastique (Goldberg et coll., 2002).

Après une lésion au niveau de la dent mature, le devenir des odontoblastes varie en fonction de la gravité de la lésion. Principalement, quand une lésion affect le complexe dentino-pulpaire : trois conditions physiopathologiques pourraient être observés à la frontière entre la dentine et la pulpe (Figure 1.3) :

1. Une lésion mineure (carie d'email, carie sans cavité, carie à progression lent, érosion, irritation mécanique ou chimique) peut activer les odontoblastes conduisant à une sécrétion localisée de matrice dentinaire réactionnelle (Lesot et coll 1992, Tziafas et coll 2000),
2. Une lésion plus importante sans exposition pulpaire (lésion carieuse à progression rapide, perte tissulaire importante suite à la préparation de cavité, effet toxique causé par la restauration) peut induire la mort des odontoblastes. Une cascade de manifestations inflammatoires se fait rapidement au niveau des odontoblastes détruits. Initialement, les fibroblastes *like cells* s'alignent contre la dentine résiduelle et une fibrodentine atubulaire est formée à la frontière dentino-pulpaire. Ensuite, des cellules progénitrices pulpaires se multiplient et migrent vers la dentine circumpulpaire (Farges et coll., 1997). Une nouvelle génération d'*odontoblaste-like cells* se différencie et forme une dentine tertiaire tubulaire (dentinogénèse réparatrice).
3. Dans le cas d'une exposition pulpaire, la pulpe peut se régénéré naturellement ou après l'utilisation d'un matériau de coiffage. Une exposition pulpaire provoquée par une lésion carieuse a un potentiel de réparation dentinaire limité à cause de la possibilité d'infection bactérienne qui compromet la réaction de défense. Les conditions favorables pour la réparation pulpaire suite à une exposition pulpaire sont : l'absence de bactérie, l'absence de modifications hémodynamiques importantes et l'absence d'infiltration de cellules inflammatoires. En cas de condition favorable, le potentiel dentinogénique des cellules pulpaire s'exprime par la prolifération, la migration et la différenciation des cellules souches qui donnent naissance à une nouvelle génération de cellules dentinaires sécrétoires (*odontoblaste-like cells*) qui permettent la formation d'un pont dentinaire (Smith, 2003).

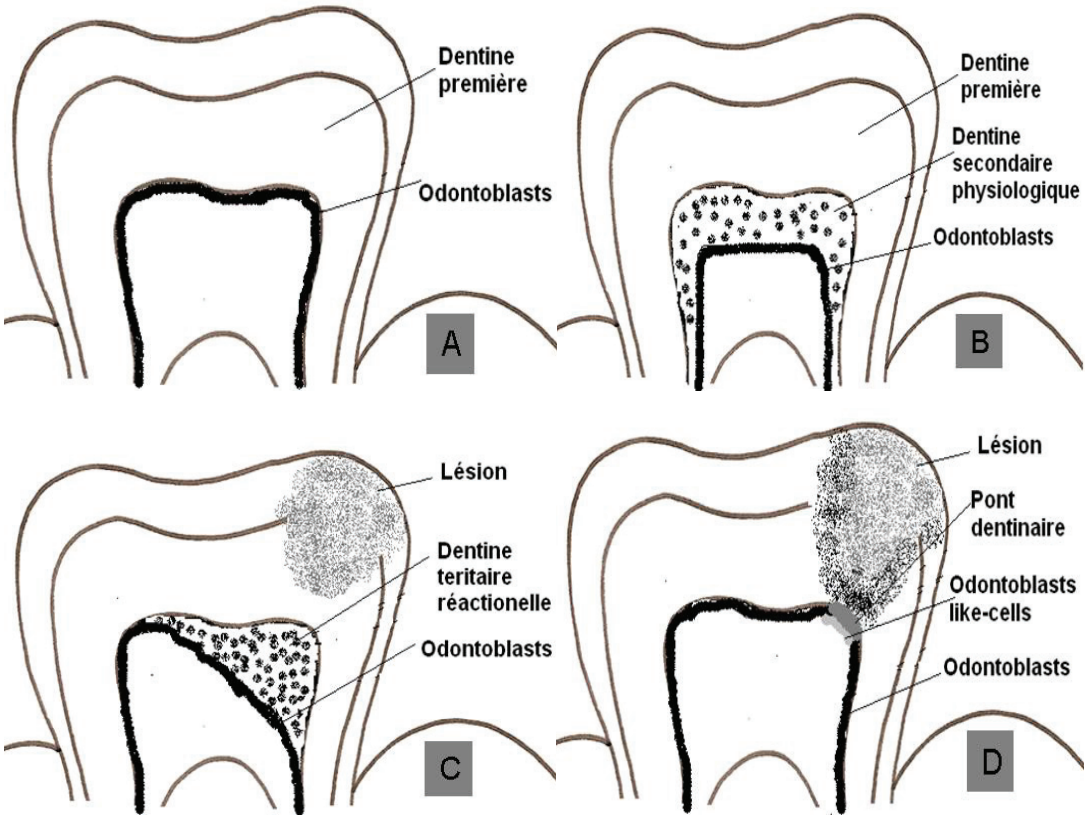

Figure (1.3) La dentinogénèse physiologique et en cas de lésion. a) dent jeune avec activité sécrétoire normale. b) dentine secondaire physiologique dans dent veille. c) après une lésion modéré sans exposition, sécrétion de la dentine tertiaire réactionnelle pour isoler la pulpe. d) après une lésion avancée une exposition pulpaire avec destruction des odontoblastes, ce qui provoque la migration des cellules progénitrices et la différenciation en *odontoblasts like-cells* et la sécrétion de la dentine réparatrice (pont dentinaire).

4. Les facteurs de croissance :

Les facteurs de croissance sont des polypeptides regroupés en différentes familles ou superfamilles qui seront développés dans le chapitre suivant, on va s'intéresser aux deux facteurs utilisés dans ce travail le TGF-β1 et le FGF-2

4.1 Le TGF-β1 :

La famille du facteur de croissance transformant de type bêta (Transforming Growth Factor bêta TGF-β) contient plus de 40 membres, dont une trentaine est retrouvée chez les mammifères.

Le TGF-β1 sous sa forme mature, c'est-à-dire sa forme biologiquement active, se présente sous la forme d'un homodimère de 25 Kda formé de 2 sous-unités identiques de 112 acides aminés chacune.

Le TGF-β1 est un médiateur biologique multifonctionnel qui participe au contrôle des activités cellulaires fondamentales comme la prolifération, l'apoptose, la migration, l'adhésion et la différenciation cellulaire. Ses effets varient en fonction du tissu et du type cellulaire considéré (Roberts et Sporn 1996). Nous citerons quelques-unes de ses fonctions générales, puis celles qu'on lui prête dans la formation et le fonctionnement des organes dentaires

4.1.1 Fonctions de TGF-β1

- Prolifération cellulaire : le TGF-β1 stimule la prolifération des fibroblastes, des ostéoblastes et des chondrocytes (Lawrence, 1996). Le TGF-β1 apparaît comme un stimulateur ou un inhibiteur de la prolifération selon le type cellulaire considéré (Spom, 1999). Le TGF-β1 peut avoir des effets opposés (stimulation ou inhibition) sur un même type cellulaire en fonction de différents paramètres.
- Chimiotaxie : le TGF-β1 possède un fort pouvoir chimiotactique qui s'opère à des très faibles concentrations. Il peut stimuler la migration de nombreux types cellulaires comme les fibroblastes et les cellules de l'inflammation, notamment les monocytes (Wahl et coll., 1987)
- Angiogenèse : parmi les cytokines impliquées dans l'angiogenèse, le TGF-βl semble intervenir plus spécifiquement lors de la phase de formation et de maturation du vaisseau. Le TGF-β1 peut agir directement en régulant la prolifération et l'activité protéolytique des cellules endothéliales, ou indirectement en interagissant avec d'autres cytokines qu'il potentialise ou inhibe comme le FGF ou le VEGF (Pepper, 1997).
- Réponse immunitaire : le TGF-β1 est considéré comme un puissant immunosuppresseur. Toutefois, son rôle est double : dans un premier temps, il a un rôle pro-inflammatoire en attirant par chimiotaxie les macrophages et les cellules dendritiques présentatrices d'antigènes immatures dans les sites subissant une agression. Ultérieurement, il réduit l'inflammation en désactivant les macrophages, en inhibant leur prolifération ainsi que celle des cellules dendritiques, des lymphocytes T et B (Wahl et coll., 2004).

4.2 Le FGF-2

Les facteurs de croissance des fibroblastes (FGF) sont une famille comportant 22 protéines identifiées à ce jour chez l'Homme. Ce sont des protéines qui activent la migration et la multiplication des cellules cibles. Ces facteurs sont généralement sécrétés par des fibroblastes. Le rôle le plus important des fibroblastes, cellules du tissu conjonctif, est de maintenir la matrice extracellulaire des tissus conjonctifs, et de réparer les lésions dues à un traumatisme. Ils servent aussi à réguler l'organisation et la différenciation des cellules des tissus environnants.

Le mécanisme d'action des FGFs, sur leurs cellules cibles, passe par leur liaison à des récepteurs spécifiques à la surface des cellules. Ces récepteurs aux FGF (ou FGF-R) constituent un groupe de molécules capables de recevoir des signaux de l'extérieur et de les transmettre à l'intérieur de la cellule et induire de nombreux messages cellulaires secondaires. Ces récepteurs ont généralement une activité tyrosine kinase dans leur domaine intracellulaire (Gerwins et col, 2000).

Basic fibroblast growth factor, aussi connu comme le bFGF ou FGF-2, est un membre de la famille des *fibroblast growth factor*. bFGF a été la première molécule pro-angiogénique à être identifiée (Cross et col, 2001).

Dans les tissus normaux, FGF-2 est présent dans les membranes basales et dans la matrice extracellulaire sous-endothéliale des vaisseaux sanguins. Il reste lié à la membrane tant qu'il n'y a pas de signal peptidique.

Pendant la cicatrisation des tissus normaux et le développement tumoral, la dégradation de l'enzyme de sulfate d'héparane provoque l'activation de bFGF, ce qui stimule la formation de nouveaux vaisseaux sanguins, un processus appelé angiogenèse. Le rôle de FGF-2 dans la régulation de la croissance diversifiée et la différenciation cellulaires a suggéré une implication dans la cicatrisation des plaies, la croissance tumorale et le cancer.

4.3 Le rôle des facteurs de croissance dans le développement dentaire et la synthèse de la matrice dentinaire

Les dents se développent à partir de l'ectoderme via des interactions séquentielles et réciproques entre les tissus épithéliales et mésenchymateuses. Pendant ces interactions 4 stades de développement sont distingués : le bourgeon, la cupule, la cloche et le stade de différenciation terminale.

Au niveau moléculaire, ces interactions sont médiés par des molécules appartenant pour la plupart au TGF-βs, FGFs, et les familles Wnt (Miletich et Sharpe, 2003).

Plusieurs membres de la famille des (FGF) ont été associés à la morphogenèse de la dent. FGF (int-2) a été le premier à être localisé (Wilkinson et al, 1989).

Le TGF-β1 a été localisé par immunohistochimie dans les germes dentaires de souris dès le stade du bourgeon, principalement dans les cellules du réticulum étoilé et en plus faible quantité dans les cellules de la papille mésenchymateuse (Cam et coll., 1990).

Les récepteurs de TGF-β1 sont présents dans l'ensemble des cellules de la pulpe mature, le signal étant plus important dans les odontoblastes que dans les cellules sous-odontoblastiques et les cellules de la pulpe centrale (Sloan et coll., 1999). Ces résultats soulignent l'aptitude des cellules odontoblastiques et pulpaires à répondre au signal TGF-β1.

Les études *in vitro* ont montré que les membres de la famille du TGF-β peuvent induire la polarisation et peuvent stimuler la sécrétion d'une matrice par les pré-odontoblastes (Lesot et al., 2001, Unda et al., 2001). L'utilisation combinée de FGF-1 et TGF-β1 favoriserait la différenciation cytologique et fonctionnelle des odontoblastes, alors que FGF-2 combiné avec le TGF-β1 stimuleraient seulement la différenciation cytologique (Unda et al., 2001). Aucun de ces facteurs n'induit seuls la différenciation terminale des odontoblastes.

4.4 Le rôle des facteurs de croissance dans la stimulation de la réparation du complexe pulpodentinaire :

Les facteurs de croissance et les protéines de la matrice extracellulaire normalement exprimés au cours de la dentinogénèse primaire jouent un rôle dans la réparation et la régénération de la dentine (Iohara et al, 2004; Tziafas et al. 2000; Unda et al. 2001).

Les cellules endothéliales lésées libèrent des molécules bioactives qui participent à l'initiation de la réaction inflammatoire et la cicatrisation.

Ces molécules semblent également être impliquées dans le recrutement d'odontoblaste-*like cells* sur le site de lésion. Parmi ces molécules, les facteurs de croissance (TGF-β1 et FGF-2) sont supposés jouer un rôle dans la signalisation des événements biologiques responsables du processus de la réparation dentinaire (Smith, 2003).

Les effets des facteurs de croissance implantés au niveau des cavités dentinaires ou en coiffage direct sur la pulpe, ont été examinés afin de fournir de nouveaux outils thérapeutiques de la pulpe vivante (Tziafas et coll., 2000, Nakashima 2005)

Les facteurs de croissance TGF-β1, FGF-2 et BMP ont été appliquées sur les pulpes exposées *in vitro* (Six et al. 2002; Sloan et Smith, 1999) ou *in vivo* (Lesot et al. 2001; Unda et al. 2001), ils ont démontré le potentiel de la signalisation de réparation des événements dentinogénétiques.

4.4.1 Le rôle de TGF-β1 dans la formation de la dentine réparatrice

La stimulation de la dentinogénèse réactionnelle a été obtenue par l'utilisation directe de certains membres de la famille TGF-β et par l'utilisation de fractions de matrices dentinaires isolées contenant ces mêmes facteurs (Smith et al, 2001). Dans l'étude de Smith et coll. (2001), le TGF-β1 a été mis en évidence parmi les molécules présentes dans la fraction solubilisée de la matrice dentinaire. De même, l'utilisation de billes d'agarose imprégnées de TGF-β1 sur la région odontoblastique dans des tranches de dents de rats en culture induit une stimulation de l'activité sécrétoire des odontoblastes (Sloan et Smith, 1999). Unda et al. (2001) ont montré que l'utilisation de FGF-2 sur les pré-odontoblasts induit leur différenciation terminale agissant en synergie avec le TGF-β1

In vitro, les cultures de cellules pulpaires ont montré le rôle du TGF-β1 dans la différenciation odontoblastique et dans la régulation de la synthèse des principaux constituants de la matrice dentinaire. Goldberg et coll., (2006) ont montré qu'il existe des récepteurs pulpaires de TGF-β. Et ils ont avancé l'hypothèse que l'implantation de ces molécules bioactives serait susceptible de provoquer la formation de dentine réparatrice, et pourrait aller jusqu'à provoquer la minéralisation plus ou moins totale de la pulpe.

Lucchini et coll., (2002) ont montré que dans la pulpe coronaire stimulée par le TGF-β1, la densité cellulaire est augmentée et une accumulation de matrice extracellulaire est observée sous la couche odontoblastique.

Les études de Sloan et coll., 1999 ont démontré que TGF-β1 et -β3 peut stimuler les odontoblastes à la sécrétion de la matrice extracellulaire et que TGF-β3 peut avoir des effets inductifs sur les cellules pulpaires

In vivo, les mêmes conclusions ont été retrouvées. Des amputations partielles de la pulpe ont été réalisées chez l'animal et divers matériau de coiffage contenant des facteurs de croissance ont été mis au contact de la pulpe exposée (Tziafas et coll., 1998, Lesot et al, 2001; Unda et al, 2001).

4.4.2 Le rôle potentiel de FGF-2 dans l'étape précoce de la régénération dentinaire (voie de signalisation)

Les cellules progénitrices ont été identifiées dans de nombreux tissus adultes hautement vascularisés et plus récemment dans la pulpe dentaire. Les cellules périvasculaires ont été proposées comme étant l'une des sources de cellules responsables de la régénération dentinaire. En effet, la présence dans la pulpe de cellules progénitrices à haut potentiel de prolifération, localisées au niveau de la micro vascularisation, a été démontrée grâce à l'utilisation d'anticorps anti-STRO-1 et 3G5 (Shi et coll 2003). Ces cellules sont capables de se différencier en différents types cellulaires (adipocytes, cellules nerveuses, chondrocytes). Elles peuvent aussi entraîner l'apparition d'un complexe pulpodentinaire.

Dans la pulpe dentaire, la stimulation de ces cellules est observée lors des lésions carieuses sévères ou traumatiques impliquant la dégénérescence des odontoblastes (cellules post-mitotiques responsables de la dentinogénèse).

Des travaux réalisés par (Tecles et coll, 2005) sur culture de dents humaines entières ont montré, à la suite d'une effraction pulpaire, une prolifération périvasculaire et une migration cellulaire vers la zone lésée. Lors de coiffages pulpaires directs avec des matériaux utilisés couramment en clinique ($Ca(OH)_2$, MTA), des foyers de minéralisation sont observés à proximité des lésions.

Ces observations démontrent une migration des cellules progénitrices et une production de dentine réparatrice. Cette migration des cellules progénitrices en direction de la lésion a une importance capitale. Elle semble due à une lésion des cellules endothéliales et des fibroblastes pulpaires. Les travaux sur la co-culture de fibroblastes pulpaires et de cellules endothéliales ont révélé l'importance des interrelations entre ces deux types cellulaires dans les processus de cicatrisation pulpaire. En effet, une lésion physique des cellules endothéliales est impliquée dans le recrutement des cellules progénitrices (Tran-Hung et col, 2008).

Par ailleurs, les fibroblastes pulpaires sécrètent des facteurs angiogéniques (VEGF, FGF-2, PDGF-AB), sécrétion qui augmente rapidement (5 heures) après une lésion artificielle des fibroblastes favorisant ainsi la néo-vascularisation (Tran-Hung et col, 2008).

La néoangiogenèse, qui se produit 24 heures après mise en culture des fibroblastes pulpaires avec des cellules endothéliales, précède la migration des cellules progénitrices (1-2 semaines) et pourrait donc constituer une voie de migration de ces cellules vers la lésion permettant l'élaboration de la dentine réparatrice.

Partie bibliographique

Chapitre 2

Publication 1

Les systèmes de délivrance colloïdaux biocompatibles utilisés pour l'ingénierie tissulaire de la pulpe dentaire

Chapitre publié dans un ouvrage scientifique

Colloidal carriers for drug delivery in dental tissue engineering, Chapter 23, p: 527-544

Colloids in drug delivery, edited by: Monther Fanun, surfactant science series volume 148, CRC Press Taylor & Francis, London 2010

L'utilisation des facteurs de croissance comme le (FGF, EGF, PDGF) pour favoriser la régénération tissulaire et la cicatrisation a été approuvée par la FDA avec quelques limitations (Fu et coll 2005).

Comme expliqué dans le chapitre précédent, l'utilisation exogène des facteurs de croissance (FGF-2 et TGF-ß1) sur la pulpe dentaire exposée a démontré leur potentiel dans la régénération dentinaire.

L'application de ces facteurs de croissance nécessite l'utilisation d'un système de délivrance ou véhicule pour différentes raisons :

- Les facteurs de croissance sont sensibles et leur demi-vie est courte (Nakashima et al 2005).
- L'utilisation directe des facteurs de croissance donne des résultats insatisfaisants, car la quantité est très élevée au début de son utilisation avec des effets imprévisibles.
- L'utilisation d'un système de délivrance permet d'incorporer une quantité réduite de facteurs de croissance, ceci est très important en raison de leur prix élevé.
- Les facteurs de croissance doivent être présents pendant une longue période afin d'être efficace, ceci peut être assuré par des systèmes à libération prolongée.

Un travail de bibliographie a été effectué sous la forme d'un chapitre dans un ouvrage international. Ce chapitre passe en revue les systèmes de délivrance biocompatibles utilisés comme véhicules ou comme systèmes de libération des facteurs de croissance dans l'ingénierie tissulaire de la pulpe dentaire. Dans ce chapitre l'accent a été mis sur les systèmes colloïdaux à base de microparticules qui seront détaillés dans le chapitre suivant.

Le chapitre commence par un rappel sur l'anatomie et la structure dentaire, suivi d'une description des différents types de facteurs de croissance impliqués dans le développement et la régénération dentinaire. Ensuite, les différents matériaux utilisés dans l'ingénierie tissulaire de la pulpe dentaire (billes d'agarose, collagène, hydrogel, polymères biodégradables naturelles ou synthétiques) sont résumés.

Les microparticules comme système de libération contrôlé des facteurs de croissance dans la régénération dentinaire sont présentées. Enfin, les principales utilisations des scafold ou des microparticules chargées en facteur de croissance dans la régénération dentinaire sont passées en revue.

23 Colloidal Carriers for Drug Delivery in Dental Tissue Engineering

Nader Kalaji, Nida Sheibat-Othman, and Hatem Fessi

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23.1 INTRODUCTION

The functional life of the tooth is determined by the vitality of the dentin–pulp complex. The importance of maintaining the vitality of the dental pulp after pathological changes in the hard tissue of a tooth caused by mechanical trauma or chronic irritation (carious lesions) is thus well accepted. The pulp maintains tissue homeostasis after tooth development and underpins the defense reactions taking place in response to injury and the reparative events leading to tissue regeneration (Smith, 2003).

Typically, pulp capping is applied to protect the exposed pulp (to avoid bacterial invasion of the pulp from the external environment) to allow it to recover and preserve its vitality and its functional and biologic activity (Olsson et al., 2006). The better understanding of the molecules involved in pathological processes such as growth factors and extracellular matrix molecules offers significant benefits in tissue engineering (TE). Growth factors are a group of molecules responsible for signaling a variety of cellular processes and various aspects of tissue regeneration. Controlled tissue repair and regeneration is stimulated by the application of recombinant growth factors in pulp capping (Lovschall et al., 2001; Tziafas et al., 2000) by forming a mineralized barrier (reparative dental bridge). This new therapy in TE is called vital pulp therapy (Iohara et al., 2004; Nakashima, 2005; Tziafas et al., 2000).

The application of growth factors in TE necessitates the use of a delivery system. Most growth factors have a relatively low half-life. For instance, the half-life of transforming growth factor β1 (TGF-β1) is on the order of few minutes in its free form during which it is capable of signaling regenerative events. Therefore, it is important to protect growth factors against denaturation during the treatment. The direct application of growth factors would give unsatisfactory results because the applied dose would be very high at the beginning of the treatment and might have some side effects. The drug might also diffuse to nontarget sites. The subsequent enzymatic digestion or deactivation of the growth factor leads to a rapid decrease of the available dose that becomes insufficient for the treatment and fails to induce the intended effects on target cells and tissues. Controlled release provides a sustained effect where a protected bioactive factor is released to act for a longer time. The use of a delivery system also allows the incorporation of a reduced amount of the growth factors that is crucial because of their high price. A supplementary advantage of drug delivery is the possibility of transporting the bioactive factors to the desired medium.

These benefits are conditioned by the use of biocompatible carriers for drug delivery and an adequate encapsulation technique that preserves the protein bioactivity during the preparation and release. Notable efforts are made to provide longer-term release of growth factors in TE and to ensure appropriate concentrations of therapeutic molecules. A wide range of drug delivery carriers has been applied to accommodate different molecules to regenerate dental tissues by this new pulp therapy.

This chapter reviews the biocompatible carriers used for drug delivery in dental TE with a specific emphasis on colloidal carriers. A brief presentation of the teeth structure is proposed followed by a description of the different types of growth factors involved in teeth development and healing. Then, the different materials used in dental TE are summarized as well as a promising drug delivery system: microspheres produced by the water-in-oil-in-water (W/O/W) method. Finally, the main applications of scaffolds or capsules loaded with growth factors in dental TE are reviewed.

23.2 TEETH STRUCTURE

Enamel (cf. Figure 23.1) is the hardest part of the tooth and the highest mineralized tissue of the body (approximately 96% mineral with the remainder water plus organic material). It protects the exposed part of the tooth, called the crown. It is as hard as steel with a Knoop hardness number of 343 (Summitt et al., 2001). Therefore, materials used to replace enamel are strong glass-, ceramic-, or metal-based substitutes (e.g., amalgam and composites).

Dentin is the tissue underneath the enamel that forms the main mass of the tooth (composed of 70% mineral, 20% organic material, and 10% water). It supports the tooth enamel and absorbs the pressure of eating because it is less mineralized and less brittle than the enamel. It consists of a number of microfibers, called dentinal tubules, imbedded in a dense homogeneous matrix of collagenous proteins (unlike enamel that does not contain collagen). The formation of dentin, known as dentinogenesis, is initiated by odontoblasts.

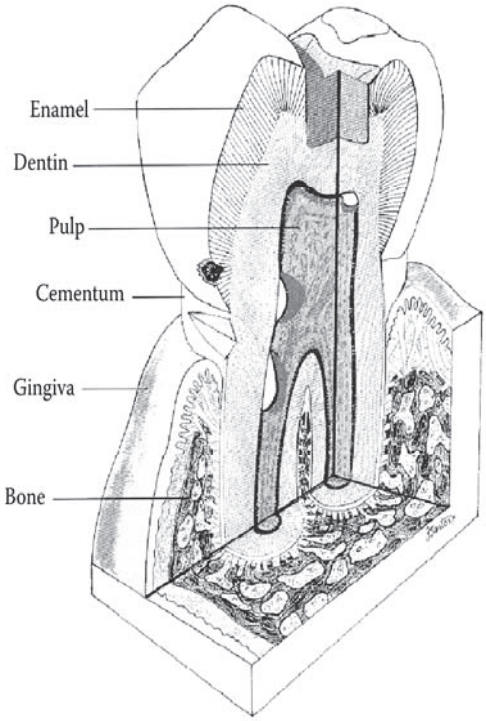

FIGURE 23.1 Structure of the human tooth. (From Summitt, J.B., Robbins, W., and Schwartz, R.S. 2001. *Fundamentals of Operative Dentistry, A Contemporary Approach*, 2nd ed. Quintessence Publishing, Chicago. With permission.)

Cementum is an anatomic part that covers the dentin outside the root (under the gum line) and is attached to the jawbone with a few elastic collagen fibers. It is as hard as bone but not as hard as the tooth enamel and dentin (composed of approximately 45% inorganic material, 33% organic material, and 22% water). Cementum is typically thinner and more permeable than enamel. Therefore, it is subject to abrasion upon root exposure to the oral environment. It is formed by cementoblasts.

Dental pulp is the most internal structure of a tooth, surrounded by the dentin. It is found in the soft center of the tooth inside the pulp chamber and the root canal. It is a living soft tissue containing nerves and blood vessels that nourish the tooth.

The tooth is therefore composed of distinct yet connected tissues. For tooth treatment, multidirectional engineering perspectives are to be achieved using composite materials that satisfy the numerous functionality requirements. The anatomy of the tooth also reveals the importance of preserving the dental pulp during the treatment because of its crucial functions such as dentin formation by the odontoblasts (as well as formation of reparative dentin), nutrition of the surrounding tissues, and sensing (trauma, pressure, and temperature).

23.3 GROWTH FACTORS

Growth factors are peptide molecules that have a central role in the regulation of a variety of cellular processes. They act as signaling molecules between cells to stimulate and inhibit growth and proliferation and modulate the differentiation state.

The interaction and binding between growth factors and specific receptors on the cell surface lead to a chain of intracellular signals as a result of signal transduction to the cell nucleus. The effect of growth factors on gene expression in the cell nucleus influences the cell behavior and activity, thus affecting intra- and extracellular events. Therefore, growth factors may regulate the genes controlling cell proliferation, differentiation, or products of the cell secretion. Because of their high potential, they are employed in very low concentrations (Smith, 2003).

23.3.1 Families of Growth Factors

Different families of growth factors exist. A noncomprehensive list of growth factors is provided in Table 23.1. There are at least 100 members in the TGF-β family and 22 members in the fibroblast growth factor (FGF) family. Growth factors can be found in a variety of cells. For example, the principal sources for TGF-β are activated T-helper cells and natural killer cells and sources for platelet-derived growth factors (PDGFs) are platelets, endothelial cells, and placenta. Some growth factors act upon specific types of cells while others are versatile and act upon a variety of cells. Most of the signaling molecules involved in tooth development belong to the TGF-β and FGF families.

23.3.2 Growth Factors Involved in Tooth Development

The early development of teeth resembles (morphologically and molecularly) other organs such as hair, nails, feathers, and glands. Teeth develop from the ectoderm via sequential and reciprocal interactions between epithelial and mesenchymal tissues during which the ectoderm undergoes distinct bud, cap, bell, and differentiation stages.

At the molecular level, these interactions are mediated by signaling molecules mainly belonging to the TGF-β, FGF, Hedgehog, and Wnt families (Miletich and Sharpe, 2003). In addition to FGF and members of the bone morphogenetic protein (BMP)/TGF-β family, nerve growth factor (NGF) was found, by immunohistochemistry or *in situ* hybridization, to be expressed in the developing tooth germ (Lesot et al., 2001; Miletich and Sharpe, 2003; Unda et al., 2001). In addition, insulin-like growth factor 1 might promote proliferation of many cell types particularly in bone growth. It stimulated cytological (but not functional) odontoblast differentiation and might have a primary role in the growth and differentiation of pulp cells (Lesot et al., 2001; Lovschall et al., 2001).

In vitro experiments have shown that members of the TGF-β family can induce polarization and stimulate matrix secretion by preodontoblasts (Lesot et al., 2001; Smith et al., 2001a; Unda et al., 2001). The combined use of FGF1 and TGF-β1 promoted cytological and functional differentiation of odontoblasts, whereas FGF2 combined with TGF-β1 only stimulated cytological differentiation (Unda et al., 2001). None of these factors alone induced the terminal differentiation of odontoblasts.

BMP constitutes a subfamily of TGF-β consisting of about 20 members (note that BMP1 does not belong to the TGF-β family of proteins). Members of the BMP family are implicated throughout embryonic tooth development, initiation, morphogenesis, cytodifferentiation, and matrix secretion. They were used to stimulate differentiation of the bovine and human adult pulp cells into odontoblasts in monolayer cultures and in three-dimensional pellet cultures (Iohara et al., 2004). In addition, they were utilized to stimulate differentiation of odontoblasts in organ cultures of mouse dental papillae cells and induction of reparative and regenerative dentin formation (Lesot et al., 2001; Nakashima, 1994a).

23.3.3 Role of Growth Factors during Dental Repair

Many growth factors and extracellular matrix proteins normally expressed during primary dentinogenesis are assumed to play a role in dental repair and dentin regeneration (Iohara et al., 2004; Tziafas et al., 2000; Unda et al., 2001). Injured endothelial cells are also known to release signaling molecules that participate in initiating the inflammatory reaction and the healing process. These molecules also appear to be involved in the recruitment of odontoblast-like cells at the injury site.

Among these molecules, growth factors are assumed to play a role in signaling the biological events responsible for the processes of reactionary and reparative dentinogenesis (cf. Figure 23.2; Smith, 2003). In reparative dentinogenesis, recruitment of progenitor or stem cells leads to differentiation of a new generation of odontoblast-like cells to replace the dead cells due to the injury.

TABLE 23.1
Families of Growth Factors

Superfamily	Family	Some Known Functions
TGF-β	TGF-β exists in three isoforms: TGF-β1, TGF-β2, and TGF-β3	Proliferation, differentiation of most cells: tissue regeneration, embryonic development, regulation of the immune system
	Inhibins (inhibin/activin)	Regulation of the menstrual cycle
	BMP	Osteogenic activity; induces cartilage and bone formation
PDGF	PDGF	Regulation of cell growth and division; angiogenesis (growth of blood vessels); migration and proliferation of endothelial and mesenchymal stem cells
	VEGFs	Vasculogenesis (formation of the embryonic circulatory system) and angiogenesis
	CTGFs	Skeletogenesis; wound healing; mediates profibrotic action of TGF-β
EGF	EGF	Growth, proliferation, and differentiation of epithelial cells, cancer cells
	TGFα	Epithelial development, neural cell proliferation
Other large peptide growth factor families	FGF1–FGF23: aFGF (FGF-1) and bFGF (FGF-2)	Mesoderm induction and patterning; cell growth, migration, and differentiation; angiogenesis, wound healing, and embryonic development
	IGFs	Regulation of most cells: bone and cartilage metabolism; muscle; nerve cells
	NGF	Differentiation and survival of particular nerve cells
	TNF-α and TNF-β, classified either as proinflammatory cytokines or sometimes as growth factors	Regulation of immune cells

Source: Smith, A.J. 2003. Vitality of the dentin–pulp complex in health and disease: Growth factors as key mediators. *J Dent Educ* 6: 678–689; Issa, M.J.P., Tiossi, R., Pitol, D.L., and Mello, S.A.S. 2006. TGF-β and new bone formation. *Int J Morphol* 2: 399–405.

Note: VEGFs—Vascular EGFs; CTGFs—connective tissue growth factors; aFGF or FGF-1—acidic FGF; IGFs—insulin-like growth factors; TNF-α and TNF-β—tumor necrosis factors α and β.

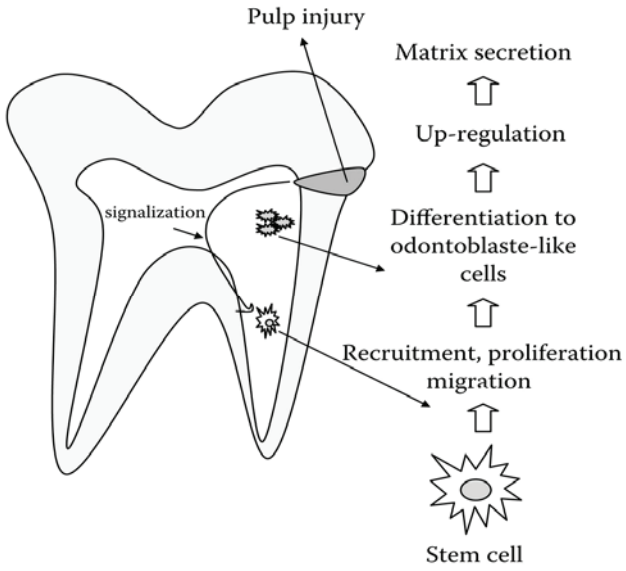

FIGURE 23.2 Schematic representation of reparative dentinogenesis.

Induction of cytodifferentiation of odontoblast-like cells from these progenitor cells is determined by the signaling role of growth factors.

Signaling can proceed with different growth factors because many growth factors are versatile and can act on different cells. However, in tooth healing, only a few growth factors are efficient. Even though a number of growth factors stimulated reparative responses in pulp capping, the reparative dentin matrix secreted was of variable structure ranging from a tubular matrix to osteodentin-like matrices (About and Mitsiadis, 2001; Tran-Hung et al., 2007). Q1

The growth factors TGF-β, FGF, and BMP were applied on exposed pulps *in vitro* (Six et al., 2002; Sloan and Smith, 1999) or *in vivo* (Lesot et al., 2001; Nakashima, 1994b; Unda et al., 2001); they demonstrated the potential of signaling reparative dentinogenic events. Transdentinal or direct application of TGF-1, FGF, and BMP-7 to the odontoblasts of unexposed pulps in cultured tooth slices demonstrated the ability of these growth factors to signal reactionary dentinogenesis (Sloan and Smith, 1999; Sloan et al., 2000). Application of soluble isolated dentin matrix Q2 extracts, containing endogenous growth factors, in unexposed or exposed cavity preparations confirmed these findings (Smith, 2001a, 2001b). Smith (2001b) and Lucchini et al. (2002) found that TGFs were able to reproduce the normal gradients of odontoblast differentiation and morphology of the tissues in dental papilla isolated from tooth germs. Unda et al. (2001) showed that the addition of FGF-β1 to preodontoblasts induces their terminal differentiation by synergistically acting with TGF-β.

23.4 MATERIALS USED IN DENTIN REGENERATION

Efforts are being made to create TE scaffolds that mimic the chemical composition, physical structure, and biological function of the native extracellular matrix for cell proliferation and differentiation. Porous structures are being researched for cellular adaptation and sufficient nutrient permeation for which control of the pore size, porosity, and interconnectivity is fundamental. Solid scaffolds, with a pore size of 50–300 μm and a porosity of about 90%, are used in applications where mechanical strength is important and necessary, especially in bone regeneration. Hydrogel scaffolds can also be used if the pore size allows cell and tissue penetration. Gradual degradation of the scaffold to nontoxic products within a predefined time could be researched in some applications.

The activity of these biomaterials or scaffolds can be improved by the synchronous incorporation of bioactive molecules. The scaffold might contain a drug that can be incorporated by different methods:

1. Imbibing or coating the scaffold with the drug (followed by drying or lyophilization)
2. Incorporating the drug in collagen or agarose beads
3. Encapsulating the drug in colloidal microspheres that are then incorporated directly into a hydrogel scaffold or first in a gel that is then introduced in a solid scaffold

Distinct release profiles are obtained in each case. Controlled delivery of growth factors has been demonstrated to promote pulp cell activation for tissue regeneration.

The scaffolds can also contain stem cells to undergo differentiation and regenerate the tissue. The triad notion is therefore used in TE that implies the use of extracellular matrix scaffolds, responding progenitor or stem cells, and indicative morphogenetic signals (using growth factors) in order to converge to the formation of regenerative tissues suitable for rapidly established functionality after intervention (Kuo et al., 2007).

Consequently, in addition to the requirements of biocompatibility and appropriate shape, esthetics, porosity, mechanical properties, and potential biodegradability, the materials used to prepare the scaffold should ensure the ability to incorporate cells and growth factors. It is important that the incorporation of cells is not indispensable because the dental pulp contains progenitor cells, which can proliferate and differentiate into dentin-forming odontoblasts, a process that can be stimulated by the use of growth factors (Nakashima, 1994a).

This chapter focuses on the colloidal materials used as delivery systems in dental TE (dentin regeneration or pulp healing), so the materials used as scaffolds and the incorporation of cells will not be discussed. The different natural and synthetic (organic or inorganic) materials used (with different shapes and patterns) to deliver growth factors *in vivo* or *in vitro* are summarized below. A good review of controlled drug delivery systems in TE is given by Biondi et al. (2008).

23.4.1 Cements

Before the emergence of the idea of TE, including the application of growth factors and cells in pulp therapy, capping by cements was practiced. These cements might be beneficial when applied to an exposed pulp and allow the pulp to recover and maintain its vitality and function. They can be bioactive themselves.

The most widely used capping agent to keep teeth alive since the early 1940s is calcium hydroxide [$Ca(OH)_2$] because of its low-grade irritation of the traumatized pulp tissue. It was used for the first time by Hermann in 1930 (Ingle and Bakland, 2002). However, the use of $Ca(OH)_2$ is not without disadvantages including its gradual degradation, tunnel defects in dentinal bridges, and inappropriate sealing properties (Hasheminia et al., 2008). Its high pH might create superficial obliteration zones. It has also been reported that $Ca(OH)_2$ has a high toxic effect on cells in tissue culture and provokes some pulp tissue destruction (Asgary et al., 2008).

Mineral trioxide aggregate (MTA) was successfully introduced in 1995 for pulp capping (Hasheminia et al., 2008), and it is approved by the U.S. Food and Drug Administration (FDA) for use in pulpal therapy. MTA has superior biocompatibility and sealing ability and is less cytotoxic compared to the conventional root-end filling materials. It prevents microleakage, resists bacterial leakage, and promotes regeneration of the original tissues allowing continued pulp vitality in teeth. When compared to $Ca(OH)_2$, it has shown less inflammation and more predicable results after pulp capping (Asgary et al., 2008) and provided a superior seal when compared to amalgam. MTA's disadvantages are its delayed setting time, poor handling characteristics, and off-white color, as well as its higher price.

Numerous efforts have been made to develop new materials that can induce reparative dentinal bridge formation with less undesirable effects. The application of adhesive systems as pulp

capping materials is suggested for their suitable physical and mechanical properties (de Souza Costa et al., 2000).

23.4.2 Agarose Beads

Agarose is a gelatinous porous medium that is obtained from agar (a natural polymer of the sugar galactose), and it used in many applications such as gel electrophoresis. It consists of linear polysaccharide chains cross-linked to provide a porous structure and mechanical stability. The degree of cross-linking defines the amount of water adsorbable by the agarose beads (they may contain up to 80% water).

The pores between crosslinked chains are typically large enough to allow proteins with molecular weights up to 1 MDa to penetrate, whereas small molecules move freely through the network. Therefore, agarose beads can be used as solid elastic protein carriers.

23.4.3 Collagen Sponge

Collagen is a protein-based natural polymer. It is the main protein of connective tissue in animals and possesses great tensile strength (present in skin, cartilage, tendons, and ligaments). A collagen sponge is a collagen-based porous biodegradable device used for cell culture. In TE, cells can penetrate into the sponge and proliferate three-dimensionally. The properties of the collagen sponge are determined by its cross-linking, degradation, porosity, and sterilization. The collagen sponge is the most intensively tested drug delivery carrier in animal and clinical studies (as wound dressing materials, matrices for bone and cartilage repair, surgical tampons, and implants for drug delivery; Chvapil, 1976). It is the only FDA-approved carrier for many proteins (such as BMP; Chen et al., 2005). However, because of its xenogeneic origin, it might have some disadvantages.

23.4.4 Hydrogels

Hydrogels are networks of hydrophilic polymer chains that are cross-linked. They are water insoluble but can absorb an important amount of water (up to 99%). Natural (such as agarose, methyl cellulose, and hylaronan) and synthetic polymers [poly(vinyl alcohol) (PVA), sodium polyacrylate, and acrylate polymers] can be used to form a hydrogel via chemical or physical cross-linking. Cross-linking avoids dissolution of the hydrophilic polymer chains and determines the mechanical properties of the hydrogel and its flexibility. A hydrogel can be used as a scaffold to repair tissues if the macropores are large enough to contain human cells. However, the main use of biodegradable hydrogels is as peptide and protein delivery matrices because of their biocompatibility and the possibility of manipulating the permeability of the solutes. It is important to note that the release of drugs from hydrogels is relatively faster and less controlled than microspheres.

Drug-loaded hydrogels are prepared by incorporating the drug either before or after cross-linking. Encapsulating the protein before cross-linking usually gives higher encapsulation efficiencies and lower release rates. Chemical cross-linking ensures high mechanical strength but, because of the chemical reaction, the protein might denaturate. For this reason, physical cross-linking is proposed in some works. In physical cross-linking, there is no need for a cross-linking agent and only physical interactions take place. For instance, the dextran-*co*-gelatin hydrogel is based on physical interactions (Chen et al., 2005).

The most widely investigated system to form hydrogels is poly(ethylene glycol) (PEG) because of the ability to confer significant functionalities of the native extracellular matrix and the possibility of controlling their chemical and mechanical properties. Gelatin (a protein produced by partial hydrolysis of collagen extracted from skin and bones of animals) is also commonly applied to form hydrogels because of its bioadhesive properties, which allow the maintaining of the drug delivery system at the target and to its biodegradability. Gelatin can also adsorb different growth factors and has considerable

desorption when biodegraded. For instance, acidic proteins can be adsorbed to basic gelatin with isoelectric points (Chen et al., 2005). Dextran and alginate hydrogels are also commonly used.

23.4.5 Biodegradable Polymers

Natural and synthetic polymers can be used to produce scaffolds, hydrogels, and microspheres. Natural polymers such as bovine serum albumin (BSA), human serum albumin, collagen, gelatin, hemoglobin, carboxymethyl cellulose, and alginic acid are expensive and their purity and reproducibility are not assured (Jain, 2000). Biodegradable synthetic polymers, such as poly(*p*-dioxanone), polylactones, polyanhydrides, polyurethanes, polyphosphazenes, and polyesters, and thermoplastic aliphatic polyesters, such as polylactide [also called poly(lactic acid) or PLA], polyglycolide [also called poly(glycolic acid) or PGA], and their copolymer poly(lactide-*co*-glycolide) (PLGA), have a lower cost and more reproducible properties with the possibility of controlling their mechanical properties, degradation kinetics, and physical structures to suit various applications. Some common biodegradable polymers used in scaffold or microsphere preparation are discussed below and classified as natural or synthetic polymers.

23.4.5.1 Biodegradable Natural Polymers

Dextrans are the most abundant and naturally occurring (by certain bacteria) biodegradable glucose polymers. They are hydrophilic, relatively inert, and nontoxic. Dextran-derived materials are considered to be compatible matrices for protein and bioactive drugs because of their hydrophilic properties and ability to control drug dissolution and permeability (Chen et al., 2007). They have also been used as plasma volume expanders over the last 50 years.

Chitosan {poly[-(1,4)-2-amino-2-deoxy-D-glucopiranose]} is a natural hydrophilic cationic polysaccharide biopolymer obtained by the hydrolysis of chitin, and it can be found in arthropod exoskeletons or shellfish (the second most abundant polysaccharide after cellulose). The solubility of the polymer and its rheological properties are determined by the degree of hydrolysis (deacetylation). It is biodegradable, nontoxic, and biocompatible but is not bioactive itself. In the pharmaceutical industry, chitosan microspheres are promising in oral and mucosal administrations (Niu et al., 2009). They are also introduced in scaffolds to make the material stronger and antiwash-out.

Hyaluronic acid, or hyaluronan, is a natural polysaccharide that is abundant in cartilage, vitreous, and skin. It is a versatile, biocompatible, and bioresorbable material, which is enzymatically degraded. It is known to contribute to cell proliferation and migration especially in skin tissue repair.

23.4.5.2 Biodegradable Synthetic Polymers

PLA and PGA are biodegradable, thermoplastic, aliphatic polyesters. They can be used to form fibers and films. The glass transition temperature of PLA is between 60°C and 65°C. Because of the methyl group in PLA, it is hydrophobic and has slow degradation (>24 months; Gunatillake and Adhikari, 2003). PGA has a glass transition temperature between 35°C and 40°C. It is crystalline and insoluble in most organic solvents. It is insoluble in water that increases the degradation times (6–12 months). By integrating different ratios of PLA and PGA in the PLGA copolymer and by controlling the copolymer molecular weights, distinct release and degradation profiles can be obtained to satisfy various applications in TE and to accommodate different growth factors (Moioli et al., 2006). The use of PLGA is approved by the FDA. These copolymers have been used to prepare various drug-loaded devices (vaccines, peptides, proteins, and micromolecules) because of their excellent biocompatibility and biodegradability. PLA, PGA, and their copolymers have been used to form both scaffolds and microspheres. A good review of PLGA-based drug delivery systems is given by Jain (2000).

One of the main disadvantages of PLGA is the need for an organic solvent to dissolve the polymer to prepare the scaffold or the microspheres. In addition, the degradation of PLGA leads to a reduction in the pH, which might affect protein integrity during the release. Even if degradation of PLGA-based scaffolds might be of benefit in some applications, it is annoying in others because this

affects the mechanical strength of the scaffold. Note however that PLGA-based scaffolds support cellular viability, attachment, and proliferation during *in vitro* culture.

Polycaprolactone (PCL) is a biodegradable polyester with a low glass transition temperature of about −60°C. It is approved by the FDA as a drug delivery device in the human body. It degrades slower than PLA (approximate degradation time is >24 months; Gunatillake and Adhikari, 2003) because of its higher resistance to hydrolysis. PCL-based scaffolds were found to support the viability, proliferation, and differentiation of cells. Because of their lower degradation rate, PCL matrices maintain a mechanically stable three-dimensional architecture during *in vitro* culture (Weert et al., 2000). Li et al. (2005) used PCL to form a nanofibrous scaffold that was seeded with bone marrow derived human mesenchymal stem cells to mimic the architecture of natural tissue. The ultrafine nanofibers provided improved mechanical strength and more extensive substrate for cellular attachment as compared to larger fibers.

23.5 PREPARATION OF MICROSPHERES

The main objectives of encapsulating the drug in microspheres consist of the protection against degradation and controlling the release rate. Microspheres employed as drug delivery devices are known to improve the bioavailability of the drugs and to give controlled release, which allows a reduction of the side effects of the drugs because of the controlled dose. They can be designated to ensure drug targeting to specific tissues or for self-regulated therapeutic action. Therefore, many pharmaceutical delivery systems are based on the use of microspheres.

In many applications microspheres are first prepared and sterilized and then loaded with the growth factor and lyophilized (e.g., for BMP, Woo et al., 2001; for TGF, Zhang et al., 2007). This avoids the degradation of the growth factor during the synthesis of microspheres or during sterilization. The growth factor in this case is incorporated by adsorption on the polymer matrix.

The formulation and sterilization conditions can be adapted to minimize the denaturation of the growth factor during the preparation. *In situ* encapsulation of the growth factor leads to better protection of the drug from environmental invasion and ensuring a controlled release profile. The use of organic solvents in the formulation must be chosen wisely. Weert et al. (2000) presented the main parameters that might lead to the denaturation of proteins during encapsulation that happen to be principally the interfaces between the drug and the organic solvent, which lead to aggregation of the protein, added to the effects of heat and loss of hydration that affect the protein stability. They outline that even when the recovery of native protein is high, the presence of low amounts of degraded protein may be harmful and cause side effects.

The sterilization should also be optimized to maintain the bioactivity of the drug and the microsphere architecture to preserve the predefined release kinetics. Different sterilization techniques can be used: ultraviolet (UV) light, ethylene oxide gas, radiofrequency glow discharge, and γ irradiation. UV light sterilization induced surface damage of basic FGF (bFGF)-encapsulated PLGA microspheres and reduced the rate of growth factor release (Moioli et al., 2006).

Drug release from such matrices takes place by diffusion through the polymer matrix (through the pores of the microspheres and through the polymer) and by erosion of the polymer material in case of simultaneous degradation of the microsphere. The release medium is supposed to penetrate inside the matrix during the release. Weakly adsorbed drugs might be desorbed rapidly from the surface of the matrix that causes a burst effect.

Different objectives have to be kept in mind during the preparation of microspheres or capsules:

- The stability of the drug during the preparation and the release (avoid the protein denaturation) needs to be ensured.
- The colloidal stability of the microspheres or capsules (avoid aggregation of particles) must be ensured.

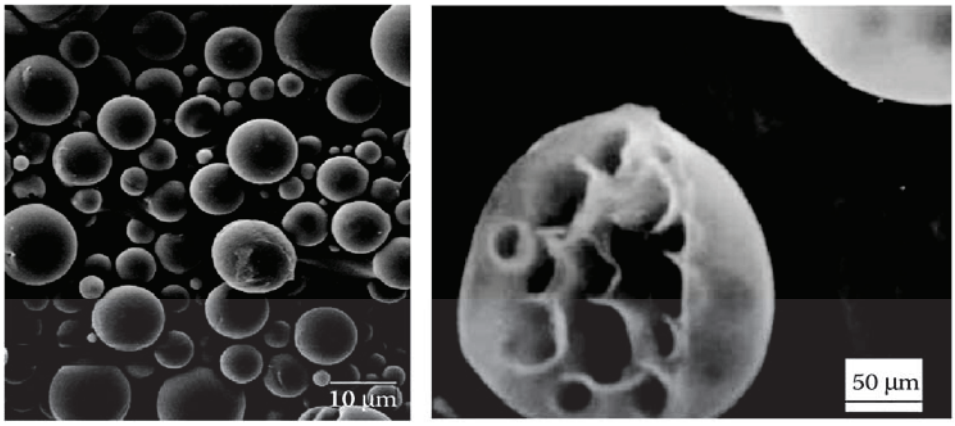

FIGURE 23.3 Scanning electron microscope micrographs of microparticles produced with the W/O/W double emulsion: (left) the exterior structure and (right) the interior structure.

- The encapsulation efficiency has to be maximized and the loading should be appropriate to the application.
- The choice of the method of encapsulation is determined by the solubility of the drug and the duration of the treatment (determined by the release profile).
- The polymers selected for pharmaceutical applications should be biocompatible and drug compatible and should possess the desired mechanical properties and biodegradation kinetics (related to the molecular weight of the polymer).

Various encapsulation methods are practiced to produce pharmaceutical carriers, such as the single-emulsion process, double-emulsion process, phase separation (coacervation), and spray drying. The oil-in-water (O/W) and W/O/W techniques are usually used for the production of nano- and microspheres. The O/W technique is best adapted to encapsulate water-insoluble drugs such as steroids and the W/O/W technique is adapted for water-soluble drugs such as peptides, proteins, and vaccines. Solvent evaporation and extraction methods are applied for solvent evacuation from the microspheres.

Because of their hydrophilic nature, growth factors are usually encapsulated by the W/O/W method. This fabrication procedure for microspheres allows better protection of the growth factor against denaturation because it reduces the contact of the organic solvent.

In the W/O/W technique, the polymer is dissolved in an organic solvent (immiscible in water, such as dichloromethane) that consists of the organic phase. The growth factor is dissolved in distilled water (internal aqueous phase). Both phases are emulsified to produce a stable W/O emulsion. This emulsion is then introduced under stirring in an aqueous phase (external aqueous phase) containing a colloidal stabilizing agent (e.g., PVA). This forms the W/O/W emulsion. Afterward, the solvent is extracted from the particles and evaporates. This leads to the solidification of the polymer, thus protecting the internal aqueous phase and the growth factor forming the solid nano- or microspheres. These microspheres are usually collected on a filter, rinsed and dried, or lyophilized. Figure 23.3 shows micrographs of microspheres produced by a W/O/W double emulsion obtained by a scanning electron microscope. Spherical microspheres obtained by this method have a smooth surface. The porosity of the internal structure is directly related to the ratio of the internal aqueous phase to the oil phase (Deloge et al., 2008).

23.6 *IN VITRO* AND *IN VIVO* DELIVERY OF GROWTH FACTORS IN DENTAL TISSUE ENGINEERING

This section reviews the main applications of growth factor delivery systems in dental TE. These carriers can be classified into three categories. First, the scaffolds or collagen can be impregnated

with growth factors and applied on the tissue. Second, colloidal microspheres are used to encapsulate the growth factor (either *in situ* or just impregnated after microsphere preparation). Third, these microspheres are then applied directly for tissue regeneration or incorporated into a scaffold that usually contains a gel to retain the microspheres. The use of colloidal microspheres presents several advantages related to the good characterization of their physicochemical properties and controlled release rate.

A recent review of drug delivery scaffolds used in dental, oral, and craniofacial TE is provided by Moioli et al. (2007).

23.6.1 Drug- or Cell-Loaded Scaffolds

The scaffold can be impregnated with a drug solution and then dried or lyophilized and used as a drug carrier. For instance, derivatized hyaluronic acid has been tested as a delivery scaffold for BMP-2 (Kim and Valentini, 2001). The release profile of BMP-2 from derived hyaluronic acid scaffolds was slower (32% of total encapsulated BMP-2 released in 28 days) than in both collagen gels (14 days) and PLA scaffolds. The released BMP-2 conserved its bioactive form in all the delivery systems that were studied. Tziafas et al. (1998) used Millipore filters as implants impregnated with bFGF and TGF-β1 to evaluate the effects of the growth factors on dog dental pulp cells *in vivo*. A deposition of a tubular matrix surrounding the implants and highly elongated odontoblast-like cells was observed after 3 weeks of implantation of the Millipore filters.

The incorporation of growth factors is not systematic in all scaffolds because they might be bioactive themselves or contain stem cells, which allows cell regeneration. Kuo et al. (2007) developed a gelatin–chrondroitin–hyaluronan tricopolymer to form scaffolds containing swine dental bud cells for tooth regeneration. The dental bud cells survived and regenerated tooth structures in the swine jaw, including dental pulp, odontoblasts, dentin, cementum, and periodontal ligaments, but the regenerated teeth were smaller than normal. The authors suggest adapting the scaffold size for a better control of the morphology of the regenerated tooth. Duailibi et al. (2008) found that using PGA/PLLA and PLGA scaffolds seeded with cultured 4-day postnatal rat tooth bud cells can form organized and bioengineered dental tissues containing dentin, enamel, pulp, and periodontal tissues. Xu et al. (2008) developed solid and porous calcium phosphate cement composite scaffolds by incorporating chitosan and mannitol in the scaffold. Because of its rapid dissolution, mannitol provides initial macropores and the subsequent degradation of chitosan offers the possibility of increasing the scaffold porosity to integrate cell ingrowth. Chitosan also adds mechanical strength to the scaffold. These scaffolds can act as carriers for osteogenic cell and growth factor delivery for bone TE.

23.6.2 Drug-Loaded Agarose Beads or Collagen

One of the early delivery systems of growth factors consisted of using agarose beads that were soaked into a drug solution and then placed on the tissue. Sloan and Smith (1999) used agarose beads for the delivery of TGF-β. They noted that the presence of empty agarose beads did not induce tissue changes. Local stimulation of the extracellular matrix by TGF-β isoforms was demonstrated. TGF-β1 and TGF-β3 stimulated dentinogenic effects in odontoblasts *in vitro* when applied on agarose beads. Sloan and Smith used agarose beads for the delivery of BMP-7. They showed that the use of agarose beads enhances predentine growth. The use of BMP-7 stimulated secretion of extracellular matrix *in vitro* but with a lower rate than TGF-β1 at similar concentrations.

Similarly to agarose beads, growth factors can be applied after impregnation in collagen that does not operate as scaffolds during the release study. For instance, Six et al. (2002) studied the ability of BMP-7 to induce dentinogenesis in the dental pulp of rat upper first molars and studied the effect of the dose on the treatment. A collagen carrier was applied with and without BMP-7. The results were compared to those obtained with direct pulp capping by $Ca(OH)_2$. They concluded that BMP-7 has the potential to provide an alternative to conventional endodontic treatments. Bergenholtz

et al. (2006) used an absorbable collagen sponge as a carrier for recombinant human BMP-2 to evaluate its benefits in bone healing. Evidence for the enhancing effect of bone formation by recombinant human BMP/absorbable collagen sponge carrier could not be found.

23.6.3 Drug-Loaded Microspheres

Only micron-sized spheres are used in dental TE, as revealed by the literature investigated in this work. In the early applications using microspheres for drug delivery in TE, blank microspheres were first produced and sterilized and then loaded with the drug and lyophilized. By enhanced optimization of the preparation conditions (choice of solvent, temperature, and sterilization method), the encapsulation of drugs during capsule formation (*in situ* encapsulation) can now be done while ensuring the protection of the protein against denaturation. The W/O/W encapsulation technique has mainly been used to prepare such microspheres because of the hydrophilic nature of most growth factors.

23.6.3.1 Drug-Loaded Microspheres Alone

The release rate from the microspheres reviewed in this section was evaluated by applying pure microspheres in the release medium (either *in vitro* or *in vivo*). Note that the same microspheres could be integrated in hydrogels or scaffolds, but the release rate would be altered and has to be preevaluated.

The first generation of drug-loaded microspheres formed by *in situ* encapsulation was bigger in size. Capsules with a diameter of few millimeters were developed by Isobe et al. (1996) to encapsulate BMP by W/O/W using dichloromethane to dissolve PLGA and an aqueous gelatin solution for particle stabilization. They concluded that the use of biodegradable polymers was a promising method to induce cartilage and bone formation (70% of BMP was released from the capsules in 5 days). Similar capsules were produced by Weber et al. (2002) who gave them the name foamspheres (because of their big size). They compared the release results to a gelatin-based hydrogel and found that the slow release of BMP from foamspheres led to the formation of larger amounts of bone than the hydrogels with their faster release of BMP.

Péan et al. (1998) studied the formation of PLGA microspheres by a W/O/W emulsion solvent evaporation/extraction method to encapsulate NGF and tried to optimize the formulation parameters in order to control the release rate and reduce the intensity of the burst effect. A mixture of dichloromethane and acetone was used as an organic solution to dissolve the polymer, and PVA was used as a colloidal particle stabilizer. The use of salt and the type of PLGA affected the morphology of the microspheres, which provides a means to control the release rate. However, employing a salt had a negative effect on the protein stability.

Lu et al. (2000) encapsulated TGF-B1 (and BSA) in PLGA/PEG by a W/O/W double-emulsion solvent extraction technique (using dichloromethane as a PLGA solvent and PVA to stabilize the produced microspheres). They found that increasing the PEG ratio in the polymer and decreasing the pH resulted in decreased protein release.

Cleland et al. (2001) used PLGA microspheres to encapsulate epidermal growth factor (EGF) for the treatment of ischemia. The microspheres were produced by a spray freeze-dried technique to yield a powder, using ethyl acetate as a polymer solvent. Then disks were produced using these microspheres. A lower release rate was obtained using the disks (40 days) than the microspheres (21 days) that was attributable to the lower surface area to volume of the disks compared to the microspheres. The release profile from the disks was characterized by two subsequent phases of release, indicating an important requirement of PLGA hydrolysis to generate sufficient pores for diffusion of EGF.

Moioli et al. (2006) encapsulated TGF-β3 in PLGA microspheres by a W/O/W double-emulsion solvent extraction technique using dichloromethane to dissolve PLGA and PVA as a colloidal stabilizer of the microspheres. The authors outline that UV light sterilization reduces the release of TGF-β3, which was explained either by direct degradation of TGF-β3 because of the light or

degradation of the polymer surface that causes a decrease in the amount of TGF-β3. TGF-β3 was released during about 42 days, and a burst effect was observed.

Niu et al. (2009) recently created chitosan microspheres to encapsulate BSA and oligopeptide. The microspheres were produced according to an emulsion-ion cross-linking method using acetic acid as a solvent and Span 80 and triphosphate for stabilization. The microspheres were collected after precipitation and washed with petroleum ether and isopropyl alcohol. The encapsulation efficiency was higher than 80% for both proteins. They found that the released oligopeptide conserved its biological activity. BSA had a faster release rate than oligopeptide (6–8 days for both proteins).

23.6.3.2 Drug-Loaded Microspheres within Implants or Cements

The incorporation of growth factors in implants or cements gives the possibility for these implants to become bioactive in order to stimulate tissue healing. The use of microspheres ensures a controlled release of the growth factors and its protection against denaturation whereas the implant assures the maintenance of the desired mechanical properties and impermeability.

Woo et al. (2001) compared immediate and sustained release of BMP from implants of sodium carboxymethyl cellulose (an anionic water-soluble polymer derived from cellulose) containing PLGA microspheres. Blank microspheres were formed by a W/O/W double-emulsion method followed by solvent evaporation and extraction. The microspheres were then collected, rinsed, dried, sterilized, and loaded with BMP to evaluate the induced local bone growth. Release of BMP decreased quickly from the immediate release system after 7 days and the released quantity was insufficient to maintain the osteoinductive effects of BMP *in vivo*. However, both systems resulted in faster and more complete bone healing in the animal model.

For the encapsulation of TGF-β1, Zhang et al. (2007) prefabricated blank microspheres by a W/O/W double-emulsion solvent evaporation technique and then impregnated the microspheres with TGF-β1. The produced microspheres were incorporated in calcium phosphate cements and used in pulp capping. The PLGA–calcium phosphate composite enhanced tertiary dentin formation that was further enhanced by the addition of TGF-β1. The introduction of biodegradable microspheres in the cements creates new pores after their degradation that gives the possibility for new tissue ingrowth and replacement, given that the degradation rate of calcium phosphate cements is very low.

23.6.3.3 Drug-Loaded Microspheres within Scaffolds

The combination of microspheres and scaffolds comes from the necessity to ensure distinct functionalities during the same treatment (mechanical properties, permeability, release rate, and protection of the drug). Employing scaffolds is necessary to ensure the desired mechanical properties and porosity for cell development. Drug-loaded microspheres are incorporated into scaffolds to enhance tissue regeneration as a result of the controlled release.

Kim et al. (2003) developed a porous chitosan scaffold containing microspheres loaded with TGF-β1 for cartilage TE. Acetic acid was used to dissolve the chitosan, to which TGF-β1 was added. Microspheres were formed by adding this solution to a hydrogenated caster oil solution. Tripolyphosphate was then used to stabilize the produced microspheres. The microspheres were subsequently washed with isopropyl alcohol and water prior to lyophilization. Then they were dispersed in 90% aqueous ethanol and incorporated into the previously formed chitosan scaffolds (by freeze-drying), after which the scaffolds were lyophilized. The chondrocytes rapidly propagated on the chitosan scaffolds, which presents a potential to be used as implants to treat cartilage defects.

DeFail et al. (2006) encapsulated TGF in PLGA microspheres by a double-emulsion technique for applications in TE of articular cartilage (using dichloromethane to dissolve PLGA and PVA as a colloidal stabilizer). The produced microspheres were then incorporated into a PEG–hydrogel scaffold. TGF-β1-loaded PLGA microspheres showed a high burst release whereas hydrogels containing microspheres showed a delayed burst release. This shows that the combination of microspheres and scaffolds affects the release rate and has to be optimized before *in vivo* applications.

Chen et al. (2005, 2007) proposed the use of hybrid hydrogel scaffolds containing microspheres as new BMP carriers. They developed dextran-*co*-gelatin hydrogel microspheres by radical cross-linking and low-dose γ irradiation. BMP was either incorporated in the microspheres before their preparation (in a dextran–glycidyl methacrylate solution before the addition of the gelatin) or after their formation. They found that this matrix could prolong the release of BMP and promote the proliferation and osteoblastic differentiation of periodontal ligament cells better than using BMP aqueous solution.

Liu et al. (2007) fabricated biodegradable chitosan–gelatin microspheres containing bFGF that were then incorporated into a porous chitosan–gelatin scaffold to improve skin regeneration efficacy and to promote vascularization. The chitosan–gelatin microspheres were prepared by the W/O emulsion method using olive oil and acetone. The microspheres were washed with acetone and isopropyl alcohol, collected by centrifugation, and air dried. They were then impregnated with a bFGF solution and incorporated into a chitosan–gelatin scaffold. The scaffold pore sizes varied from 95 to 160 μm, which is appropriate for TE. The scaffolds incorporating the bFGF-loaded microspheres significantly increased the cell proliferation when compared to empty chitosan–gelatin scaffolds.

Kikuchi et al. (2007) used collagen sponges as a scaffold and gelatin hydrogel microspheres as a carrier for FGF2. Microspheres were prepared from a gelatin solution through cross-linking of gelatin using glutaraldehyde. After dropwise addition in olive oil, acetone was added and the microspheres were collected by centrifugation and added into a Tween 80 solution. They were then impregnated with FGF2 (not *in situ* encapsulation). These FGF-loaded microspheres were incorporated into a collagen sponge scaffold and used for the formation of dentin in exposed rat molar pulps. These results were compared to FGF2-loaded collagen scaffolds that did not contain gelatin hydrogel microspheres. They noted that, during the early phase of pulp healing, pulp cell proliferation and invasion of vessels into dentin defects above the exposed pulp were induced in both groups; but in the later phase, the induction of dentin formation was distinctly different between the two types of FGF2 release. A controlled release profile was obtained when the FGF2 was incorporated in the gelatin hydrogel microspheres and induced the formation of dentin-like particles with dentin defects above exposed pulp. A noncontrolled release was obtained with the collagen sponge scaffold that induced excessive reparative dentin formation in the residual dental pulp (although dentin defects were not noted).

23.7 CONCLUSIONS

This chapter presented a review of the biocompatible carriers used in dental TE with emphasis on colloidal carriers (biodegradable polymer-based nano- and microspheres, gels such as hydrogels and alginate gels, gelatinous carriers). Dental TE is practiced in conservative dentistry to restore the original tooth (structure, function, and esthetics) while preserving pulp health.

The use of nano- and microsphere colloids as delivery systems for growth factors was found to be promising in pulp healing and dentin regeneration. These colloids improve the stability of growth factors and provide sustained release that is due to the continuous dosing. They proved reproducible delivery of growth factors because of the possibility to produce microspheres with specific physicochemical and colloidal properties in large volumes (Kalaji et al., 2009).

The ultimate objective of dental TE is to completely regenerate in the jaw a previously lost or missing tooth with similar physical properties and functions that would provide a remedy for all problems encountered with false teeth and implants. The term regenerative TE has recently been used to designate this branch of dental TE involving the triad (three key elements): responding progenitor or stem cells, indicative morphogenetic signals (growth factors), and extracellular matrix scaffolds (Kuo et al., 2007).

A variety of dental tissues have to be regenerated to form the whole tooth. This necessitates a rigorous study of the scaffold structure, the incorporation of the stem cells, and the growth factors

(Duailibi et al., 2008). The scaffolds have to contain hybrid functional microspheres with distinct release rates of a variety of growth factors, which are necessary to maintain the different tissue ingrowths, and the molecules necessary to overcome changes in the release medium (such as buffers).

ABBREVIATIONS

bFGF	Basic FGF
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
$Ca(OH)_2$	Calcium hydroxide
EGF	Epidermal growth factor
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
MTA	Mineral trioxide aggregate
NGF	Nerve growth factor
O/W	Oil-in-water
PCL	Polycaprolactone
PDGF	Platelet-derived growth factor
PEG	Poly(ethylene glycol)
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
PLGA	Poly(lactide-*co*-glycolide)
PVA	Poly(vinyl alcohol)
TE	Tissue engineering
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor β
UV	Ultraviolet
W/O/W	Water-in-oil-in-water

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Partie bibliographique

Chapitre 3

La microencapsulation des protéines type facteur de croissance : méthode de préparation, caractérisation, modélisation de libération et méthodes d'évaluation biologique

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1. Introduction

Dans ce travail des microparticules sont fabriquées par une méthode de formulation physicochimique et utilisées comme système de libération contrôlé des facteurs de croissance pour l'ingénierie du tissu de la pulpe dentaire.

Dans ce chapitre, nous allons aborder la microencapsulation et ses méthodes avec les protéines comme principes actifs. Ensuite nous allons détailler la méthode de double émulsion qui est la méthode adaptée pour la microencapsulation des protéines. Nous abordons aussi les différents composants qui entre dans le procédé d'encapsulation (polymère, solvant et émulsifiant). Ensuite, les méthodes utilisées pour la caractérisation physico-chimique des microparticules fabriquées seront présentées ainsi que la modélisation mathématique de la libération des principes actifs à travers ces microparticules. Enfin la méthode de vérification de l'activité biologique des facteurs de croissance encapsulés sur une modèle de dent humain extraite sera présentée.

2. La microencapsulation

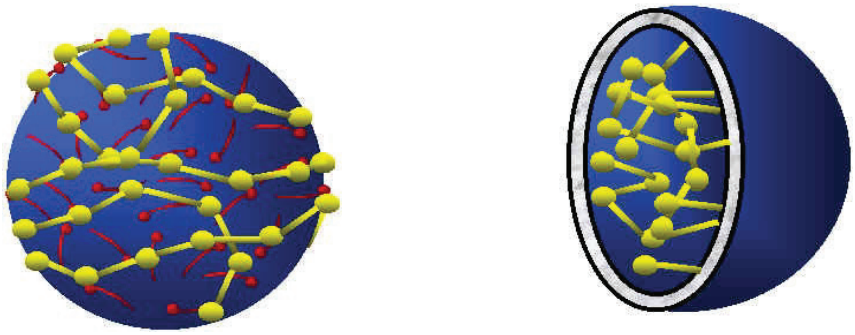
L'encapsulation peut être définie comme un ensemble de techniques conduisant à l'inclusion de substances solides, liquides ou gazeuses au sein d'un matériau support. Ces techniques permettent d'obtenir des microparticules individualisées dont la taille peut être comprise entre quelques nanomètres (10 nm) et quelques millimètres (2 mm). Elles présentent les avantages de pouvoir maîtriser à la fois la protection, le transport et le relargage de l'entité encapsulée.

2.1. Les microparticules

Les microparticules sont des formes pharmaceutiques destinées à contrôler la libération d'un principe actif. Ce terme (microparticule) regroupe à la fois les microcapsules et les microparticules.

L'appellation microparticules se réfère aux sphères dans lesquelles le principe actif est dispersé de façon homogène au sein de la matrice (figure 3).

Le terme microcapsules est utilisé pour caractériser des microparticules présentant une structure de type réservoir (figure 3.1), c'est-à-dire que le principe actif se trouve dans le cœur des microparticules, entouré par une membrane de polymère.



Microparticules Microcapsules

Figure 3.1 : les 2 types des microparticules

Les paramètres de formulation des microparticules déterminent la structure des microparticules (microparticules ou microcapsules). Le choix de type de particule fabriqué est déterminé par la future utilisation de ces microparticules ; les microparticules sont choisies si une libération prolongée est souhaitée et les microcapsules sont fabriquées pour une libération déclenchée.

L'utilisation des polymères biodégradables permet la délivrance prolongée de principe actif d'une manière programmée sur plusieurs heures, jours, semaines ou mois pour une utilisation unique.

2.2. Objectifs de microencapsulation

1- Augmenter la stabilité chimique

2- Retarder ou prolonger l'activité sur les tissus cibles.

3- Réduire la toxicité

4- Augmenter la tolérance immunologique

5- Diriger l'activité vers des tissus ou des cellules spécifiques.

2.3. Procédés de microencapsulation

Quelle que soit l'utilisation ultérieure des microparticules fabriquées, Il existe plusieurs techniques de la microencapsulation (Jain, 2000) (Dubernet et Benoit, 1986) :

- Les procédés physicochimiques
- Les procédés chimiques

- Les procédés mécaniques

Procédés chimiques	Procédés physico-chimiques	Procédés mécaniques
- polymérisation interfaciale - polymérisation en milieu dispersé :(émulsion, suspension…..) par voie radicalaire ou anionique	- Evaporation-extraction de solvant - Gélification thermique d'émulsion (hot melt) - Séparation de phase ou coacervation (simple ou complex)	- Nébulisation/séchage (spray-drying) - Enrobage en lit fluidisé (spray-coating) - Gélification ou congélation de gouttes (prilling)

Tableau 3.1 : Les trois grandes classes de procédés de microencapsulation (Dubernet et Benoit, 1986)

Les procédés de microencapsulation conduisent à envelopper d'une paroi polymérique (synthétique ou naturelle) un solide, un liquide ou un gaz qui forme ainsi un système réservoir ou une matrice.

Le choix de la méthode dépend de la nature du polymère, du principe actif, de l'intention et de la durée d'utilisation et de la libération désirée.

Afin de choisir le meilleur procédé d'encapsulation de protéine, il est indispensable de présenter un rappel sur les propriétés et les problèmes liés aux protéines.

3. Rappel sur les protéines:

Les peptides et les protéines sont formés par des enchaînements d'acides aminés liés entre eux par des liaisons peptidiques. A partir de quatre unités, on parle de polypeptides (protéines).

Les acides aminés dans la chaîne peptidique sont liés les uns aux autres dans un ordre précis et forme ainsi la séquence. Cette séquence conduit la protéine à adopter son organisation tridimensionnelle.

L'activité biologique complète des protéines est assurée par quatre niveaux conformationeles : structures primaires, secondaires, tertiaires et quaternaires. Les protéines sont des molécules très variées. L'être humain en fabriquerait plus de 100 000 différentes avec pour chacune une forme tridimensionnelle et une activité qui lui sont propres. Elles remplissent de nombreux rôles dans la cellule (d'hormones, d'enzyme, de transporteurs de substances, de fonctions de structure, de mouvement, de défense de l'organisme).

Les protéines sont considérées comme agents thérapeutiques car elles sont actives et efficaces au site d'action spécifiques avec peu d'effets secondaires et certaines sont des molécules endogènes donc elles ne sont pas allergisantes (Cullander et Guy, 1992).

Le traitement de certaines pathologies humaines importantes telles que le diabète, l'hémophilie, certains déficits hormonaux n'est possible que par l'administration de protéines thérapeutiques.

Pendant les dernières décennies, il y a eu un intérêt considérable pour le développement de nouveaux médicaments à base de protéines, cela en partie grâce aux progrès rapides dans le domaine des biotechnologies qui ont conduit à une disponibilité plus importante des protéines recombinantes (Cohen et al, 1991).

Actuellement, 130 protéines thérapeutiques sont utilisées et approuvées par le FDA et 1000 nouveaux principes actifs peptidique pour plus de 100 maladies (comme le cancer, le sida, les maladies auto-immunes, etc.) sont en phase d'essais cliniques humains ou à l'étude par la FDA (Tauzin, 2006 et Reichert et all 2003).

Les protéines ont, en général, une masse moléculaire élevée, leur capacité d'absorption à travers les membranes gastro-intestinales est faible. Elles sont très sensibles aux conditions ambiantes (enzymes, pH, température ....) ce qui les rend de très mauvaises candidates pour l'administration orale (Dai et al. 2005). Longtemps la formulation de ces molécules n'a été possible que sous forme de solutions injectables afin d'éviter les barrières biologiques. Cependant, les recherches récentes se sont portées sur de nouvelles formes galéniques et de nouveaux modes d'administration (Dai et al. 2005).

Malgré ces avancées, l'usage de cette classe potentielle de médicaments reste limité. L'obstacle majeur de l'utilisation pharmaceutique des protéines reste leur instabilité chimique et physique.

3.1 Quelques propriétés des protéines

Parmi les propriétés physico-chimiques des protéines, nous allons développer quelques propriétés qui sont intéressantes à prendre en compte dans le procédé d'encapsulation.

3.1.1 La solubilité :

Certaines protéines sont solubles dans l'eau pure (albumines), d'autres - notamment les globulines- ne se dissolvent qu'en présence de sels neutres ou en faisant d'autres modifications. Un certain nombre de facteurs peuvent influencer la solubilité (électrolytes, PH, solvants organiques et température).

3.1.2 Masse moléculaire :

La masse moléculaire est une propriété fondamentale qui affecte la diffusion des molécules peptidiques à travers les membranes, cela peut être très important dans les études de diffusion et de la libération des protéines à travers des matrices polymérique. Plus que la masse moléculaire est grande plus que la diffusion est lente. La masse moléculaire varie de quelques milliers à un million de daltons ou plus (Weil J.H, 1990).

3.1.3 Stabilité des protéines :

Les protéines sont des molécules fragiles et lors de l'exposition à des conditions qui ne leur conviennent pas, elles peuvent subir différentes altérations chimiques ou physiques.

3.1.3.1 L'instabilité chimique : une altération chimique comme la déamidation, l'oxydation, la protéolyse, l'échange entre ponts disulfure résulte en une modification de la protéine par un clivage ou par la formation d'une liaison conduisant à la formation d'une nouvelle molécule (Manning et al. 1989).

3.1.3.2 L'instabilité physique : se caractérise par un changement dans la structure secondaire et plus.

3.1.3.3 Type d'instabilité physique :

- La dénaturation : indique la perte de la structure tertiaire et fréquemment de la structure secondaire. Cette dénaturation est causée par l'augmentation ou la diminution de la température, par les pH extrêmes et par l'ajout de solvants organiques (Manning et al. 1989). Elle peut être réversible ou irréversible.
- L'agrégation est rencontrée avec les protéines présentant des états intermédiaires partiellement dépliées à cause de l'exposition des régions

hydrophobes contigües qui sont protégées dans la protéine native ou absentes dans la protéine dénaturée (Fields et Alonso, 1992).

- La précipitation est une agrégation macroscopique (Manning et al. 1989).
- L'adsorption des protéines sur les surfaces (Twardowski et a1. 1983).

La dégradation des protéines peut conduire à une perte de l'activité biologique et à une réponse immunitaire indésirable. Les produits de dégradation des protéines peuvent affecter le profil de libération des protéines depuis les systèmes particulaires par exemple (Sinha et Trehan, 2003).

3.1.3 .4 Causes de l'instabilité des protéines :

Les agents dénaturants sont très variés et leurs effets peuvent relever de mécanismes physiques, chimiques ou mixtes. Tous les facteurs capables d'entraîner la rupture des liaisons hydrogènes et hydrophobes peuvent rendre la protéine instable ou dénaturé (Percheron F et coll., 1991).

- La chaleur favorise la rupture des liaisons hydrogènes. La température pour une stabilité maximale est entre -10 et 35°C pour la majorité des protéines.
- Les variations de pH : certains acides (nitrique, trichracétique, perchlorique, etc.) et certaines solutions acides de métaux lourds (Hg, Pb) sont utilisés en analyse biologique pour éliminer les protéines du milieu. Les pH extrêmes entraînent la rupture de liaisons électrostatiques et hydrogènes. La stabilité maximale des protéines est obtenue lorsque leur charge nette est égale à zéro.
- L'urée et le chlorure de guanidinium : ces petites molécules, en solution concentrée entraînent une dénaturation réversible par formation de nombreuses liaisons hydrogènes avec la protéine.
- Les tensioactifs anioniques (effet réversible sans précipitation) dissocient les structures quaternaires.
- Les réactifs rompant les ponts disulfure, de façon réversible, tels les réducteurs ou de façon irréversible, tels les oxydants.

3.2 Conclusion sur les protéines

Nous pouvons conclure que les problématiques de la délivrance d'une protéine dont les facteurs de croissance font partie sont :

- Les protéines sont des molécules relativement grosses avec différents niveaux de structure. Elles ont des structures secondaires, ternaires et même quaternaires avec des chaînes latérales qui contiennent des groupements réactifs. Des modifications de ces structures ou de ces chaînes peuvent conduire à la perte de l'activité biologique.
- Les protéines sont des molécules fragiles et la fabrication d'un système de délivrance des protéines peut entraîner une modification des structures des protéines et de leurs activités (Sinha et coll., 2003).
- le système de délivrance des protéines doit répondre aux exigences suivantes:
    1. Conservation de la stabilité physique et chimique
    2. Prolongation de l'effet biologique
    3. Augmentation de l'absorption
    4. Diminution de l'antigénicité et du métabolisme (Sinha et coll. 2003).

4. Les procédés physicochimiques (procédé d'extraction de solvant)

4.1 Les émulsions et les émulsions multiples :

Une émulsion est préparée par une dispersion d'un liquide immiscible dans un autre, cette émulsion est stabilisée par un troisième composant qui est l'agent stabilisant. Il y a deux types d'émulsions : soit la phase interne est de l'eau et on parle alors d'émulsions W/O soit l'eau constitue la phase externe et on parle d'émulsions O/W (figure 3.2).

D'autres systèmes plus complexes résultant de la dispersion d'une émulsion dans une autre phase continue sont appelés émulsions multiples ou double émulsions. Par conséquent, on a W/O/W ou O/W/O (figure 3.2).

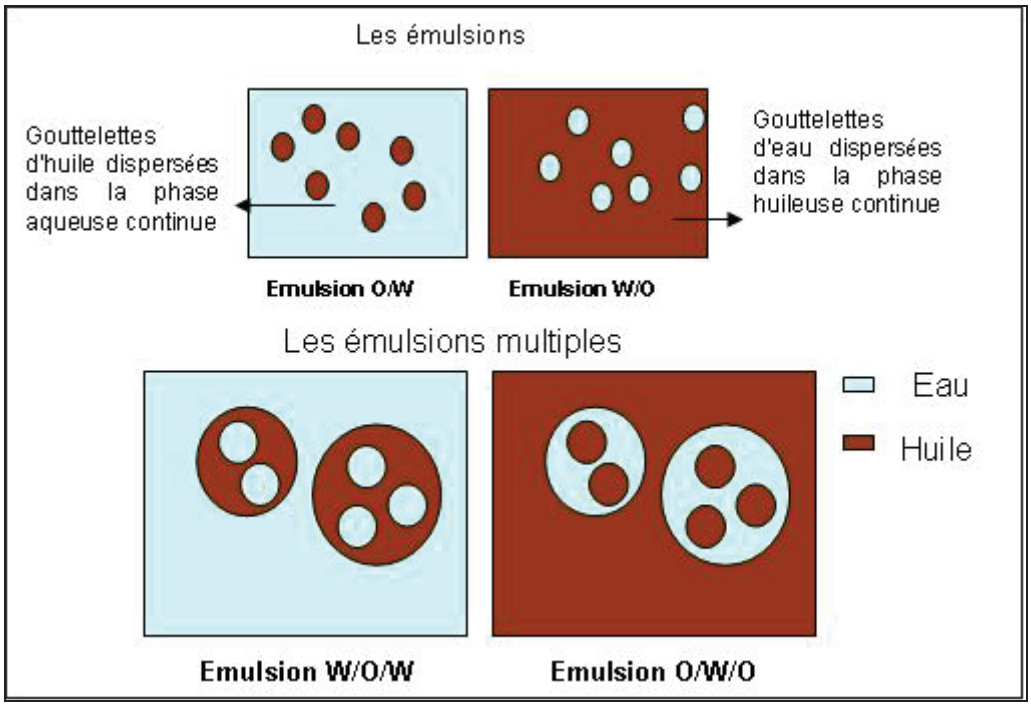


Figure 3.2 : Les émulsions et les émulsions multiples

4.2. La méthode d'émulsion multiple avec évaporation de solvant pour l'encapsulation des protéines

La méthode d'émulsion multiple/évaporation de solvant représentée dans la figure (3.3) consiste à dissoudre le principe actif dans l'eau distillée ou dans une solution tamponnée (phase aqueuse interne) et à dissoudre le polymère dans un solvant organique non miscible avec l'eau. La phase aqueuse interne est versée dans la phase organique. Ce mélange est agité pour former l'émulsion primaire W/O. Cette émulsion W/O est alors versée dans une solution aqueuse d'un tensioactif approprié (phase aqueuse externe) sous agitation. Cette phase externe doit contenir un agent stabilisant pour former une double émulsion W/O/W. L'émulsion résultante est agitée de façon permanente ce qui permet au solvant de s'évaporer et au polymère de précipiter autour des gouttelettes formées pendant la phase d'émulsification primaire. Le résultat est la formation des microparticules solides contenant le principe actif.

Il est également possible d'utiliser un alcool qui a une affinité avec le solvant organique pour accélérer son extraction (Alonso et coll., 1993).

Les microcapsules sont récupérées par filtration ou par centrifugation, puis sont lavées et séchées.

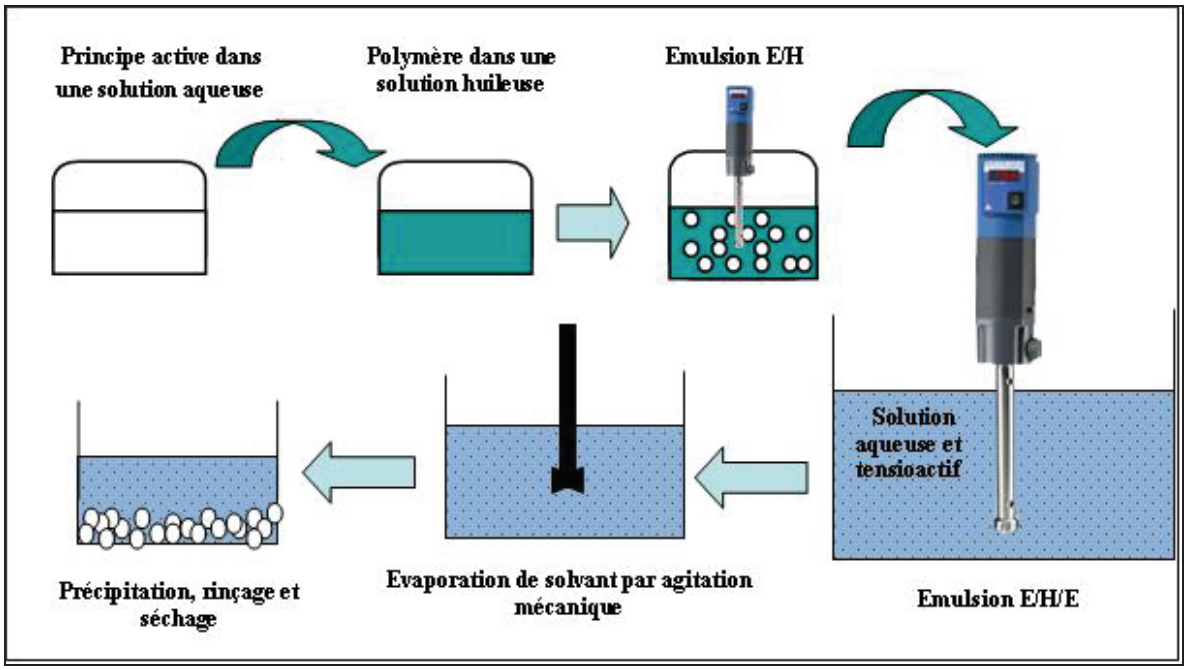


Figure 3.3 : Procédé de microencapsulation par évaporation de solvant à partir d'une émulsion W/O/W

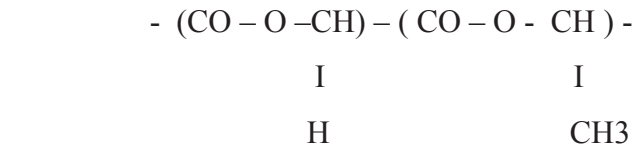
4.3. Les polymères biodégradables :

Les microparticules polymériques biodégradables et biocompatibles ont connu un essor important ces dernières décennies. Les polymères biodégradables sont d'origine naturelle ou synthétique, sont dégradés *in vivo* de façon enzymatique ou non enzymatique, produisant des substances biocompatibles, non toxiques, qui seront éliminés par le métabolisme de l'organisme.

Les principes actifs encapsulés seront relargués soit par diffusion à travers la "barrière" de polymère, soit par érosion du matériau polymère, soit par une combinaison simultanée des deux phénomènes.

Les poly(esters) aliphatiques thermoplastiques comme l'acide poly-lactique (PLA), l'acide poly-glycolique (PGA), ou plus particulièrement le copolymère d'acide lactique et glycolique, nommé acide poly lactique-co-glycolique (PLGA) ont généré un réel intérêt à exploiter grâce à leur biocompatibilité et leur biodégradabilité.

4.3.1. Propriétés physico-chimiques et biologiques du copolymère PLGA :



Formule chimique de l'Acide Poly(lactique-glycolique) (PLGA)

La connaissance de propriétés physico-chimiques et biologiques du polymère, (tout comme celles caractérisant le principe actif à encapsuler, mais aussi les divers paramètres de formulation comme la vitesse d'agitation, le choix du stabilisant et le choix du solvant) influence largement le choix de la technique idéale d'encapsulation, le relargage de la molécule, etc.

Lors de l'utilisation de PLGA comme dispositifs pour délivrer un principe actif, le polymère doit avoir une résistance mécanique contre les changements éventuels subits à cause de la méthode d'encapsulation (température, précipitation, dispersion, etc.). Dans ce contexte, différentes propriétés propres au polymère contrôlent cette résistance. Parmi ces propriétés, on peut citer entre autres le poids moléculaire, la composition de polymère (ratio PLA / PGA), la cristallinité, et la régularité géométrique des chaînes individuelles.

4.3.1.1. La composition du polymère (ratio PLA / PGA)

Constitué par la polymérisation de l'acide lactique et l'acide glycolique, la proportion de chacun influence les caractéristiques du PLGA. En effet, l'acide lactique est plus hydrophobe que l'acide glycolique. Ainsi en général, les copolymères PLGA riches en acide lactique sont moins hydrophiles, absorbent moins d'eau, et donc se dégradent moins rapidement.

4.3.1.2. Le poids moléculaire

Le poids moléculaire influence le relargage par la différence de la perméabilité.

4.3.1.3. La cristallinité et la régularité géométrique des chaînes individuelles

La cristallinité du polymère PLGA influence directement la résistance mécanique, la capacité à supporter l'hydrolyse, et par conséquence la dégradation de celui-ci. Cette cristallinité dépend du type et du ratio molaire des composants monomères :

- les copolymères de PLGA dont le rapport PLA / PGA est de 50 / 50, s'hydrolysent plus rapidement que ceux contenant une proportion plus importante de l'un des deux monomères.
- la présence de la forme L-PLA associé au PGA permet d'obtenir un copolymère cristallin, alors que l'association D, L-PLA avec PGA donne un copolymère amorphe dans la nature.

4.3.1.4. La dégradation biologique du PLGA

De même, la dégradation biologique du PLGA dépend du ratio molaire en acide lactique et glycolique dans les chaînes polymériques, du poids moléculaire, du degré de cristallinité, et de la Tg du polymère.

La mise en jeu d'enzymes ou non (hydrolyse simple) au cours de la dégradation est très controversée (Jain, 2000).

Le PLGA se dégrade donc :

- En acide lactique entrant dans le cycle de l'acide tricarboxylique, serait métabolisé avant d'être éliminé sous forme de CO2 et H2O,
- En acide glycolique qui peut être éliminé tel quel par les reins, ou peut entrer dans le cycle de l'acide tricarboxylique et éventuellement être éliminé sous forme de CO2 et de H2O.

Un mécanisme de dégradation à trois phases du PLGA a été proposé (Jain, 2000) :

1°) D'abord, une cassure aléatoire des chaînes a lieu. Le poids moléculaire du polymère diminue significativement, mais aucune perte de poids n'est appréciable et aucun produit monomère soluble ne se forme.

2°) La phase intermédiaire se caractérise par une diminution du poids moléculaire accompagnée par une perte rapide de poids, et par une formation de produits monomériques et oligomèriques solubles.

3°) Des éléments monomériques solubles sont ainsi formés à partir de fragments oligomèriques. C'est au cours de cette phase qu'a lieu la solubilisation complète du polymère.

4.4. Le stabilisant (l'alcool polyvinylique PVA)

Au cours du procédé d'évaporation/extraction, on assiste à une diminution progressive du volume, et donc à une augmentation consécutive de la viscosité des gouttelettes d'huile dispersées. L'effet produit est alors la tendance à la coalescence des gouttelettes ou des microparticules, aboutissant à la formation d'agrégats. Pour remédier à ce problème, il suffit d'ajouter dans la phase continue, une petite quantité de produit « stabilisant » les microparticules (émulsifiant). Celui-ci crée une fine pellicule autour des gouttelettes ou des microparticules empêchant leur rapprochement pendant l'élimination du solvant mais aussi après.

Les propriétés physico-chimiques et la concentration de stabilisant influencent considérablement la taille, la forme et l'efficacité d'encapsulation du principe actif. Pour la plupart des stabilisants, la taille des microparticules formées diminue avec l'augmentation de la concentration de celui-ci.

Dans ce procédé, plusieurs stabilisants peuvent être utilisés comme le polyvinylpirrolidone, l'alginate de sodium, le méthylcellulose et la gélatine, mais le stabilisant le plus utilisé est le poly vinyl alcool (PVA) (Ardashy et coll., 1991).

L'Alcool Polyvinylique (PVA) est le produit de l'hydrolyse du Poly (acétate de vinyle). Le PVA présente l'avantage d'être relativement non toxique, non carcinogène, tout en ayant des caractéristiques bio-adhésives (DeMerlis, 2003).

D'après Jeffery et coll., 1993 l'augmentation de la concentration de PVA dans la phase aqueuse, aboutit à une réduction de la taille des microparticules. L'encapsulation du principe actif n'est pas influencée par la variation de cette concentration. Les auteurs attribuent ce constat aux gouttelettes instables formées lors de l'émulsion, aboutissant à la formation de grosses microparticules, en présence de concentration de PVA faible.

4.5. Le solvant

Le solvant organique utilisé doit avoir un point d'ébullition faible afin d'éviter les problèmes liés aux résidus de solvant et surtout d'éviter de chauffer pour l'évaporer. Parmi les solvants que nous pouvons utiliser, nous trouvons l'acétonitrile, l'acétate d'éthyle, le chloroforme, le benzène et le dichlorométhane DCM (Blanco-Prieto MJ et coll., 1994).

Le DCM est le solvant le plus utilisé pour la préparation des microparticules par émulsion multiple W/O/W grâce à ses bonnes propriétés physiques. En effet, le dichlorométhane est un solvant capable de dissoudre un grand nombre de polymères, il a une solubilité faible dans l'eau (2.0%, w/v) et un faible point d'ébullition (39.8°C) (Fan Tao Meng et coll., 2003).

En effet, on doit enlever toutes les traces du solvant des microparticules car les solvants sont généralement toxiques. Les résidus de solvant sont considérés toxiques dans les limites de 60 ppm pour le chloroforme et 600 ppm pour DCM, (pharmacopée européenne 2002, Serota et col 1986) et la présence de résidus de solvant peut affecter la stabilité des microparticules.

Pendant l'étape de l'extraction de solvant (appelé aussi étape de la solidification car suite à cette étape les microparticules polymérique solide sont fabriqués), le solvant organique doit d'abord diffuser dans la phase aqueuse externe puis, il s'évapore à l'interface air/liquide par un des protocoles suivants :

- La préparation des microparticules est maintenue sous agitation à température ambiante jusqu'à l'évaporation complète du solvant (Blanco-Prieto MJ et coll., 1994).
- L'émulsion multiple est transférée dans un rotavapor et chauffée à 30°C sous pression réduite (Ogawa Y et coll., 1988).
- L'évaporation à température ambiante est arrêtée quand le solvant n'est pas totalement éliminé et les microparticules partiellement solides sont transférées dans une solution contenant un stabilisant ou simplement dans une quantité d'eau (Cohen S et coll., 1991).
- Quelques processus modifiant cette méthodologie sont proposés comme l'extraction de DCM par l additionnant d'un alcool qui a une affinité avec le DCM comme l'isopropanol. L'isopropanol va donc accélérer la diffusion de DCM et son extraction (Alonso et coll., 1993).

5. Les caractéristiques des microparticules chargées en protéines

Une préparation satisfaisante résulte en l'obtention de microparticules sphériques sans déchets (microparticules déformées ou précipitées de polymère). Les microparticules sont caractérisées par leur taille, leur morphologie externe et interne, leur efficacité d'encapsulation et la vitesse de libération du principe actif.

5.1. Taille des microparticules :

La distribution de la taille est contrôlée par la vitesse d'agitation de deuxième émulsion (Y.Y Yang et coll., 2001).

La taille des microparticules est à fixer en fonction de la voie de délivrance désirée. La taille peut aussi affecter les autres caractéristiques des microparticules comme la libération et le taux d'encapsulation par effet de la surface exposée à l'eau.

Contrairement aux autres voies d'utilisation comme l'injection ou l'inhalation où des microparticules de petite taille sont recherchées, pour une utilisation locale, des microparticules de petite taille (10µm) ou de grande taille (150µm) peuvent être appliqués.

Pour effectuer les mesures de la taille des microparticules, la technique de granulométrie laser est utilisée dans ce travail.

5.2. Morphologie des microparticules :

5.2.1 Morphologie interne

La morphologie interne des microparticules joue un rôle très important dans la libération du principe actif à travers la paroi de polymère.

Il y a beaucoup de paramètres qui peuvent avoir une influence sur la morphologie interne des microparticules.

En diminuant le volume de la phase interne, la porosité interne diminue. En effet, la structure poreuse des microparticules est le résultat de la phase aqueuse interne donc, la diminution de cette phase interne conduit à une structure moins poreuse.

La vitesse d'agitation pendant la première émulsification joue également un rôle très important sur la taille des pores. Puisque une vitesse importante donne des gouttelettes plus petites avec une taille des pores seront plus petites.

La morphologie des microparticules peut être affectée par la présence d'additifs comme polyéthylène glycol PEG (40000) (Sah H et coll, 1994), vitamine E TPGS (tocophéryl polyéthylène glycol 1000 succinate (Péan J.M. et coll., 1998).) et NACL (Gang Ruan et coll 2002).

5.2.2 Morphologie externe

La morphologie externe des microparticules est important pour vérifier la forme sphérique et les aspects de surface (laisse, poraux ….).

L'optimisation de la procèdes de l'encapsulation et surtout de l'étape de l'extraction de solvant permet d'avoir des microparticules séparés, sphériques et laisses, ce qui permettra une libération maitrisée

L'observation microscopique par plusieurs techniques (optique ou électronique) a toujours été la façon de vérifier la forme, morphologie de surface.

5.3 Efficacité d'encapsulation:

Le facteur le plus important pour une bonne efficacité d'encapsulation des principes actifs hydrophiles est la stabilité de l'émulsion primaire. La perte de protéine encapsulée durant la fabrication est essentiellement le résultat de la diffusion de la protéine de la phase aqueuse interne vers la phase aqueuse externe (Gang Ruan et coll., 2002).

L'efficacité d'encapsulation peut être modifiée par les facteurs suivants :

- Stabilité de l'émulsion W/O et de l'émulsion W/O/W
- Vitesse d'évaporation du solvant
- Taille des microparticules.

Donc, le choix judicieux du polymère, du solvant et des additifs peut aider à améliorer l'efficacité de l'encapsulation des protéines au sein des microparticules.

L'efficacité d'encapsulation est généralement présentée en pourcentage et calculée comme suivant :

Efficacité d'encapsulation = quantité du principe actif encapsulé/quantité de principe actif totale*100

Le taux d'encapsulation ou « *loading* » présente le continue du principe actif dans les microparticules et calculé comme suivant :

Le taux d'encapsulation ou « *loading* » = quantité du principe actif encapsulé/ poids totale des microparticules.

5.4 Libération du principe actif :

5.4.1 Les systèmes à libération déclenchée et à libération prolongée :

5.4.1.1 Les systèmes à libération déclenchée : sont généralement des microcapsules formées d'une membrane étanche qui vont libérer brutalement leur contenu par

éclatement de la membrane sous l'effet d'une pression (mécanique ou osmotique) ou par fusion sous l'effet de la température, ou par dissolution sous l'effet de pH.

5.4.1.2 Les systèmes à libération prolongée : se distinguent les uns des autres par les mécanismes de libération mis en jeu (diffusion passive, dégradation/dissolution du matériau enrobant, échange d'ions, etc.) et par leur structure (microcapsule ou microparticule) qui influence directement la cinétique de libération résultante.

Pour les microcapsules, deux possibilités de profil de libération existent :

- la vitesse de libération initiale est très rapide (*«burst effect»*) en raison de la présence d'un excès de matière active dans la membrane
- un temps de latence précède la libération : temps nécessaire pour que le principe actif diffuse à travers la membrane avant d'atteindre le milieu extérieur.

A partir des microparticules (microsphères), le phénomène de diffusion de la molécule active à travers une matrice est décrit par une cinétique obéissant à la loi d'Higuchi. La quantité libérée de matière active est directement proportionnelle à la racine carrée du temps (Walstra P 1985). La loi de Fick est également utilisée pour étudier la diffusion à travers une matrice.

Les deux lois ne représentent pas l'effet *burst* ou le délai de libération à moins de varier le coefficient de diffusion avec le temps

5.4.2 Les facteurs qui influencent le profil de la libération

La structure poreuse des microparticules a un effet très important sur la libération de principe actif. En diminuant la porosité dans les microparticules, la libération du principe actif se ralentit. De même, plus les pores sont gros, plus la libération est rapide. Une porosité interne élevée est indispensable pour la libération des protéines d'un poids moléculaire élevé ou pour la libération des protéines ayant une forte affinité avec la matrice de polymère. En revanche, des microparticules avec une porosité faible peuvent être utilisées pour retarder la libération des médicaments hydrophiles (Andre-Abrant A et coll., 2001).

La morphologie externe influence le taux de dégradation des microparticules et elle détermine les interactions éventuelles entre les microparticules et les tissus.

En plus de cette structure interne et externe qui détermine le profil de libération, on trouve d'autres facteurs modifiant la libération comme la taille des microparticules et la distribution du principe actif dans la matrice.

6. Cahier des charges de la préparation de microparticules chargées en protéines :

Derrière n'importe quelle préparation de microparticule, nous cherchons :

1. Efficacité d'encapsulation élevée.
2. Reproductibilité.
3. Profils flexibles de libération.
4. *«burst effect»* faible qui aide à la libération prolongée de protéine.
5. Le ratio protéine/polymère doit permettre l'encapsulation d'une quantité importante de protéine par rapport au polymère. Cela diminue la masse de microparticules administré.
6. Le processus d'encapsulation doit conduire à des microparticules de taille désirée.
7. la Stabilité et l'activité biologique des protéines encapsulées doit être maintenue durant et après la formulation des microparticules.
8. Minimum de contact entre la protéine et le solvant organique utilisé pour dissoudre le polymère, ce qui minimise la dénaturation de protéine.

7. Modélisation de la diffusion du principe actif à travers les microparticules

La modélisation permet une meilleure maîtrise du procédé. Elle permet d'anticiper l'évolution du procédé et de l'optimiser. Elle consiste à relier les qualités du produit

(taille des particules, épaisseur) aux conditions opératoires (agitation, concentrations, forme du réacteur).

Les modèles sont généralement basés sur des lois chimiques ou physiques ou parfois empiriques. Ils prennent souvent la forme d'équations (ordinaire ou différentielles).

Dans le cadre de ce travail, nous avons étudié la vitesse de libération des protéines à travers la matrice polymérique. La loi de Fick à été employée pour réaliser cette modélisation en utilisant le logiciel Matlab.

7.1. LA LOI DE FICK :

La diffusion est représentée par la loi de Fick de diffusion ou l'équation de diffusion qui prend les formes suivantes en 1, 2 et 3 dimensions :

1 Dimension :

$$\frac{1}{D}\frac{\partial C}{\partial t} = \frac{\partial^2 C}{\partial x^2} \quad (1)$$

2 Dimensions :

$$\frac{1}{D}\frac{\partial C}{\partial t} = \frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} \quad (2)$$

3 Dimensions :

$$\frac{1}{D}\frac{\partial C}{\partial t} = \frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} + \frac{\partial^2 C}{\partial z^2} \quad (3)$$

Où,

t : temps (s)

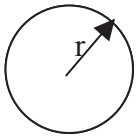
C : concentration de principe diffusant (mole/cm3)

D : coefficient de diffusion (cm2/s)

L'équation de diffusion en géométrie sphérique devient :

$$\frac{1}{D}\frac{\partial C}{\partial t} = \frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial C}{\partial r}\right) + \frac{1}{r^2\sin\theta}\frac{\partial}{\partial\theta}\left(\sin\theta\frac{\partial C}{\partial\theta}\right) + \frac{1}{r^2\sin^2\theta}\frac{\partial^2 C}{\partial\phi^2} \quad (4)$$

avec r : rayon de la particule (cm)



Puisque lors de la diffusion dans une particule sphérique les conditions initiales et les conditions de limites ont une symétrie sphérique, la concentration C dépend seulement de r et ne dépend pas de θ (Co-latitude) et ϕ (Longitude). L'équation de diffusion dans une géométrie sphérique se réduit à :

$$\frac{1}{D}\frac{\partial C}{\partial t} = \frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial C}{\partial r}\right) = \frac{\partial^2 C}{\partial r^2} + \frac{2}{r}\frac{\partial C}{\partial r} \quad (5)$$

7.2. SOLUTION ANALYTIQUE DE L'EQUATION DE DIFFUSION :

Cette équation possède une solution analytique. Cette solution peut-être est obtenue en procédant par séparation de variables.

$$C(r,t) = R(r)T(t) \quad (6)$$

Avec r et t définis comme précédemment.

En remplaçant C par ces fonctions dans l'équation 5 nous obtenons :

$$\frac{1}{DT(t)}\frac{dT(t)}{dt} = \frac{1}{R(r)r^2}\frac{d}{dr}\left(r^2\frac{dR(r)}{dr}\right) \quad (7)$$

Chaque côté de cette équation est sûrement égale à une constante, disons -b2 ce qui donne ces deux équations :

$$\frac{1}{r^2}\frac{d}{dr}\left(r^2\frac{dR}{dr}\right)+b^2R=0 \qquad (8)$$

Et

$$\frac{dT(t)}{dt}+b^2DT=0 \qquad (9)$$

Nous pouvons effectuer un changement de variable pour équation 8, R(r) = f/r et nous obtenons ainsi l'équation suivante :

$$\frac{d^2f}{dr^2}+b^2f=0 \qquad (10)$$

Les solutions de cette équation sont f=sin(br) et f=cos(br). Cependant, R(r) doit être égale à zéro si r=0, donc :

$$R(r)=\frac{1}{r}\sin(br) \qquad (11)$$

La solution de l'équation 9 est de la forme :

$$T(t)=\exp\left(-b^2Dt\right) \qquad (12)$$

La valeur de b dépend des conditions aux limites. Si on suppose que C=0 pour r=r0 (à la surface de la particule ce qui est relativement vrai si la libération se fait dans un milieu dilué et sous une bonne agitation), on obtient :

$$0=\sin(br_0)$$

ce qui donne les solutions suivantes pour b :

$$b=\frac{n\pi}{r_0}\text{, où } n=1\rightarrow\infty$$

La solution analytique de l'équation 5 est donc la suivante :

$$C(r,t) = \sum_{n=1}^{\infty} a_n s \frac{in(n\pi r / r_0)}{r_0} \exp\left(-n^2\pi^2 Dt / r_0^2\right) \qquad (13)$$

La masse de principe actif libéré peut-être calculé à partir de cette équation en considérant qu'elle est égale à la masse à la surface de la particule (r=r0).

$$C(r_0,t) = \frac{M_\infty - M_t}{M_\infty} = \sum_{n=1}^{\infty} a_n s \frac{in(n\pi)}{r_0} \exp\left(-n^2\pi^2 Dt / r_0^2\right)$$

où M_∞ est la masse totale de principe actif encapsulé et M_t est la masse de principe actif libérée à l'instant t ce qui donne la solution analytique de la deuxième loi de Fick de diffusion :

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp\left(-n^2\pi^2 Dt / r^2\right) \qquad (14)$$

Nos microparticules étant sphériques, nous pouvons donc faire une simulation de la diffusion de principe actif dans la particule, soit en utilisant le modèle défini par l'équation 3, soit par l'équation 5.

8. Méthodes d'évaluation de l'activité biologiques de microparticules chargées en facteurs de croissance

Pour valider les microparticules comme système de libération contrôlée pour les facteurs de croissance, l'étape essentielle est de vérifier si l'activité biologique de ces facteurs de croissance est maintenue. Plusieurs méthodes peuvent être utilisées pour cette vérification.

8.1 L'étude de la prolifération cellulaire

Dans ce travail, dans une première approche les microparticules sont appliquées sur des cellules pulpaires extraites de pulpes des troisièmes molaires immatures et mises en

culture. Le test MTT est utilisé pour étudier la prolifération cellulaire en présence de ces microparticules chargé en facteurs de croissance. Les méthodes de culture et l'explication du test MTT seront abordées dans la partie expérimentale.

8.2 Les modèles expérimentaux pour l'évaluation des matériaux de coiffage pulpaire direct

De nombreuses études *in vitro*, et *in vivo* ont tenté d'expliquer les mécanismes qui contribuent à la réparation ou mieux, à la régénération pulpodentinaire. Tous les modèles expérimentaux ont leurs limites, mais tous contribuent à une meilleure connaissance de ces phénomènes complexes.

8.2.1 Les modèles expérimentaux *in vivo*

Les expérimentations *in vivo*, chez l'homme sont de plus en plus difficiles à réaliser pour des raisons éthiques dictées par la loi Huriet (1988 modifié en 2000 et 2004). Ces expérimentations menées il y a quelques années et encore aujourd'hui dans certains pays où ces considérations n'existent pas, ont apporté des résultats très importants et constituent un cadre de référence pour toutes les autres études.

Les expérimentations *in vivo* chez l'animal permettent des études à plus grande échelle. La taille des échantillons, la durée des études, leur reproductibilité, l'utilisation de souris transgéniques (Braut et al., 2003), d'animaux germ-free (Tsuji et col 1987) et de souris immunodéprimées pour des études de transplantations (Batouli et al., 2003) ont permis des avancées considérables dans la compréhension de l'odontogénèse, et des processus de migration, d'interaction et de différenciation cellulaires.

Ainsi, de nombreux biomatériaux de coiffage direct ont été testés chez les rongeurs (Andelin et al. 2003 ; Goldberg et al. 2001), les chiens (Tziafas et al. 1995; Iohara et al. 2004), les singes (Cox et al., 1996), et les porcs (Nakamura et al.. 2004). Ces études ont permis de comparer les effets des matériaux utilisés sur la pulpe, et de mieux comprendre la chronologie des mécanismes impliqués. Les progrès de la biologie moléculaire permettent de révéler l'expression de marqueurs spécifiques à certains types cellulaires, et à certains stades fonctionnels (McLachlan et al. 2003).

Même si les avantages de l'expérimentation animale sont indéniables, les résultats de ces études ne sont pas forcément transposables chez l'homme. En effet, la morphologie des dents et les systèmes de défenses sont différents. Le mode de croissance continu de certaines dents chez les rongeurs, nécessitent une grande prudence dans l'extrapolation des résultats chez les humains. Enfin, l'inconvénient majeur des expérimentations animales réside dans leur coût élevé, et l'infrastructure nécessaire pour les mener à bien.

8.2.2 Les modèles expérimentaux *in vitro*

Pour toutes les raisons abordées dans le paragraphe précédant, des modèles expérimentaux *in vitro* ont été élaborés dans un double perspectif : la recherche fondamentale, et la première phase d'évaluation des biomatériaux avant l'étape d'essais cliniques. Plusieurs modèles d'évaluation des biomatériaux ont donc été développés.

- Le premier consiste en la mise en culture *in vitro* de cellules d'explants pulpaires (About et al.. 2000) (figure 3.4). Ces cellules extraites de pulpes des troisièmes molaires immatures et mise en culture en présence de β-glycérophosphate forment des nodules de minéralisation dont la composition organique et minérale est identique à la dentine. L'évaluation d'un biomatériau dans ce modèle correspond à sa mise en place directe sur les fibroblastes pulpaires *in vitro*. Ce système permet de simuler les effets du biomatériau sur les fonctions spécifiques des cellules cibles en situation de coiffage pulpaire direct.

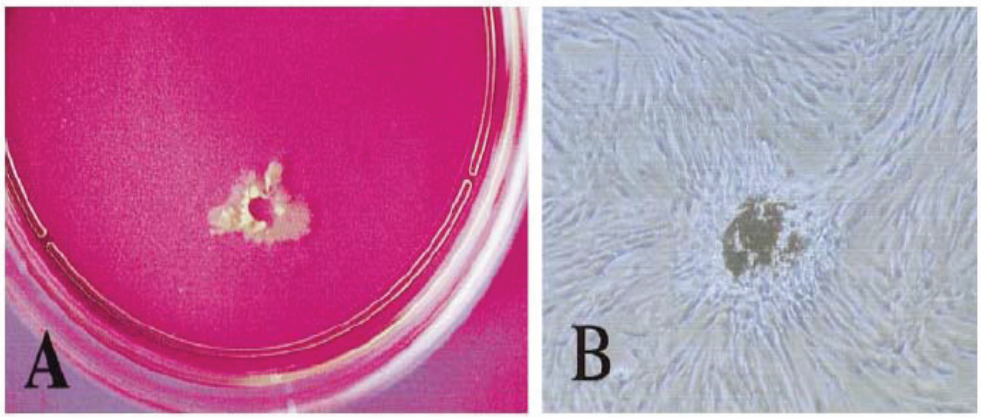


Figure 3.4 : Formations de nodules de minéralisation sur culture *in vitro* des cellules d'explants pulpaire. D'après (About et al.. 2000)

- Le deuxième modèle consiste en la mise en culture de tranches de dents humaines immatures comportant des cavités dentinaires (Magloire et al, 1996) (figure 3.5). Les tranches de 750 µm d'épaisseur sont réalisées à l'aide d'une scie, elles intéressent couronnes et racines dentaires et comportent le tissu pulpaire sain et la cavité. En utilisant ce modèle, Magloire et al, 1996 ont démontrés l'existence d'une prolifération cellulaire en regard de la cavité et d'une néovascularisation. Cependant, la préparation des tranches implique des dommages importants qui rendent la distinction difficile entre la lésion due à la cavité et la lésion due à la technique des tranches.

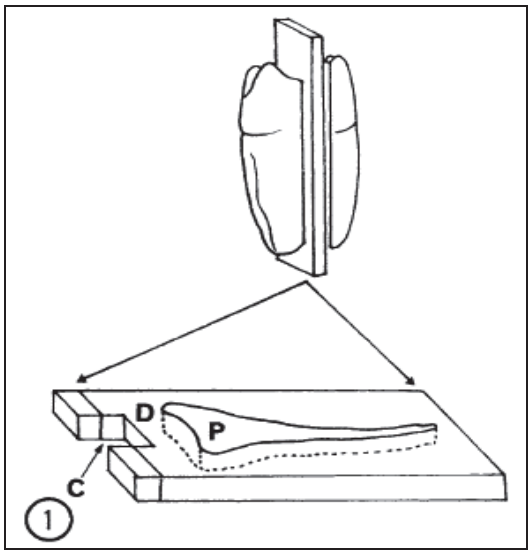


Figure 3.5 : la mise en culture de tranches de 750 µm de dents humaines immatures comportant des cavités dentinaires d'après (Magloire et al, 1996)

- Le troisième modèle correspond à la mise en culture de tranches de dents de rats comportant des cavités dentinaires et pulpaires obturées par différents biomatériaux dentaires (Sloan et al 1998). Un écueil supplémentaire est que les dents de rats sont des dents à croissance continue contrairement aux dents humaines. De plus, comme pour les tranches de dents humaines, il est difficile d'interpréter les résultats obtenus en regard des cavités puisque la préparation des tranches de dent entraîne inévitablement l'apparition de lésions pulpaires.

- Enfin, un modèle de culture de dent entière humaine *in vitro* (figure 3.6) est développé depuis 2004 dans le laboratoire IMEB-ERT 30 à la faculté d'odontologie de Marseille (Teclès et al 2007). Ce modèle a été utilisé pour démontrer la prolifération de

cellules perivasculaires, puis la migration de ces cellules à proximité d'une cavité avec effraction pulpaire.

Dans ce travail nous avons monté une collaboration avec le laboratoire IMEB à Marseille et nous avons utilisé ce modèle de culture de dent entière humaine pour vérifier l'activité biologique de facteur de croissance libéré de nos microparticules et la possibilité d'utiliser ces microparticules comme matériaux de coiffage pulpaire direct.

Travaux expérimentaux

Introduction

Ce travail de recherche tente de répondre à plusieurs questions :

La méthode de double émulsion est-elle adaptée à la fabrication des microparticules chargées en facteur de croissance type FGF-2 et TGF-ß1 ?

Quels sont les paramètres de formulation les plus adaptés pour optimiser l'encapsulation de ces facteurs de croissance ?

Le procédé de séchage des microparticules a t-il un effet sur les caractéristiques des microparticules finales ?

Les microparticules sont-elles adaptées à l'utilisation locale en contact avec le tissu pulpaire en termes de caractéristiques physicochimiques, toxicité, profil de libération et activité biologique ?

Pour répondre à ces questions, nous avons commencé une série d'expérience par la préparation des microparticules contenant une protéine modèle BSA en utilisant la méthode de double émulsion W/O/W suivi d'évaporation/extraction du solvant. Cette première approche nous a permis d'optimiser un protocole expérimental d'encapsulation et de nous familiariser avec ce procédé afin de pouvoir suivre dans les meilleures conditions l'encapsulation des facteurs de croissance FGF-2 et TGF-ß1.

L'étape de séchage est indispensable pour éviter la libération pendant la période de stockage. Les microparticules sont séchées et stockées sous forme de poudre et elles sont redispersées dans l'eau avant leur utilisation. Nous avons donc effectué une étude de comparaison entre les caractéristiques physico-chimiques des microparticules avant et après séchage et redispersion dans l'eau pour vérifier la stabilité de ces microparticules et l'effet de séchage sur celui-ci.

Dans la troisième partie de ce travail, l'encapsulation des facteurs de croissance a été effectuée en utilisant le protocole d'encapsulation optimisé dans les parties précédentes. Les microparticules ont subi une caractérisation profonde. Une étude de modélisation mathématique de la cinétique de libération pour relier les caractéristiques de formulation à la vitesse de libération est effectuée. A la fin de cette partie nous sommes arrivés à valider les

microparticules comme forme galénique pour l'encapsulation et l'utilisation locale des facteurs de croissance FGF-2 et TGF-ß1. Une étude de modélisation mathématique de la libération de principe actif à travers les microparticules est faite pour mieux comprendre le mécanisme de la libération et relier les caractéristiques des microparticules avec leur profil de libération.

Dans la quatrième partie, des études biologiques sur des cellules pulpaires *in vitro* ont été effectué pour vérifier la toxicité des microparticules ainsi que des tests de prolifération des fibroblastes pulpaires humains pour vérifier la conservation de l'activité biologique des facteurs de croissance encapsulés.

La dernière partie de ce travail traitera l'utilisation de microparticules comme un matériau de coiffage dentaire direct afin de confirmer leurs activités biologiques *ex-vivo*. Un modèle de culture de dent entière humain a été utilisé. Ce modèle a été validé récemment par l'équipe du Professeur About de la faculté d'odontologie de Marseille.

Des études de colorations histologiques et immunohistochimiques seront effectuées sur des coupes de dents humaines après leur mise en culture pour évaluer les effets moléculaires et tissulaires de l'utilisation de microparticules en contact d'une effraction pulpaire.

Travaux expérimentaux

Chapitre 1

Publication 2

Optimisation de procédés de préparation des microparticules libérant un principe actif biologique (protéine) : études des effets des paramètres de formulation sur les caractéristiques des microparticules

Journal of Nanoscience and Nanotechnology (2009) 9(1):467-74.

Le but de cette partie est d'optimiser les moyens techniques mis en œuvre pour réaliser l'encapsulation des facteurs de croissance type FGF-2 et TGF-ß1 à l'aide de l'acide poly (lactique-glycolique) PLGA, en prenant l'albumine bovine comme protéine modèle, et d'étudier l'influence des différents paramètres de formulation (la vitesse d'agitation mécanique, les concentrations et les rapports de concentration des réactifs utilisés) sur les caractéristiques des microparticules.

Dans ce travail, nous avons pu mettre au point une méthode fiable de fabrication des microparticules encapsulant un principe actif peptidique (BSA). Cette méthode est basée sur la réalisation d'une émulsion multiple W/O/W suivie de l'extraction du solvant de la phase huileuse.

La méthode modifiée choisie au cours de ce travail limite les contraintes extrêmes auxquelles les protéines peuvent être exposées pendant la fabrication.

Au cours de ce travail, différents paramètres ont été modifiés pendant la préparation des microparticules

Ces paramètres sont :

- la vitesse d'agitation (500, 1000, 6500 rpm),
- la concentration du stabilisant (PVA) dans la phase externe (0.25, 0.5, 1, et 2%),
- le volume de la phase interne dans l'émulsion primaire.

Des mesures de la taille, de l'efficacité d'encapsulation et du profil de libération ont été effectuées pour étudier les effets de ces modifications sur la caractérisation des microparticules et pour optimiser le protocole de préparation selon le mode d'utilisation désiré.

Les paramètres qui peuvent moduler la taille des microparticules sont la vitesse d'agitation pendant la seconde l'émulsification et la concentration du stabilisant dans la phase aqueuse externe. Parmi les facteurs qui peuvent avoir un effet sur la morphologie interne, on

trouve que la diminution du rapport principe actif-eau/polymère conduit à une structure moins poreuse.

Une modélisation mathématique est effectuée, dans laquelle, le coefficient de diffusion et la vitesse de libération ont été calculé en fonction des conditions opératoires pendant la formulation. La seconde loi de Fick est utilisée pour estimer le coefficient de diffusion de l'albumine à travers la matrice des microparticules. La vitesse d'agitation, la concentration de PVA dans la phase aqueuse externe et le ratio eau/polymère dans la phase aqueuse interne ont été variées.

Les résultats de modélisation montrent que la vitesse de libération diminue quand la taille des particules diminue. Donc, tous les paramètres de formulation qui ont un effet sur la taille des particules (vitesse d'agitation et concentration de PVA) sont supposés avoir un effet sur la vitesse de libération. Enfin, les résultats montrent aussi que l'augmentation de ratio eau/polymère dans l'émulsion première augmente la porosité interne des microparticules et augmente ainsi la vitesse de libération.

Le modèle mathématique présenté dans cette étude peut être utilisé pour prédire la libération du médicament avec différentes concentrations du principe actif encapsulé. Ce modèle permettra de poursuivre les études avec les molécules bioactives (les facteurs de croissance).

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Preparation Conditions Effect on the Morphology and Release Kinetics of Biodegradable Particles: A Mathematical Approach

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Poly(lactic-co-glycolic acid) particles encapsulating albumin were produced by the double emulsion technique (Water in Oil in Water). The diffusion coefficient and therefore the release rate were quantified as a function of the operating conditions. The second Fick's law of diffusion was used by applying the entire particle size distribution to estimate the diffusion coefficient. The stirring speed, polyvinyl alcohol concentration in the external water phase and the water to oil ratio in the internal emulsion were varied. It was found that the release rate increased while decreasing the particle size. Therefore, all the process parameters that are assumed to affect the particles size—such as the stirring speed or marginally the polyvinyl alcohol concentration—are supposed to affect the release rate. Finally, decreasing the water to oil ratio in the internal emulsion was found to increase the particle porosity and therefore to increase the diffusion coefficient.

Keywords: Release Rate, Microparticles, Encapsulation, Biodegradable Polymers, Mathematical Model.

RESEARCH ARTICLE

1. INTRODUCTION

Encapsulation and particulate formulations are known to achieve better pharmaceutical profiles of a variety of drugs by increasing their solubility and bioavailability. Particulate delivery systems can improve the therapeutic efficacy of the drug and decrease its side effects. Poly(lactic-co-glycolic acid) (PLGA) is one of few biodegradable and biocompatible polymers, authorized by the Food and Drug Administration (FDA) for drug delivery[1] and is therefore widely used for the preparation of nanoparticles and microparticles.

A number of encapsulation techniques are available for drug encapsulation. The choice of a technique is determined by the nature of the drug, the polymer, the intended administration and the duration of the therapy. For instance, the oil/water simple emulsion solvent evaporation/extraction technique is usually used for encapsulating hydrophobic drugs where as the water/oil/water (W/O/W) double emulsion technique is preferred for the encapsulation of hydrophilic drugs. The W/O/W double emulsion technique was developed by Ogawa et al.[2] for the production of microparticles with sustained delivery of hydrophilic active agents. In order to ensure the stability of the particles and their bio availability at the end of the preparation, a complete extraction of the solvent must be achieved. The influence of different process parameters (i.e., stirring speed, concentration of polyvinyl alcohol (PVA) in the external or internal phases, the water to oil ratio in the internal emulsion and the type of polymers) on both the size and morphology of the microparticles as well as on the drug release kinetics have been investigated for several applications (see for instance[3–9]). These studies represent an adequate comprehensive literature data on microparticles fabrication process and suggest enhanced protocols to increase protein encapsulation efficiency or modulate its release kinetics.

Mathematical modelling is usually employed for a better comprehension of the release profile and a quantitative estimation of the release rate. The drug release is usually represented by the Fick's law of diffusion or the Higuchi equation (see for instance[10–13]). Good reviews on mathematical modelling of drug release are given for the diffusion from microspheres by Arifin et al.[14] and for the

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diffusion through thin films and cylinders by Siepmann et al.[15] Using empirical relationships, some models were adapted to take into account the polymer degradation (see for instance[16]) or the Monte Carlo technique.[17] In such models, the release of the entrapped active agent is supposed to occur first through pores of the matrix that become larger under the degradation of polymer. In some applications, the diffusion coefficient was quantified and related to the amount of an agent. Siepmann et al.[18] estimated the diffusion coefficient by fitting experimental results while varying the concentration of a plasticizer in the fabrication of microparticles.

The aim of this study is to relate the diffusion coefficient of the encapsulated substrate to the operating conditions. Taking the bovine serum albumin (BSA) as a protein model and PLGA as a biodegradable polymer and using W/O/W double emulsion technique as a microparticles fabrication method. First, the effect of the formulation parameters employed during the preparation of the microparticles, such as the stirring speed, the PVA concentration in the external phase and the water to oil volume ratio in the internal emulsion, was studied in regards to the particle size, morphology and release profiles. Second, the release profile was analysed over 6 days using the second Fick's law of diffusion to estimate the diffusion coefficient in the different experiments and to relate this coefficient to the operating conditions.

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2. MATERIALS AND METHODS

2.1. Materials

Poly(D,L lactic-co-glycolic acid) (PLGA) is RESOMER® RG 502H with a copolymer lactide-glycolide ratio of 48:52 to 52:48 was purchased from Boehringer Ingelheim. Polyvinyl alcohol (PVA) was obtained from Fluka Company. Bovine serum albumin (BSA) (fraction V, 60 kDa) was supplied by Sigma Chemical Company. Methylene chloride and isopropyl alcohol from Carlo Erba Reagents.

2.2. Methods

2.2.1. Preparation of Microparticles

Microparticles were produced according to a modified W/O/W double-emulsion solvent extraction/evaporation method.[19] On one hand, the PLGA polymer is dissolved in methylene chloride. This phase is referred to as the oil phase. A BSA/PBS (phosphate saline buffer) solution (referred to as the internal aqueous phase) is then emulsified for 1 min with the oil phase using a vortexer, which results in a water-in-oil (W/O) emulsion. The resulting emulsion constitutes the internal emulsion. On the other hand, PVA is dissolved in a 100 ml water solution and introduced into a 250 ml reactor. This is referred to as the external aqueous phase. The reactor is equipped with a stirrer and baffles. A jacket allows the reactor temperature to be controlled. All preparations were performed at ambient temperature. The W/O phase is then introduced into the reactor containing the PVA solution under constant stirring, in order to produce the double W/O/W emulsion. The resultant solution is stirred during 10 min. In order to extract the methylene chloride to the external phase, 100 ml of isopropyl alcohol 5% is added to the solution. This solution is then stirred for 2 hours. The resulting microspheres are collected using a filter and washed three times with deionised water. The particles are then vacuum-dried overnight and stored at 4 °C.

Several factors that influence the particles size, the encapsulation efficiency, the particles morphology and the release profiles were investigated. These factors include the PVA concentration in the external emulsion, the stirring speed of the external emulsion and the water to oil volume ratio in the internal emulsion.

2.2.2. Characterization of Raw Materials

Differential Scanning Calorimetry. The Differential scanning calorimetry (DSC) technique was accomplished using a calorimeter, connected to a refrigeration system DSC TA 125 (TA instrument®, USA). Sample analysis was performed by weighing 5–10 mg of PLGA copolymer and sealing it in an aluminium sample pan. An empty sealed aluminium pan was used as a base line reference. Under a nitrogen purge, the sample and the reference were heated from 25 °C to 80 °C at a rate of 5 °C/min, then cooled at a rate of 10 °C/min to 25 °C and finally heated up to 65 °C at a rate of 5 °C/min. Heating the polymer once past the glass transition temperature (T_g), then cooling the polymer below the T_g at a faster rate than that used for heating any history of the polymer can be eliminated.[20]

Water Contact Angle Measurement. In order to study the influence of the PVA concentration on the adsorption of water in the PLGA microparticles, different solutions were prepared. First of all, the adsorption of water by the PLGA copolymer was studied in the presence and absence of the BSA. Therefore, on one hand, 0.2 g of PLGA were dissolved in 2 ml of dichloromethane (DCM) and on the other hand, 0.2 g of PLGA with 0.1 g of BSA were dissolved in 2 ml of DCM. Approximately, 1 ml of each of the prepared solution was poured into separate glass slides (2.5 cm–7 cm). The slides were levelled to a horizontal position to obtain uniform film thickness. The solutions were left for 24 hours at room temperature to allow the DCM to evaporate.

Secondly, the same films (PLGA alone and PLGA with BSA) were prepared and immersed into a 1%-PVA solution for 24 hours then dried for another 24 hours at room temperature. Three replicates were determined for each experimental condition.

Films with thickness < 100 μm were formed in all cases. Contact angles of polymer films were measured by

dropping 1 μl of distilled water on the different films. The droplet was observed using a light microscope unit from (KRUSS, GERMANY) and the contact angle was determined by a Drop Shape Analysis programme from the same company. Analyses were performed three times for all films.

2.2.3. Characterization of the Microparticles

Size and Morphology of the Microparticles. The particle size distribution (PSD) of the microparticles was measured using a Coulter® counter multisizer (Beckman Coulter LS 230) after dispersion of the microparticles in deionised water.

The surface morphology of the microspheres was observed by scanning electron microscopy (SEM) (FEG Hitachi® S 800). Microspheres were mounted onto metal stubs using a double sided adhesive tape. Subsequently, they were vacuum-dried, contacted with silver paint, sputter-coated with a thin layer of gold (10–150 A) and imaged with the SEM at 15 kV or 10 kV.

In Vitro Release Study. In order to study the kinetics of release, the collected microparticles, for a specific experiment, were distributed into several flasks. In each flask, 75 mg of microparticles were dispersed in 2 ml PBS (pH 7.4) at 37 °C. The suspension was stirred at a constant speed. The flasks were analysed at different times (30 min, 1, 2, 4, 6, 12 hours, 1, 2, 4, 6 days) at which times, the content was centrifuged at 14000 rpm for 10 min and analysed by high performance liquid chromatography (HPLC) (Spectra System P1000X12–Thermo Separation Products) after the supernatant was discarded. Each experiment was duplicated. A Zorbax GF-250 column (4.6 mm × 250 mm, Agilent Technologies) packed with spherical silica was used. The mobile was PBS (pH 7.4) at a flow rate of 1 ml/min. The sample injection volume was 20 μl and the UV detection was at 210 nm. The total analysis time was 8 min.

3. RESULTS AND DISCUSSION

3.1. Characterization of Raw Materials

Before the fabrication of the microparticles, the glass transition temperature (T_g) of PLGA was verified by DSC and the effect of PVA on the water absorption by the PLGA was studied by contact angle measurement.

3.1.1. Glass Transition Temperature of Polymer

Figure 1 shows the DSC curve obtained with the second heating cycle of PLGA. A Tg of 42 °C was found for the PLGA 50:50 copolymer. This indicates that the drug release mainly takes place through the pores of the microparticles and is negligible through the copolymer since the release is studied at 37 °C. It can be seen that no melting point is exhibited by the PLGA 50:50 copolymer which indicates its amorphous nature.

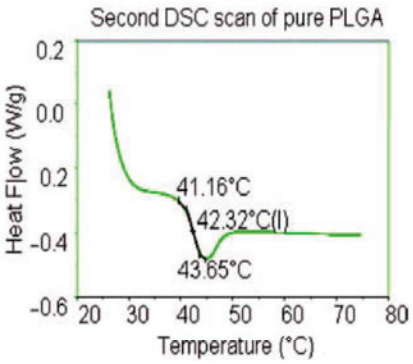

Fig. 1. DSC second scan of the PLGA powder.

3.1.2. Water Contact Angle Measurement

Different phenomena may occur during the drug release. If we consider that particles might partially be swollen with water, then it becomes interesting to study the influence of the different additives on water imbibitions. The results of the water contact angle measurement are shown on Table I. It can be seen that the addition of BSA does not affect the water contact angle. On the contrary, the addition of PVA significantly affects the water contact angle which indicates a faster wetability in the presence of PVA at the surface of the particles by decreasing the interfacial tension. Therefore, the concentration of PVA on the surface of the particles can affect the diffusion kinetics, since the particles are rapidly swollen with water in the presence of PVA on their surfaces.

3.2. Morphology of the Produced Microparticles

SEM allows for the study of the internal and external morphology of the microparticles. Figure 2 shows that spherical microparticles are obtained. The black spots on the surface of the microparticles are flattened zones which seem to correspond to interior pores close to the surface. Small openings in these zones can also be observed.

Figure 3 confirms the findings of the previous figure. It can be seen that the produced particles have a matrix form. A membrane is however visible on the surface. The matrix device contains several holes that are assumed to

Table I. Contact angle measured into water of PLGA and PLGA+BSA films with and without immersion into the PVA solution.

Polymer	Water contact angle
PLGA	72.94
PLGA+BSA	71.48
PLGA+PVA	48.24
PLGA+BSA+PVA	51.98

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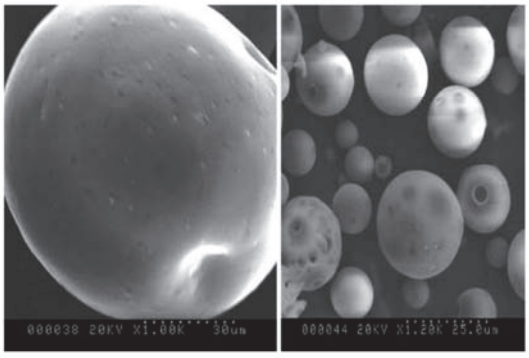

Fig. 2. SEM pictures of the obtained microparticles.

contain the internal aqueous phase. Small holes that cannot be viewed by this technique might exist in the polymer matrix. Regarding this disposition, the distribution of the drug in the matrix can be assumed to be homogeneous.

The ratio of water to oil in the internal emulsion is supposed to affect the inner morphology of the microspheres. An increased water to oil volume ratio in the internal emulsion yields to a matrix with more pores. In order to investigate this parameter, microparticles prepared with different water to oil ratios in the internal emulsion were cut into two parts and observed by the SEM as shows Figure 7. The water droplets trapped at the interior of the microspheres evaporate during the sample preparation for the SEM and leave empty spaces. It can be seen that rather than obtaining more pores of the same size when increasing the water to oil ratio, bigger pores are obtained which might be due to the high surface energy of water (72.8 mJ/m^2) that makes the internal water droplets coalesce with each other yielding to bigger holes. These photos clearly show the effect of the water to oil ratio on the internal morphology of the micropheres which is an important parameter affecting the diffusion and therefore the release rates.

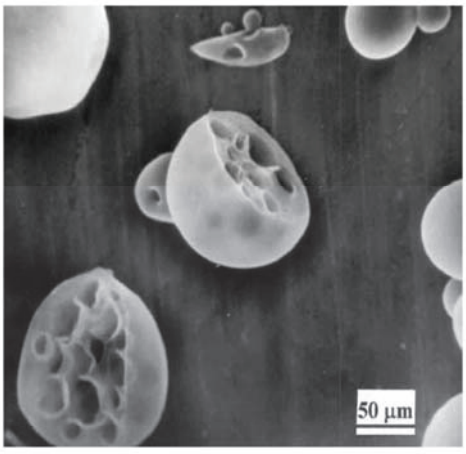

Fig. 3. SEM picture of microparticle cross-section showing the internal structure.

Table II. Effect of formulation parameters on the average particle size.

		Particle size (μm)
Studying the *stirring speed* (W/O ratio = 1/2 and [PVA] = 1%)	500 rpm	220 ± 6.5
	1000 rpm	140 ± 9.3
	6500 rpm	15 ± 1.1
Studying the *PVA concentration* (Stirring speed = 1000 rpm and W/O ratio = 1/2)	0.25%	184 ± 2.2
	0.5%	168 ± 3.7
	1%	140 ± 9.3
	2%	127 ± 2.4
Studying the *water to oil volume ratio of* in the internal emulsion (Stirring speed = 1000 rpm and [PVA] = 1%)	1/2	140 ± 9.3
	1/4	135 ± 4.8
	1/8	128 ± 11.8

3.3. Particle Size

The stirring speed is the dominant parameter affecting the particle size because it provides the energy to disperse the oil phase in the water. Therefore, high stirring speeds are supposed to give smaller microspheres, because the second emulsion is broken up into smaller droplets. Table II shows the average particle size of the microspheres obtained under different stirring rates. The stirring rates at 500 rpm and 1000 rpm (maximum possible value) were obtained with the stirrer of the 250 ml reactor whereas the stirring rate at 6500 rpm was obtained with an Ultraturax apparatus (minimum possible value by the Ultraturax used in this work). In this latter experiment, the internal emulsion was also homogenized using the Ultraturax rather than the vortexer used in the other experiments.

Figure 4 shows the obtained PSD in these experiments as measured by the Coulter. It can be seen that the particle size distribution is also narrower with higher stirring speeds. Thus, the stirring speed needs to be optimized in order to obtain the desired size and size distribution.

The PVA concentration in the external phase is known to affect the size of microspheres. The PVA plays the role of an emulsifier and allows for the stabilization of

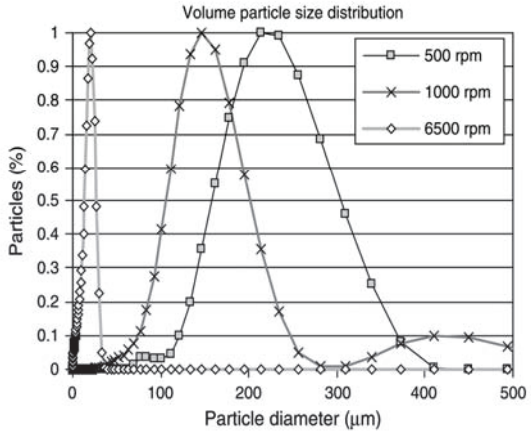

Fig. 4. The measured volume particle size distribution by the Coulter as a function of the stirring rate.

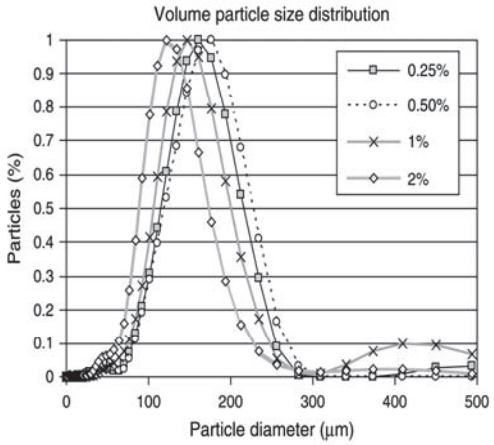

Fig. 5. The measured volume particle size distribution by the Coulter as a function of the concentration of PVA.

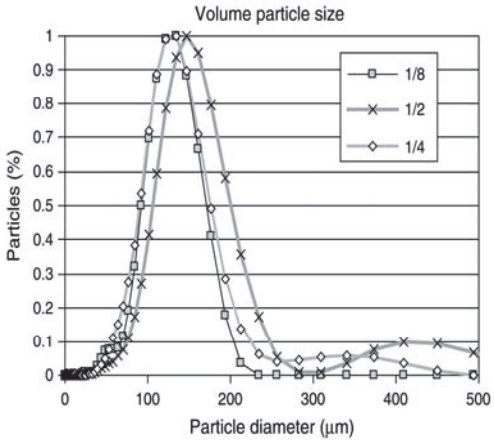

Fig. 6. The measured volume particle size distribution by the Coulter as a function of the water to oil ratio in the internal phase.

the particles. The presence of PVA is supposed to protect particles from coalescence and to ensure their stabilisation. In this work, 4 different concentrations of PVA in the external water phase where employed during the elaboration of the microspheres: 0.25, 0.5, 1 and 2%. Figure 5 shows a slight decrease in the particles size when increasing the PVA concentration. Table II depicts the average size of the obtained particles.

Discrepancies can be found in the literature regarding the PVA concentration in the external phase. Mao et al. 2007 reported that a variation range form 0.1% to 1% of the PVA concentration ad a marginal effect on the final particle size. A slight increase in the particle size was observed but 0.1% PVA was found to be sufficient to stabilize the microspheres. Yang et al.[21] found that increasing the concentration of PVA (which was varied from 0.05 to 0.5%) leads to a slight decrease in the particle size. The authors suggested that the presence of PVA in the external water phase may increase the viscosity of the double emulsion, resulting in an increased difficulty in breaking up the emulsion into smaller droplets, thus yielding bigger microspheres. However, only slight variations in the concentration of PVA where studied and probably this phenomenon would be better perceived with larger variations in the PVA concentration.

The water to oil volume ratio in the internal emulsion is not supposed to have an effect on the particle size. Table II shows the average particle size obtained with the water to oil volume ratios 1/2, 1/4 to 1/8. A very slight decrease of the particle size is observed. Figure 6 shows the PSD of these experiments. No difference between these experiments can really be observed.

3.4. Microparticles Loading

Protein loading or albumin content determines the therapeutic dose for an application with a specific amount of microparticles. The release of microparticles was studied over 6 days. It was noticed however that no release occurred after 6 hours as observed by HPLC. The amount of Albumine released at 6 hours was therefore assumed to correspond to the microparticles loading calculated by the following equation:

$$\text{Drug content} = \frac{\text{Amount of drug in microspheres}}{\text{Amount of microparticles recovered}} \times 100\% \quad (1)$$

The protein stability was not considered in this work since the loadings obtained (varied from 0.5–4% as a function of the water to oil ratio in the internal phase) were close to those found in the literature for microspheres prepared by the same method.[7]

3.5. Release Kinetics

The second Fick's law[22] can be used to describe the release from the produced microparticles. In spherical spheres, the second Fick's law of diffusion takes the following form:

$$\frac{\partial C(t)}{\partial t} = \frac{1}{r^2}\frac{\partial}{\partial r}\left(Dr^2\frac{\partial C(t)}{\partial r}\right) \quad (2)$$

where $C(t)(\text{mol/dm}^3)$ represents the concentration of the drug in the sphere, $D(\text{dm}^2/\text{s})$ is the apparent diffusion coefficient and $r(\text{dm})$ is the radius of the sphere.

An analytical solution can be derived for Eq. (2) for matrix systems assuming a homogeneous dispersion of the drug in the sphere, a constant diffusion coefficient on r and perfect sink conditions (the mass transfer resistance at the surface is negligible and the surrounding release is infinitely large implying that the concentration on the surface of the matrix is constant and equals to 0):[22]

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(\frac{-Dn^2\pi^2 t}{r^2}\right) \quad (3)$$

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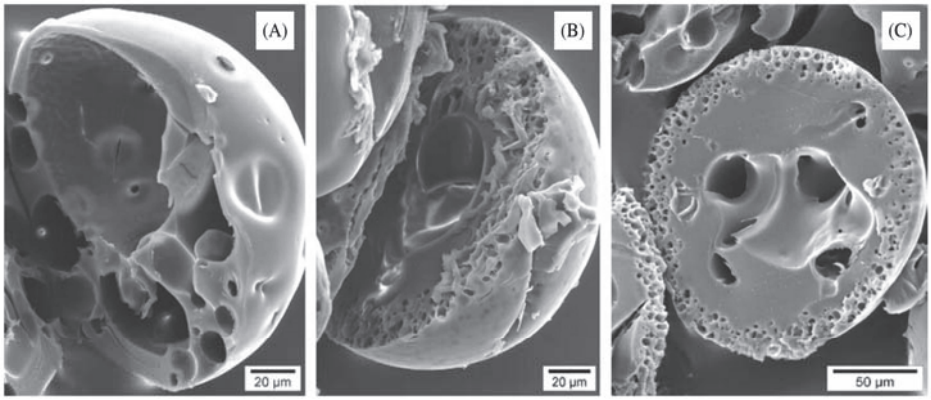

Fig. 7. SEM pictures of microparticles cross-sections with different water to oil volume ratios in the internal emulsion. A, B and C correspond to 1/2, 1/4 and 1/8 respectively.

where M_t and M_∞ are the cumulative absolute amounts of the drug released at time t and at infinite time respectively. The solution given by 3 assumes also that the drug loading is lower than the solubility of the drug inside the polymer matrix.

Equation 3 was used to estimate the diffusion coefficient during the release of the different experiments performed in this study. In order to apply Eq. (3), the PSD measured with the Coulter was used which is supposed to give an improved estimation and precision of the average particle size. Indeed, using the PSD allows us to take into account the polydispersity of the size and the presence of agglomerates while using the average particle size assumes that all particles have the same size which would affect the estimation of the diffusion coefficient (see Table II and Figs. 4–6).

The model uses the drug loading as obtained by HPLC for each experiment. Release is then conducted and the diffusion coefficient is calculated by optimization, using the Matlab® program. In all experiments, since no release occurred after 6 hours, simulations were conducted only over 6 hours. For this reason, it was not found necessary to consider a variable diffusion coefficient. Indeed, the molecular weight of the polymer as measured by gel permeation chromatography was sensitively the same for PLGA and for the particles after several days in PBS at ambient temperature. This is in accordance with previous reports from the literature. For instance, Panyam et al.[23] studied the degradation of PLGA nano- and microparticles over 100 days. The authors found that the polymer degradation had a biphasic behaviour: a rapid decrease in the polymer molecular weight is observed during the first 20 days but the particles kept their spherical structure, then, during a second phase, they are deformed and pore formation is observed. No significant changes in the polymer molecular weight were reported during the first 6 hours.

It is worthy to note that the estimated diffusion coefficient is a global coefficient that takes into account diffusion through the matrix if existing and the pores. However, the release takes place at 37 °C whereas the polymer T_g is 42 °C. Therefore, the polymer is in a glassy state and the diffusion is difficult through the polymer. Only diffusion through the pores of the matrix is therefore supposed to take place.

The estimated diffusion coefficient in all experiments is given on Figure 8. An important effect of the stirring speed on the diffusion coefficient can be observed: increasing the stirring speed decreases the diffusion coefficient. As noted previously, this can be related to a rapid stabilisation of small microspheres. The drug diffuses less easily in the matrix devices produced with higher stirring speeds. It is important to remind however that a faster diffusion took place with smaller particles as well known in the literature (Fig. 9). Indeed, the diffusion is a function of the diffusion coefficient and the surface of exchange with water (that increases while decreasing the particle size). Since a higher diffusion coefficient and bigger particles were obtained for the experiment realized with 500 rpm,

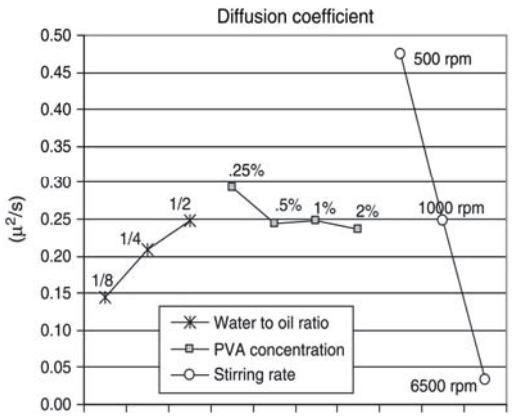

Fig. 8. The diffusion coefficient obtained by fitting the release profile to the real experimental values based on the PSD during experiments with different operating conditions.

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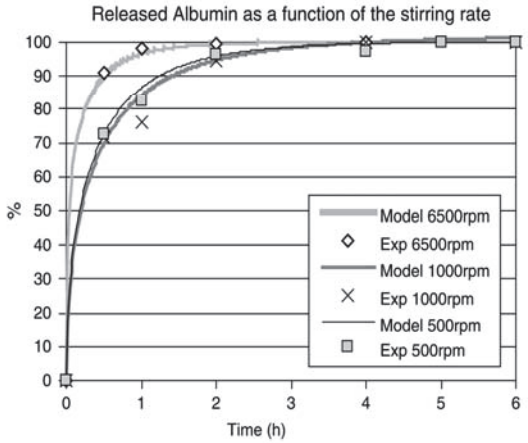

Fig. 9. Released amount of albumin as a function of the stirring rate. The continuous lines are obtained by the model during the optimization of the diffusion coefficient and the points represent the real experimental data.

the diffusion rate was between those of the experiments with 6500 and 1000 rpm (Fig. 9).

Concerning the concentration of PVA, almost the same coefficient and release rate were obtained for all experiments (Fig. 8). Water imbibition into the microspheres does not appear to depend on the PVA concentration on their surfaces as expected by the contact angle measurement. This can be due to the high porosity of the particles where water is absorbed easily. Mao et al. 2007 found that the burst effect of release was decreased by increasing the concentration of PVA from 0.1 to 1%. The hypothesis emitted by the authors was that a higher PVA concentration yields a more stable emulsion that hinders the mass transfer of the drug with the surroundings. However, the authors did not give a quantitative estimate of the diffusion coefficient in this case.

Regarding the water to oil ratio (Fig. 8), it can be seen that a higher diffusion coefficient is obtained with higher water to oil ratio reflecting therefore a higher porosity of the microsphere, as confirmed by the SEM observations. Similarly, Mao et al.[9] found that the burst effect was increased by increasing the ratios of water to oil in the internal emulsion, which is mainly due to the increased porosity of the microspheres. It is worthy to mention that estimating the diffusion coefficient based using the PSD was primordial in this series of experiments. Indeed, Figure 6 shows a slight change in the PSD while changing the water to oil ratio. Neglecting this difference in the PSD, would have leaded to an error in the estimation of the diffusion coefficient.

4. CONCLUSIONS

The double-emulsion solvent extraction/evaporation method was used to prepare PLGA microspheres. The effect of different formulation parameters, such as stirring speed, PVA concentration in the external phase and the water to oil ratio in the internal emulsion, on the particle size, morphology and release profiles were studied. The release profile was analysed over 6 hours using the second Fick's law of diffusion to estimate the diffusion coefficient in the different experiments and to relate this coefficient to the operating conditions. The diffusion coefficient was estimated by fitting the release profile to the measured one. The whole particle size distribution was used in the estimation rather than an average value of the particle size which improved the precision of estimation. It was found that increasing the stirring speed of the external emulsion increased the release rate but decreased the diffusion coefficient. These phenomena are explained by a rapid precipitation of the polymer due to the higher contact area with the external water phase. This leads to the protection of a higher amount of drug and renders the matrix more robust and therefore decreasing the release rate. This can be of interest in applications for which a high burst effect must be avoided. Therefore, the release rate is not only a function of the concentration ratios but also of the rapidity of the stabilisation of the microparticles. The effect of PVA was found to be marginal on the release rate. Finally, the water to oil ratio in the internal phase was found to affect the porosity of the microspheres and therefore to enhance the release rate.

Estimation of the diffusion coefficient enables a better comprehension of the encapsulation process and a relation of the release kinetics to the processing conditions. The diffusion coefficient appears to be the paramount parameter; although it has been shown to be related to the molecular weight of the polymer in previous literature[23] for longer release periods, our results suggest that it is primarily linked to the microparticles porosity and indirectly to the particle size. The mathematical process model presented in this study can be used to predict the drug release with different concentrations of the encapsulated molecule. This model will allow for further studies with bio active molecules.

The protocol of fabrication of microparticles optimised in this work is intended for the encapsulation of growth factors for short (few days) local applications (direct and indirect capping) in conservative dentistry and endodontics.

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RESEARCH ARTICLE

Travaux expérimentaux

Chapitre 2

Publication 3

Etude de la stabilité colloïdale est physicochimique des microparticules de PLGA encapsulant une protéine modèle avant et après leur séchage et redispersion dans l'eau

e-polymer **010**: 1-12.

Cette étude a été menée pour évaluer l'effet des procédés de séchage et redispersion dans l'eau sur les caractéristiques colloïdales et physico-chimiques des microparticules de PLGA fabriquées par un procédé d'émulsion multiple W/O/W.

La caractérisation colloïdale et physicochimique de ces microparticules a été étudiée avant et après rinçage et séchage. Le procédé de séchage consiste à employer un flux d'air comprimé doux et filtré pendant environ une heure.

La morphologie externe et interne a été évaluée par microscopie électronique à balayage et les propriétés de surface par microscopie à force atomique. Les microparticules fabriquées sont apparues assez peu rigides avec une forme sphérique et une surface lisse après séchage. La surface des microparticules devient irrégulière après un mois de conservation en suspension dans l'eau, ceci est dû à la dégradation de la matrice polymérique.

En ce qui concerne la morphologie interne, l'AFM et la MEB ont indiqué la présence de petites cavités situées juste sous la surface dans une couche fine de polymère. La MEB indique la présence de cavités plus importantes dans le centre des microparticules.

Les études électrocinétiques et les mesures de la taille des particules ont également été effectuées en fonction du pH et la salinité. Il a été déduit que la stabilité colloïdale des microparticules produites est essentiellement gouvernée par l'interaction électrostatique répulsive due à la présence de groupes carboxyliques dans le PLGA. La quantité de stabilisant (PVA) est faible mais suffisante pour assurer la stabilité de microparticules dans un milieu de forte salinité. Le processus de séchage n'a donc aucun effet sur les propriétés de surface et la stabilité colloïdale des microparticules préparées.

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Colloidal and physicochemical characterization of protein-containing poly(lactide-*co*-glycolide) (PLGA) microspheres before and after drying

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Abstract: In the present work, the double emulsion (W/O/W) method was used to microencapsulate a protein model (Bovine Serum Albumin) in a biodegradable polymer poly(lactide-co-glycolide) (50:50) (PLGA). Colloidal and physicochemical characterization of PLGA based microspheres was investigated after their "crude" preparation and after drying and redispersion processes. Particle morphology and size were studied by scanning electron microscopy and by laser diffraction analysis. Electrokinetic study and colloidal stability were also studied as a function of pH and salinity of the medium. Atomic force microscopy (AFM) was used to study the surface properties. It was found that smooth microspheres were obtained after the drying process. The Atomic force microscopy results of microspheres stored for one month in water show softness and surface irregularity caused by the microspheres degradation. The colloidal stability of theses microspheres is mainly governed by repulsive electrostatic interaction due to the PLGA charge. The used amount of stabilizing agent poly(vinyl alcohol) (PVA) is low enough to ensure the sterical stabilization. Drying process has no effect on the surface properties and colloidal stability of the prepared microspheres.

Introduction

Colloidal carriers offer some advantages for sustained delivery of protein agents such as growth factors, proteins and other peptides [1, 2, 3, 4]. These advantages include the increased bioavailability, the protection of the drug against degradation and the control of the rate and the duration of release of the entrapped protein agents [5, 6].

The preparation of such microparticulate drug delivery materials necessitates the use of hydrophilic materials to be adsorbed on their surface essentially to ensure their stability and to ensure a good biodistribution in the vascular compartment. Therefore, it is important to analyse the surface chemistry in order to have a good understanding on the stability and biodistribution of the microspheres and to relate these properties to the fabrication parameters. In this direction, various techniques and tools have been used. The microsphere surface is usually analysed by electron microscopy for the size and morphology, zeta potential and surface tension for the surface charges, atomic force microscopy (AFM) for the deflection of the microspheres' surface, X-ray photoelectron spectroscopy can provide quantifiable data on biomaterial surface chemistry such as the degree of surfactant adsorption on the surface [7].

The surfactant poly(vinyl alcohol) (PVA) is frequently used as a stabilizer in biodegradable microsphere fabrication [8]. As it is a non charged polymer, the colloidal stability is mainly governed by sterical stabilization. The contribution of repulsive electrostatic interaction is not excluded and is due to the presence of negative charges on the particles. Adsorption of PVA on biodegradable microspheres was studied by X-Ray photoelectron spectroscopy [7]. It was found that poly(lactide-co-glycolide) (PLGA) microspheres adsorb less PVA than PLA microspheres which was explained by the slight increase in hydrophilic character of the PLGA over PLA which decreases the need for PVA stabilization. In addition, it has been pointed out that the adsorption of PVA onto PLGA containing PEG moieties was reduced. It has also been shown that a fraction of PVA forms a stable network on the polymer surface, which cannot be removed during the washing procedure [9,10]. Also, since PLGA microspheres are negatively charged due to the presence of carboxylic groups, the presence of PVA on their surface is supposed to shield their surface charge which was verified by zeta potential [11]. It was found however that the incorporation of PVA did not significantly influence the zeta potential [12]. This means however that the stability of PLGA particles is not directly related to the zeta potential and a best stability might be obtained with lower zeta potential.

In this work, microspheres are prepared by the w/o/w double emulsion technique [13,14, 15]). Numerous studies have been carried out to vary the different process parameters to optimize the w/o/w double emulsion technique. The polymer molecular weight, the polymer composition, the volume of the inner water phase, the DCM and PVA concentrations, the temperature and the stirring speed were studied in terms of their effect on the physicochemical characterization of the fabricated microspheres and the encapsulation efficiency [5, 6, 16, 17, 18]. The characteristics of microspheres in these studies were always investigated after the encapsulation process and not after drying and redispersion in water.

The aim of this work is to investigate the physicochemical characteristics of PLGA microspheres encapsulating a model protein, Bovine Serum Albumin (BSA) as active agent, produced by the w/o/w encapsulating technique described above. The colloidal stability, morphology and surface properties of the PLGA based microspheres after their “crude” preparation and after drying and redispersion are studied to evaluate the influence of drying process on these characteristics and especially on the stability of the microspheres. In this manuscript, the encapsulation efficiency and the activity of the loaded protein will not be discussed.

The particle size and morphology were studied by scanning electron microscopy and laser diffraction analysis. Atomic force microscopy (AFM) and zeta potential were realised to study the surface properties of the microspheres. The colloidal stability was studied as a function of both the pH and the salinity of the medium.

Results and discussion

Microspheres particle size and morphology

In order to illustrate the internal and external morphologies of the freshly produced microspheres, scanning electron microscopy (SEM) has been employed. SEM pictures (Fig. 1) show the surfaces of the microsphere before and after drying. It can be seen that the SEM pictures are very similar before and after drying which means that the drying process has no effect on the morphology of microspheres. It is clear

that the shape of the obtained microspheres can be assumed to be spherical with smooth surfaces. However, two populations of particles can be distinguished in Fig. 1 at left whereas only the bigger population is conserved after the rinsing and drying process. The population of small size was certainly lost during the rinsing process or could not be collected from the filter.

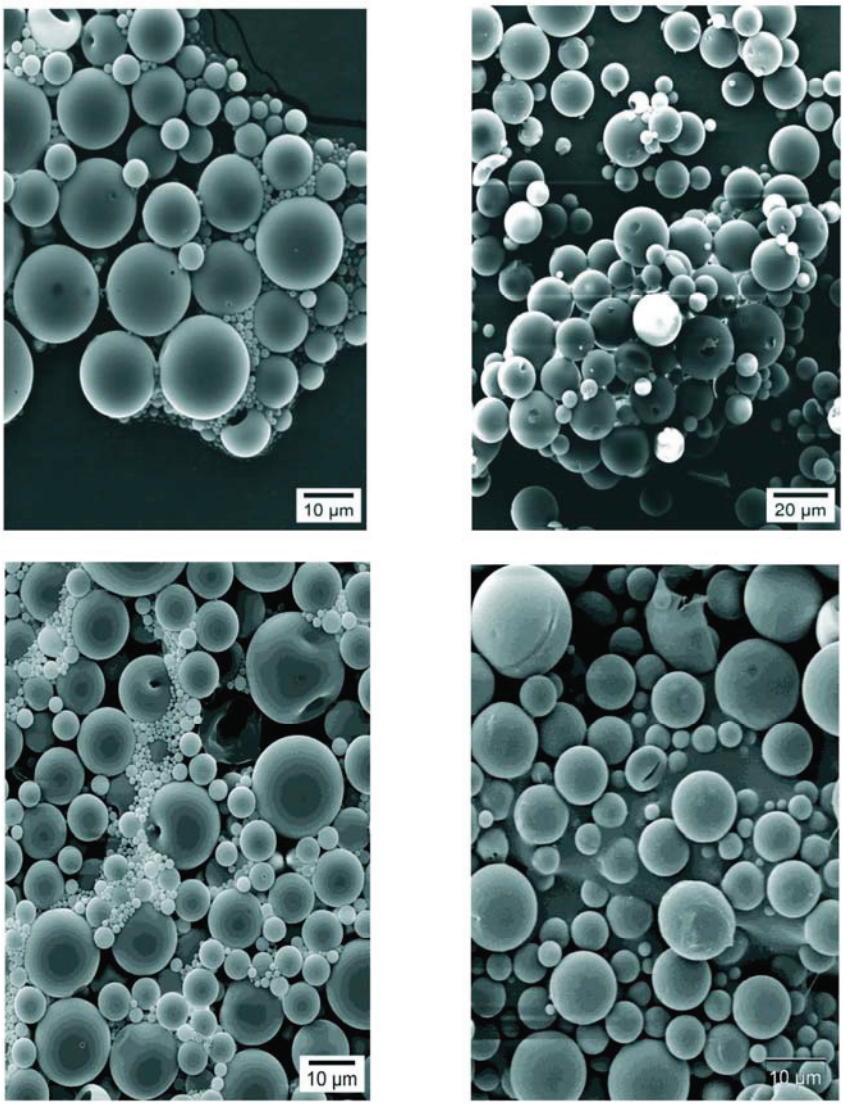

Fig. 1. SEM pictures of surfaces of microsphere left, before drying and right, after drying.

In Fig. 2 particles were cut in two parts in order to observe the internal morphology of the produced particles. It can be seen that the microspheres have a matrix form. A membrane is visible on the surface. The matrix device contains several holes that are assumed to contain the internal aqueous phase. It can be seen that small cavities are located close to the surface and bigger cavities are located in the centre of the

microsphere. Small cavities could be created during the solvent extraction process while the big cavities during the first w/o emulsion.

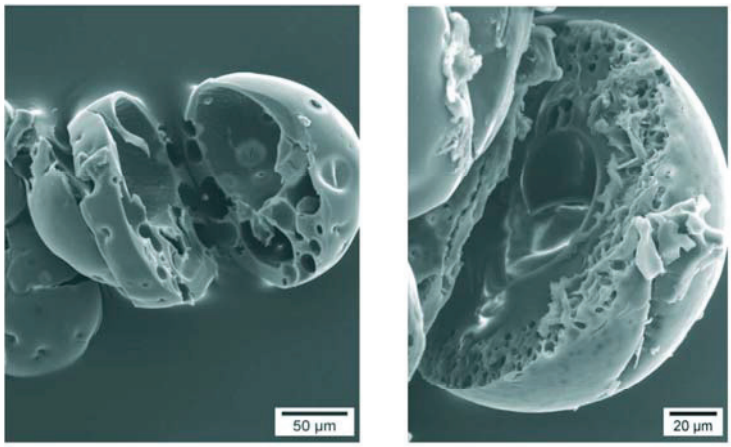

Fig. 2. SEM picture of microspheres cross-section showing the internal structure.

The particle size analysis confirms the SEM measurements. It can be seen in Figure 3 that the drying process does not affect the particles size. The mean particle size was about 8.25 µm before drying and 7.75 µm after drying. It can be seen however that small particles (≤ 1µm) disappear after rinsing and drying making the particle size distribution slightly narrower.

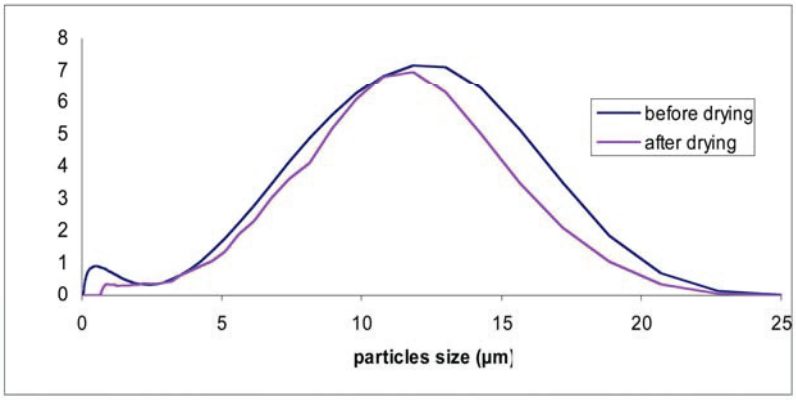

Fig. 3. Microspheres size distribution before and after drying.

Surface properties by AFM microscopy

AFM was used to analyze particles after their suspension in water for about a month. The AFM images of the microspheres surface and in height section indicate a soft particle surface in the form of vesicle spread out under the effect of its proper weight on the surface of the mica. This depression is highlighted by measuring the ratio height to width (Fig. 4). In the case of high solid particles, the above mentioned ratio is close to (or equal to) 1.

Concerning the analysis of the surface morphology, obtained cartographies show a rough and irregular surface that was not observed by SEM for newly produced particles. This can be explained by the long time during which particles were suspended in water (almost one month). In fact, it has been reported in the literature [4, 19, 20] that this period is sufficient to cause microsphere degradation. This degradation can be represented by this softness and surface irregularity.

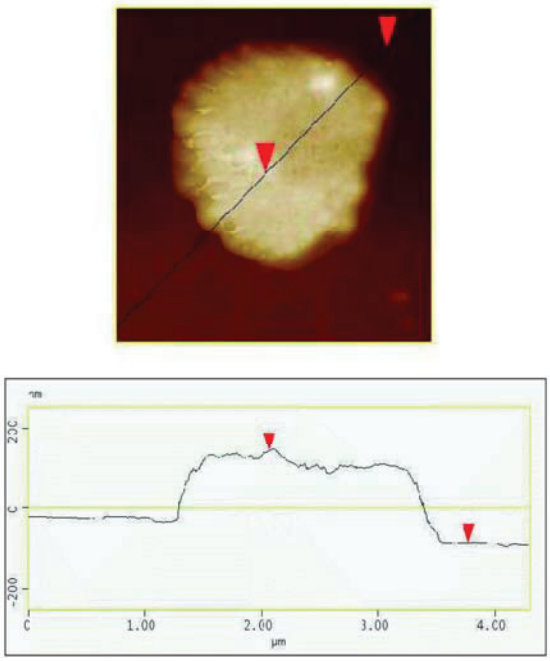

Fig. 4. AFM images in height section.

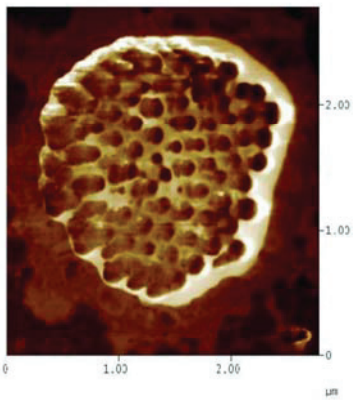

Fig. 5. AFM image of the surface of the microsphere after one month in water.

The nodules observed on the surface image (Fig. 5) show differences in the energy dissipation of AFM ultralever which indicates differences in the surface properties of the microspheres. It might indicate the presence of small cavities under a fine layer of

polymer (see Fig. 2 by SEM). These cavities are probably filled with water, which gives a nodular aspect instead of craters. The drug release should be affected by this internal structure [20].

Fig. 6 shows SEM picture of microspheres after one month in water. A slight change in the particles shape is observed. The particles are no more spherical. Also, the particles' surface is slightly irregular as also observed by AFM (Fig. 4). It is important to mention that differences in the deflection of the particle surface in Fig. 5 are not due to surface irregularity but to the presence of cavities of the order of 0.1 µm that are close to the surface. Irregularities due to the degradation of particles morphology observed by SEM are much bigger.

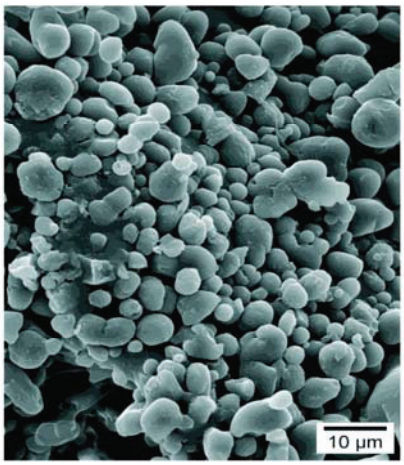

Fig. 6. Microspheres after one month in water by SEM.

Electrokinetic study

An electrokinetic study was realized to evaluate the surface properties of the prepared microspheres before and after the drying process. The electrophoretic mobility of the microspheres was investigated as a function of:

i) The pH of the medium at a concentration of NaCl of 1 mM

ii) The ionic strength at a constant pH (pH~8)

Firstly, the zeta potential was investigated in order to point out the effect of pH on the surface charge density. Fig. 7 shows the variation of the zeta potential versus pH at a constant salinity.

The magnitude of the zeta potential was found to be lower than 35 mV, revealing a relatively low surface charge density. The negative zeta potential may be attributed to the presence of carboxylic functions in the PLGA (RESOMER RG 502H) which carries predominantly free carboxylic acid groups on one of the chain ends [21, 22]. For other polymers than PLGA where the zeta potential is initially positive, the zeta potential decrease while increasing the pH and becomes negative at high pH values [23, 24, 25, 26]. The difference between the two observations (zeta potential vs. pH) can be attributed to the presence of high carboxylic amount and the coexistence of other negatively charged compounds.

During rinsing and drying processes, a big amount of PVA is taken off from the particles' surface. It is likely however that some amount of PVA remains adsorbed on the surface even after rinsing [9, 10]. Reducing the amount of PVA on the particles' surface is supposed to increase the zeta potential since the PLGA charge is no more masked by the PVA. Moreover, rinsing reduces the concentration of small particles which should contain a higher amount of PVA due to their higher surface area. It was found that the adsorption of PVA increases with smaller particles due to their higher surface area [7]. Since the concentration of small particles is slightly reduced one may expect an increase in the zeta potential. It can be seen however that the zeta potential is only slightly affected by these processes. Consequently, the drying process can be considered not to have a dramatic effect on the surface properties of the prepared microspheres for the concentrations of PVA used in this work.

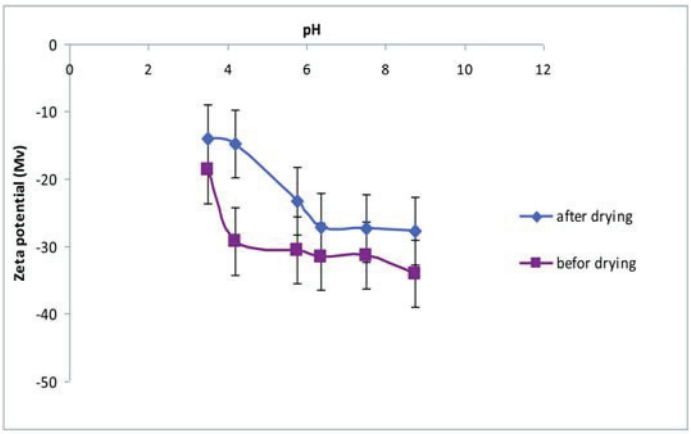

Fig. 7. Zeta potential of PLGA microspheres as a function of pH at a constant salinity before and after drying.

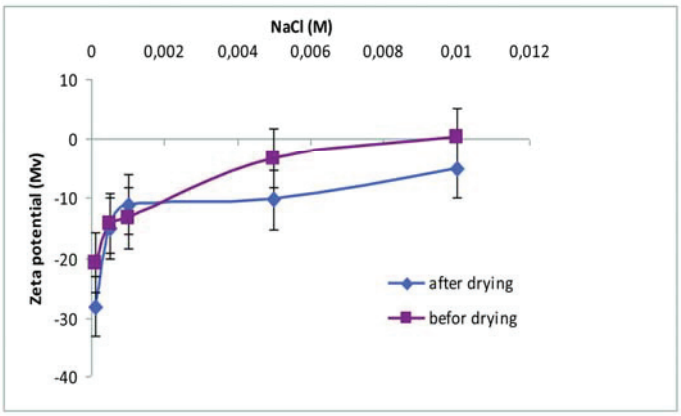

Fig. 8. Zeta potential of PLGA microspheres as a function of the ionic strength at constant pH before and after drying.

Secondly, the zeta potential was investigated as a function of ionic strength at constant pH (pH~8). It appears (Fig. 8) that the decrease in the magnitude of the zeta

potential is basically predicted by the classical electrokinetic theory [26, 27]. For smooth-like particle bearing low surface charge density, the increase in salinity generally leads to a decrease in the potential energy, therefore, a decrease in the magnitude of the zeta potential. The magnitude of the zeta potential of the microspheres before and after drying is low and closes to zero at moderate salinity. The observed slight difference can be attributed to the experimental error range. Therefore, again the drying process was found to have no effect on the colloidal stability of the prepared microspheres under different salinities.

Particles size and stability

The mean particles size as a function of salt concentration at a constant pH (pH~8) was measured until aggregation before and after the drying process. The results are shown on Table 1. Until 10 mM of NaCl, the colloidal stability of the particles was maintained. Above this critical value, the particles aggregate for both crude and dried particles. Since a small amount of NaCl (< 10 mM) was enough to induce particles aggregation, the colloidal stability should mainly be governed by repulsive electrostatic interaction. In fact, the used amount of PVA is low enough to ensure the sterical stabilization of the particles.

Tab. 1. Mean particles size as a function of the salt concentration before and after the drying process.

NaCl (mM)	Size before drying (µm)	Size after drying (µm)
0	8.52	7.75
0.1	8.78	7.71
0.5	8.85	7.62
1	9.37	7.94
5	8.83	7.96
10	Aggregation	Aggregation

Determination of PVA content

The amount of PVA adsorbed on the microspheres' surface was measured after rinsing by colorimetric titration as explained in the experimental part. This is important in order to study the colloidal stability of the particles.

The main results of the titration are the following: It should be pointed out that only a small amount of PVA was used in the microencapsulation process (0.1%). After washing twice and drying, only 1% of the initially introduced amount of PVA remained on the surface of microspheres. We could find 0.5% w/w of PVA in the microspheres after the drying process. The same percentage was found in the literature [20].

The same results of colloidal stability (size and Zeta potential) were obtained before and after drying of the microspheres, which means that both concentrations (0.1% and 0.001% of PVA) lead to the same results. This also suggests that 0.1% of PVA is

sufficient to stabilize the emulsion and microspheres but not to stabilize the microspheres in a high salinity medium.

Conclusions

This study was conducted to evaluate the effect of drying and redispersion process on the colloidal and physicochemical characteristics of PLGA microspheres manufactured by a double emulsion method w/o/w.

The colloidal and physicochemical characterization of these microspheres was investigated before and after rinsing and drying processes that consisted of employing a soft flow of compressed air for approximately one hour.

The surface and internal morphology was evaluated by scanning electron microscopy and the surface properties by atomic force microscopy. It can be concluded that the fabricated microspheres are soft and have a spherical shape with a smooth surface after drying but which becomes irregular after one month of suspension in water. Concerning the internal morphology, AFM and SEM indicated the presence of small cavities located just under a fine surface layer of polymer. SEM indicated the presence of bigger cavities in the centre of the microspheres. Electrokinetic study and particle size measurements were also performed as a function of pH and salinity. It was deduced that the colloidal stability of the produced microspheres should mainly be governed by repulsive electrostatic interaction due to the presence of carboxylic groups in the PLGA. The used amount of stabilizing agent (PVA) was low enough to assure microsphere stability in a high salinity medium. The drying process had no effect on the surface properties and colloidal stability of the prepared microspheres. In order to complete this work, the study of the encapsulation efficiency and the biological activity of the loaded protein are in progress. In fact, this work is the first part of a project aimed to encapsulate growth factor in PLGA microparticles for tissue engineering and dental applications.

Experimental part

Materials

The used poly(D,L lactic-co-glycolic acid) (PLGA) was RESOMER® RG 502H with a copolymer lactide-glycolide ratio of 48:52 to 52:48 and was supplied by Boehringer Ingelheim. Albumin, bovine BSA (60 kDa) was supplied by Sigma Chemical Company, poly(vinyl alcohol) (PVA) was obtained from Fluka Company and Methylene chloride from Carlo Erba Reagents.

Preparation of microspheres

BSA-containing microspheres were fabricated by a modified water-in-oil-in-water (W/O/W) double-emulsion solvent extraction/evaporation method as shown in Fig. 8. An internal aqueous phosphate saline buffer (PBS) solution containing 2 mg of BSA was poured into an oil phase (2 mL of methylene chloride containing 500 mg of PLGA). The mixtures were emulsified for 1 min using an Ultraturax ® High-performance disperser by IKA® apparatus (13000 rpm) to produce a primary W/O emulsion.

The emulsion was injected into a 50 mL of external aqueous solution of 0.1% (w/v) PVA under Ultraturax® stirring (6500 rpm) for 1 min to produce a double W/O/W

emulsion. The resultant solution was poured into a large volume of water (100 mL) under mechanical stirring for 2.5 hours in order to extract the solvent to the external phase. The resulting BSA-containing microspheres were collected by simple precipitation, washed twice with 50mL of deionized water, this precipitation was dried (by utilizing a gentle flow of compressed air for approximately one hour) and drying process was chosen to avoid any stress on the particles and the protein (methods such as filtration, centrifugation, freeze drying or vacuum drying were avoided). The obtained microspheres were stored under -20 °C.

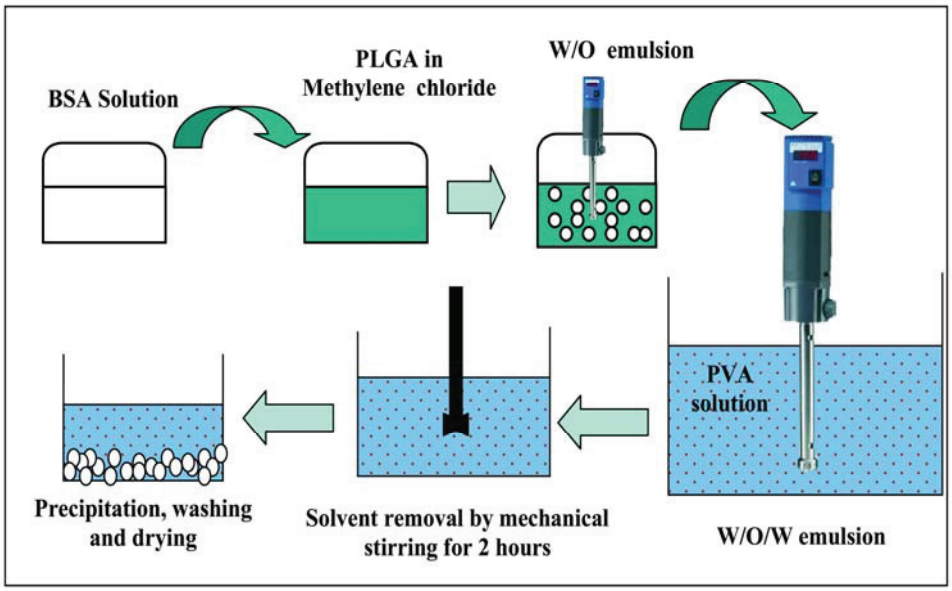

Fig. 8. Schematic diagram of microspheres fabrication.

Microsphere size analysis

The particles size was measured using a Coulter® counter multisizer (Beckman Coulter LS 230). Samples were prepared by re-dispersion of microspheres in deionized water. The results were reported as a volume size distribution.

Scanning electron microscopy (SEM)

The surface morphology of the microspheres was investigated using scanning electron microscopy (SEM) (FEG Hitachi® S 800). Particles were first rinsed on a filter with deionised water and a gentle flow of compressed air was used to realize complete drying of the microspheres. Afterwards, particles are collected from the filter. Microspheres were mounted onto metal stubs using a double-sided adhesive tape. Subsequently, they were vacuum-dried, contacted with silver paint, sputter-coated with a thin layer of gold (10-150A) and imaged with the SEM at 15 kV or 10 kV.

Atomic force microscopy (AFM)

A sample of 20 mg of microspheres was suspended in 5mL of distilled water and after one month the suspension was taken and deposited on freshly cleaved

Muscovite mica. 5 minutes after its preparation the still wet sample was observed at ambient temperature on a multimode-Veeco AFM in (Tapping mode). For technical reasons and a good resolution of image we were limited to observe the microspheres of diameter less than 5 µm.

Electrokinetic study

The electrophoretic mobility of the prepared microspheres was determined using the ZetaSizer 3000HS, from Malvern Instruments. The experiments were carried out using highly diluted microspheres dispersion in water at 1 mM NaCl. The electrophoretic mobility was determined as a function of pH at a constant ionic strength and as a function of salt concentration at a constant pH. In this study, the electrophoretic mobility (µ) values were converted to the zeta potential (ξ) [14] using the classical Smoluchowski's equation [24]:

$$\mu = (\varepsilon_0 \varepsilon_r / \eta)\, \zeta \qquad (1)$$

where η is the viscosity of the medium, ε_0 and ε_r are the permittivity of vacuum and the relative permittivity of the medium, respectively. All the measurements were at least the average of triplicate values and performed at 25 °C.

Colloidal stability Microspheres as a function of salt concentration

Samples were prepared by re-dispersion of microspheres in several salt concentrations.
The particles size was determined as a function of salt concentration using a Coulter® counter multisizer (Beckman Coulter LS 230). The results were reported as volume size distribution. Aggregation was observed by optical microscopy and by hydrodynamic size analysis.

Determination of PVA content

The residual amount of PVA in the obtained microspheres was determined using an iodine-borate colorimetric method [28] with some modification [29].

The method involves the extraction of poly(vinyl alcohol) from the sample matrix into an aqueous phase, followed by the formation of a PVA–iodine–borate complex that can be detected by visible spectroscopy. The method consists of solubilizing PVA by destroying the microspheres (10 mg) with 2 mL of 1 M NaOH for 30 min at 90 °C.

The resulting solution was neutralized with 1 M HCl. Then, 3 mL of a boric acid solution (3.7% w/v) and 0.5 mL of an iodine solution (1.66% KI + 1.27% I2 in distilled water) were added and the volume was adjusted to 10 mL with distilled water. Samples were analysed at 650 nm using a (Cary 50 spectrophotometer, Varian) in triplicate.

Known amounts of PVA added to 50 mg of PLGA were treated in the same way and used as standards.

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Travaux expérimentaux

Chapitre 3

Publication 4

Les microparticules chargées en FGF-2 et TGF-ß1 : préparation, caractérisation et modélisation.

Journal of biomedical nanotechnology, vol: 6, p: 106-116 (2010)

Dans ce chapitre, le protocole d'encapsulation optimisé dans les travaux précédents est utilisé pour l'encapsulation effective des facteurs de croissance TGF-β1 et FGF-2.

Le but de cette publication est de valider le protocole d'encapsulation par un procédé d'émulsion multiple comme méthode de préparation des microparticules chargées en facteur de croissance TGF-β1 et FGF-2. Les microparticules obtenues ont été caractérisées pour évaluer la taille, la morphologie, la toxicité, la voie de dégradation, la stabilité des facteurs de croissance après l'encapsulation, l'efficacité d'encapsulation et le profil de libération. Une étude de modélisation mathématique a été appliquée pour relier les caractéristiques des microparticules à la vitesse de libération.

Les microparticules ont été caractérisées morphologiquement par microscope à balayage et confocale, leur taille a été mesurée par granulométrie Laser. L'efficacité d'encapsulation et la cinétique de libération sont vérifiées, le dosage de chaque facteur de croissance a été effectué à l'aide d'un kit ELISA spécifique.

L'efficacité d'encapsulation est d'environ 35%, quant au taux d'encapsulation *«loading»* des microparticules il se situe entre 1 et 3 ng/mg. Les microparticules obtenues ne présentent aucune toxicité potentielle en terms de leur teneur en PVA et en solvant. Le test de stabilité des facteurs de croissance dans les microparticules montre que les microparticules renforcent la stabilité des facteurs de croissance (la matrice polymérique protège les facteurs de croissance et assurent leur libération contrôlée). Comme les facteurs de croissance ont été détectés et dosé par ELISA après l'encapsulation, nous pouvons conclure via des tests immunologiques que l'activité des facteurs de croissance encapsulés a été conservée et les microparticules sont biocompatibles et adaptées pour l'ingénierie tissulaire.

La morphologie interne des microparticules est liée au temps de précipitation du polymère pendant le procédé d'encapsulation (plus spécifiquement pendant la deuxième émulsion). Les microparticules de petites tailles sont censées se former plus rapidement en raison de leur rapport surface/masse plus importante. Il a été constaté que les microparticules les plus petites ont une matrice plus compacte. En effet, les résultats montrent que la stabilité des facteurs de croissance est plus importante dans les microparticules les plus petites.

La diminution du poids moléculaire du polymère (synonyme de dégradation) est moins importante pour les petites microparticules que pour les plus grosses et le coefficient de

diffusion de petites particules est inférieur à celui de plus grandes particules. L'efficacité d'encapsulation des petites particules (2-10µm) est plus élevée que celle des grandes. Les petites particules ont une vitesse de libération plus lente pour assurer une libération contrôlée plus longue (15 jours à 2 mois), ainsi qu'une stabilité plus élevée des facteurs de croissance dans la matrice des microparticules, ce qui assure des meilleures conditions de stockage.

Pour toutes ces raisons, la fabrication de petites microparticules est recommandée pour avoir des microparticules adaptées à notre utilisation.

L'un des points clés de ce travail est la combinaison de certaines caractéristiques avec la modélisation mathématique des processus. Cela a pu donner des informations sur la morphologie interne des microparticules surtout quand on sait que les microparticules de petite taille ne sont pas faciles à couper pour pouvoir les observer par microscopie comme pour les microparticules les plus grandes.

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Controlled Release Carriers of Growth Factors FGF-2 and TGFß1: Synthesis, Characterization and Kinetic Modelling

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The purpose of this work is to produce microspheres loaded with transforming growth factor β1 TGFβ1 and basic fibroblast growth factor FGF-2; to ensure the protein protection from degradation during the encapsulation and storage steps, to evaluate the release rate and the microspheres toxicity. The water in oil in water double emulsion technique was adapted to avoid the protein degradation during the encapsulation. The obtained microspheres were deeply characterized to evaluate their size, morphology, toxicity, the way of degradation, the protein stability and release rate. The microspheres were found to be biocompatible and the encapsulation efficiency was about 35%. It was observed that the obtained microspheres increase the shelf life of the growth factors. The diffusion coefficient was quantified using Fick's law of diffusion that was combined to an empirical equation representing the decrease in the protein stability. Such modelling helped to give indirect information about the microspheres morphology and drug distribution within the microspheres. The main conclusion consists of the formation of a higher compact polymer matrix when smaller particles are produced, which has different distinct effects: the encapsulation efficiency and the stability of the encapsulated growth factor are enhanced while both the diffusion and the polymer degradation rates decrease.

Keywords: Polymers of Lactide and Glycolide, Growth Factors, Microencapsulation, Controlled Drug Release.

RESEARCH ARTICLE

1. INTRODUCTION

Growth factors have a central role regulating a variety of cellular processes (proliferation, migration, differentiation…). Due to their short half life in solution, notable efforts were made to provide longer-term release of growth factors in tissue engineering and to ensure appropriate therapeutic concentrations. In a first approach, scaffolds,[1] collagen sponge or agarose beads[2] were directly impregnated with growth factors and applied for tissue regeneration. In a second approach, the growth factors were encapsulated in colloidal microspheres of micron size.[3] Studies also reported microspheres embedded in a gel and used in a scaffolds system.[1,4] In some cases, the microspheres were produced, sterilized then loaded with the growth factor and lyophilized.[5] In this case, the growth factor is adsorbed on the polymer matrix. Encapsulation of growth factors ensures a better protection and controlled release than posterior incorporation to preformed spheres which provides a sustained effect. Advantages of using encapsulation methods are also related to the possibility of characterizing their physicochemical properties and relating them to the release rate.

A number of microencapsulation techniques have been developed and reported to date such as stimuli-responsive microgel containing protein,[6] single emulsion process,[7,8] double (multiple) emulsion process, phase separation (coacervation), spray drying[9] and nanoprecipitation.[10,11]

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The choice of the technique depends on the nature of the polymer, the drug, the intended use, and the duration of the therapy. Due to their hydrophilic nature, growth factors (also some proteins and peptides) are usually encapsulated by the water-in-oil-in-water (w/o/w) method followed by solvent extraction/evaporation.[12–15] This microspheres fabrication procedure allows a better protection of the growth factor against degradation since it reduces the contact between the encapsulated agent and the organic solvent.

Protective biomaterials used for encapsulation of such drugs are mainly biodegradable natural polymers as dextrans,[16] chitosan[17] hyaluronic acid, and biodegradable synthetic polymers as polycaprolactone (PCL)[18] and polymers of lactide and glycolide (PLGA).[19, 20] PLA, PGA and their copolymers have been used to form both scaffolds and microspheres. The use of PLGA is approved by the U.S FDA.[19] These copolymers have been used to prepare various drug loaded devices (vaccines, peptides, proteins and micromolecules) due to their excellent biocompatibility and biodegradability.

In this work, PLGA is used to encapsulate FGF-2 or TGFβ1 using the w/o/w method. The produced microspheres were deeply characterized to investigate their toxicity, degradation rate of polymer, microspheres morphology, stability of the encapsulated drug, drug distribution, encapsulation efficiency, and the drug release rate. A mathematical law is used to describe the release rate. The combination of physical measurements and modelling estimations was found to be beneficial to investigate this system and interpret some observations.

2. MATERIALS AND METHODS

2.1. Materials

Poly (D,L lactic-co-glycolic acid) (PLGA) is RESOMER® RG 502H with a copolymer lactide-glycolide ratio of 48:52 to 52:48 was purchased from Boehringer Ingelheim. Recombinant Human Transforming Growth Factor-beta 1 (TGFß1) (25 KDa) was purchased from AbCys Company/France. Recombinant Human Fibroplast Growth factor FGF-2 (17 KDa) was kindly provided by Wenzhou Medical College/China. Albumin–fluorescin isothiocyanate conjugate bovine (FITC–BSA) (60 kDa) was purchased from Sigma Chemical. Poly(vinyl alcohol) (PVA) was obtained from Fluka and Methylene chloride (DCM) from Carlo Erba Reagents. RayBio® Human TGFß1 and FGF-2 enzyme-linked immunosorbent assay (ELISA) Kit was purchased from BioCat/Germany. Cell culture medium used for stability and release studies was a serum-free DMEM (Gibco/France) supplemented with micokill and ciprofloxacin (Bayer/France).

2.2. Preparation of Microspheres

Microspheres of PLGA were prepared by the w/o/w double-emulsion and solvent extraction/evaporation method as previously described[14, 15] (Table I). PLGA was dissolved in DCM. This oil phase was then emulsified using a high speed mixing apparatus (Ultrathurrax®, T25 basic, IKA® Werke/Germany) with an internal aqueous phase phosphate saline buffer (PBS) solution containing TGFß1 or FGF-2 always with FITC-BSA to form a w/o emulsion. All preparations were performed at ambient temperature. The resulting emulsion was added to 50 mL of external aqueous solution containing 0.1% (w/v) PVA and emulsified with Ultrathurrax® in order to produce the double w/o/w emulsion. The double emulsion was then poured into a large volume of water (100 mL) under magnetic stirring for 2.5 hours to allow removal of the organic solvent. Finally, the resulting microspheres were collected on a filter, washed twice with 50 mL of deionised water, dried and stored at −20 °C. Table II shows the different operating conditions considered to investigate the microspheres morphology.

The main precautions included in the previously developed w/o/w encapsulation method are:

—Double sterilized water was used in all solutions; beakers and glasses were sterilized; preparations take place under fume hood.

—Growth factors were stored at −20 °C; Growth factor solutions were prepared 10 min before the emulsion preparation.

—Stirring time of the first w/o emulsion was limited to 30 s to avoid heating.

—For solvent extraction, the final solution was poured into a large quantity of water under magnetic stirring without vacuum evaporation to avoid pressure aggression and temperature rising. Also, addition of isopropanol alcohol to accelerate solvent extraction was avoided.

—For protein extraction, Dimethyl sulfoxide (DMSO) was used to dissolve the microspheres, which is less aggressive than DCM used previously for PLGA dissolution.[21, 22]

Table I. Conditions of the double emulsion method.

Growth factor	Internal aqueous phase	Oil phase	First emulsion stirring time	First emulsion stirring speed	External aqueous phase	Second emulsion stirring time	Second emulsion stirring speed
FGF-2	PBS solution containing 1 mg FITC-BSA and 5 μg FGF-2	2 mL methylene chloride containing 500 mg PLGA	30 s	13000 rpm	0.1% (w/v) PVA	30 s	6500 rpm
TGFß1	PBS solution containing 500 μg FITC-BSA and 1.5 μg TGFβ1						

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Table II. Conditions of the different experiments with the resulting microspheres size and encapsulation efficiency.

Experiment	Active agent	Internal aqueous phase volume (μl)	Water to oil volume ratios	Second emulsion stirring speed	Stirring apparatus	Size (μm)	Encapsulation efficiency Method 1	Encapsulation efficiency Method 2	Loading ng/mg (Method 1)
1a	FGF-2	250	1/2	6500	ULTRA-TURRAX®	8±5	32	35.3	2.9
1b	TGFß1					11±5	33	36.3	1
2a	FGF-2	100	1/5			8±5.5	32.4	35.3	2.9
2b	TGFß1					11±5	30.1	34.4	0.9
3a	FGF-2	25	1/10			8±6.2	31.3	35.3	2.8
3b	TGFß1					11±5	31.7	38.9	0.95
4a	FGF-2	100	1/5	2000	Ika® Mechanical Overhead	93±25	24.3	28.	2.2
4b	TGFß1				Stirrers	137±40	23.5	28.1	0.71

3. MICROSPHERES CHARACTERIZATION

3.1. Particle Size and Morphology

Scanning electron microscopy (SEM) was performed using a FEG Hitachi® S 800 microscope. Microspheres were mounted onto metal stubs with a double sided adhesive tape, vacuum-dried, contacted with silver paint, sputter-coated with a thin layer of gold (10–150A) and imaged with the SEM at 15 kV or 10 kV. The size distribution was determined with a laser diffraction technique using a Coulter® counter multisizer (Beckman Coulter LS 230) after dispersion of the microspheres in deionised water.

3.2. Enzyme-Linked Immuno Sorbent Assay

The titration of growth factors was performed using a human ELISA kit specific for each growth factor. 100 μL of each sample solution were added into appropriate wells of the kit microtiter plate. After 2.5 hours of incubation at room temperature, the plate was rinsed several times with the kit buffer solution and a biotinyled antibody solution was added into each well and incubated for 1 h. After washing away the unbound biotinylated antibody, a Streptavidine solution was added to the plates and incubated for 45 min. The plates were finally washed five times and introduced into a luminescent plate reader (LabSystem). The light emission was recorded after the injection in each well of 150 μL buffer containing 200 μM of luminol, 500 μM of hydrogen peroxide and 200 μM of p-iodophenol. The calibration curves were generated for each growth factor with the appropriate kit standard solutions ranging from 0 to 100 ng/mL.

3.3. Encapsulation Efficiency and Drug Loading

Two methods were used to measure the encapsulation efficiency. Method (1) consists of measuring the amount of growth factor entrapped in the microspheres after extraction (extraction protocol). The extraction protocol consists of dissolving about 20 mg of microspheres in 1 mL of DMSO then adding 9 mL of cultural medium and analysing by ELISA. In Method (2), the encapsulation efficiency was calculated by deducting the lost quantity of growth factors in the aqueous supernatant at the end of the microspheres preparation (after solvent evaporation, before rinsing) from the initial used quantity.

3.4. Protein Distribution into the Microspheres

Protein distribution into the microspheres could be analyzed thanks to the presence of FITC–BSA in the microspheres, by confocal laser scanning microscopy (Leica Microsystems TCS SP2/Germany). The microspheres were suspended into water and spread on a cover slip. The fluorescein was excited by a 488 nm argon laser. Different sections of the microspheres were scanned. The images presented in this work were taken in a central section of the microspheres.

3.5. Residual Solvent

Gas chromatography was used to analyze the residual amount of DCM (Boiling point (BP) = 40 °C). An Agilent Model 4890 gas chromatograph was used with the program Star Chromatography Workstation and a BONDED FSOT Capillary column 30 m × 0.53 mm (i.d.). The analytical conditions were: injector temperature 250 °C; detector temperature, 280 °C; initial oven temperature 70 °C that increased at 10 °C/min to 220 °C with a final stabilization at this temperature for 2 min and flow rate of the carrier gas (nitrogen) was 13 mL/min. The calibration curve was based on different DCM concentrations in DMSO (500–31 ppm) with toluene as internal standard at a constant concentration of 100 ppm then using the ratio of DCM to toluene areas under the peak. For the measurement of DCM residual amount in the microspheres, 50 mg of microspheres were dissolved in 2 mL of DMSO (BP = 189 °C), an appropriate amount of toluene (BP = 111 °C) was added as an internal standard.

3.6. PVA Content

The residual amount of PVA in the microspheres was determined using an iodine–borate colorimetric method as proposed in Ref. [23] including some modifications proposed.[7] The method requires the extraction of

poly(vinyl alcohol) from the polymer matrix into the aqueous phase, followed by the formation of a PVA–iodine–borate complex that can be detected by visible spectroscopy.

3.7. Stability of Growth Factors in Aqueous Solution

In order to study the growth factor stability in aqueous PBS solution (pH = 7.4) or in the culture medium (usually used for *in vitro* test on cultured cells), the same amount of growth factor (50 ng) was dissolved in flasks containing 1 mL solution. These flasks were put either at ambient temperature or in the refrigerator and the growth factor content was analyzed using ELISA.

3.8. Microspheres Shelf Life

The microcapsules shelf life, or the stability of encapsulated growth factors in the PLGA microsphere matrices, was studied as follows: Flasks of 20 mg of microspheres were put at room temperature or at 5 °C. The growth factor content was analyzed at specific intervals using ELISA by applying the extraction protocol explained above.

3.9. Size Exclusion Chromatography (SEC)

PLGA Degradation during the release was studied by monitoring the polymer molecular weight using Size Exclusion Chromatography (SEC). Waters SEC system was used. This system was equipped with an isocratic pump (Waters 515) operating at a flow-rate of tetrahydrofuran (THF) of 1 mL/min, a refractive-index detector Model (Waters 410) with integrated temperature controller to maintain temperature at 35 °C, a guard column (PLgel 5 μm) and three Polymer Laboratories columns (2 × PLgel 5 μm Mixed C (300 × 7.5 mm) and 1 PLgel 5 μm 500 A (300 × 7.5 mm)), all columns working in-line and the software Empower pro. The calibration was carried out using narrow distributed polystyrene standards. After specific time intervals of suspension in PBS, the microspheres were collected and vacuum dried for 24 h to determine the PLGA molecular weight. Samples of microspheres were dissolved in THF and put in an ultrasonic bath to obtain a homogenous solution. Chromatography was carried out after sample filtration using a 0.45 μm filter.

3.10. Microspheres Morphology Alteration

The morphology of the microspheres was assessed by SEM and the evolution in the surface properties of the microspheres was analysed by atomic force microscopy (AFM) after specific intervals of suspension in water. For AFM analysis, after suspension in distilled water, the microspheres were taken and deposited on freshly cleaved muscovite mica. The still wet sample was observed at room temperature on a multimode-Veeco AFM in tapping mode.

3.11. *In Vitro* Release Study

Known quantities of microspheres were dispersed in test tubes containing 1 mL of culture medium. The suspension was gently stirred at room temperature. At specific intervals, the tubes were centrifuged at a rotating rate of 14000 rpm for 10 min and analyzed by ELISA.

4. RESULTS

4.1. Microspheres Size and Morphology

As usually observed in the double emulsion method,[14, 15, 21, 22] a broad size distribution was obtained for formulations containing TGFß1 or FGF-2 (Table II). Figure 1 shows that the obtained microspheres are quite spherical and have a smooth and regular surface as observed by SEM and confirms the polydispersity of the microspheres.

The water-to-oil volume ratio in the internal emulsion had no effect on the particle size as previously shown.[15, 21] The stirring speed, on the contrary, had a significant effect on the microspheres size that varies from about 90 to 8 μm with stirring speeds of 2000 and 6500 rpm.

4.2. Encapsulation Efficiency and Drug Loading

Using the method referred as the extraction protocol, the encapsulation efficiency was 32% for small microspheres and 24% for big ones for both growth factors (Table II). The second method consisting of dosing the growth factor lost in supernatant after collecting the microspheres estimates the encapsulation efficiency between

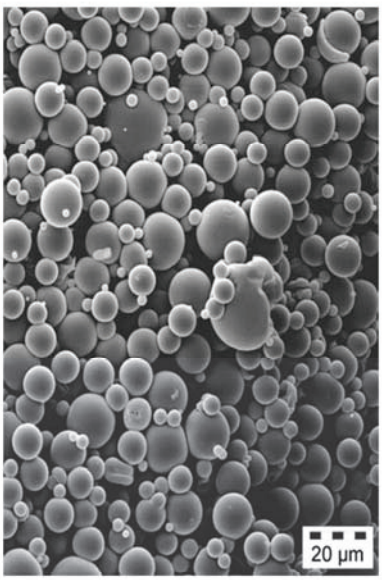

Fig. 1. SEM pictures of PLGA microspheres (experiment 2a).

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35% and 39% for small microspheres and approximately 28% for big ones. The difference between both methods can be explained by the reduction of the growth factor activity due to contact with solvent or water. On one hand, during the extraction protocol, the contact with DMSO might reduce the growth factor stability which misestimates the real loading of microspheres. On the other hand, the activity of growth factor present in the aqueous supernatant might decrease and hence the calculation leads to overestimating the real growth factor loading.

From these data, growth factors loading into the microspheres could be calculated and was found to be equal to 2.9 ng/mg for FGF-2 and approximately 1 ng/mg for TGFß1.

4.3. Protein Distribution within the Microspheres

FITC–BSA was incorporated into the microspheres together with the growth factors. The fluorescence of the albumin allows the detection of FITC–BSA in the microspheres using fluorescence confocal microscopy. The distribution of FITC–BSA in the microspheres should be comparable to that of the growth factors, but not necessarily the encapsulation efficiencies. Confocal microscopic images show a homogeneous distribution of the FITC–BSA in the microspheres at 30 min with a slightly higher density near to the surface and in the central part (Fig. 2(a)). The centre contains no FITC–BSA after 1 day (Fig. 2(b)) and after 7 days, FITC–BSA is mainly present in the periphery (Fig. 2(c)). This suggests a gradual diffusion of FITC–BSA through the microsphere with time.

4.4. Residual Solvent

Residual DCM level in the microspheres as determined by GC was always about 3 ppm of DCM per mg of microspheres (about 150 ppm of DCM in 50 mg of microspheres).

4.5. PVA Content

Blank samples (not containing protein or growth factors) were previously analyzed[14] for the PVA content before and after double rinsing and it was found that rinsing allows eliminating most of the PVA. In this work, after rinsing, 0.5% by weight of PVA was found in the microspheres. Similar results were reported in the literature.[9, 23]

4.6. Growth Factors Stability in Aqueous Solution

The stability of FGF-2 and TGFß1 in PBS buffer (pH 7.4) or in the culture medium was measured by ELISA. The decrease of FGF-2 and TGFß1 concentration with time in the PBS buffer was very fast (Fig. 3). On the other side, growth factors stability in culture medium was very good. Figure 4 shows only FGF-2 stability. Similar results were found for TGFß1 (data not shown).

4.7. Microspheres Shelf Life

Figures 5 and 6 show that the decrease in the activity of encapsulated growth factors is much lower than free growth factor in the aqueous medium which means that the

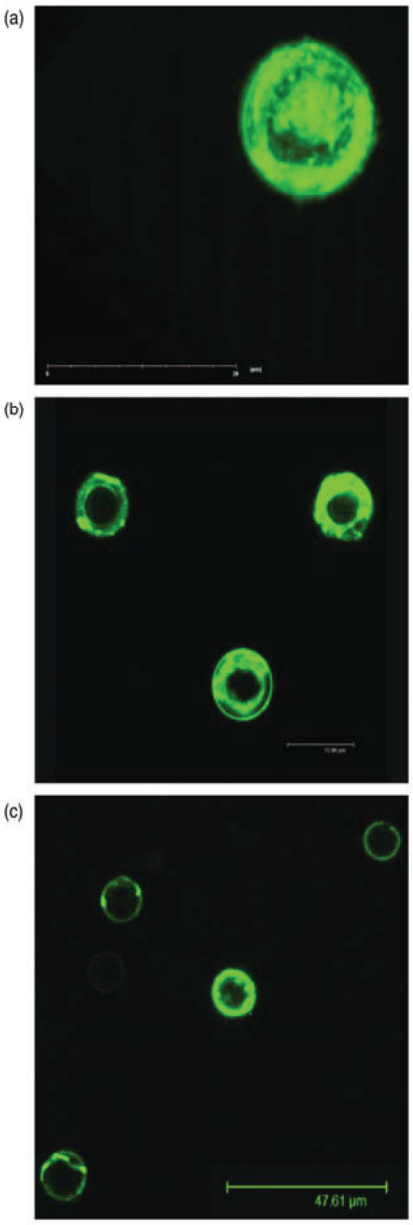

Fig. 2. Confocal microscopy micrographs (central section). The distribution of FITC–BSA within the microspheres after (a) 30 minutes, (b) 1 day and (c) 7 days.

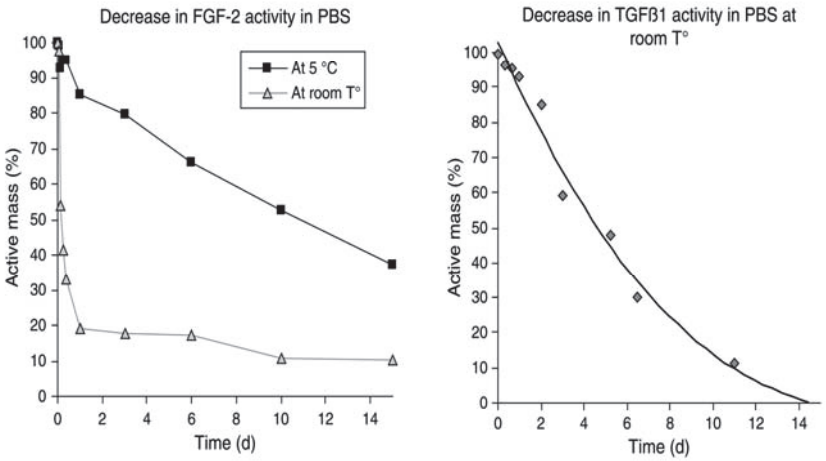

Fig. 3. Decrease in the activity of both growth factors in PBS, very fast decrease is shown at room temperature for both growth factors. At 5° slower decrease was remarked but is still fast comparing with culture medium.

polymeric membrane enhances the stability of the growth factor. It is interesting here to investigate the temperature effect and the microspheres size and permeability on the encapsulated growth factor stability. Figure 5 shows, as expected, that loading decreases more rapidly at higher temperature. It is interesting to notice also that the shelf life of big microspheres loaded with FGF-2 is somewhat shorter than smaller microspheres.

Figure 6 shows that after 21 days, the microspheres shelf life is proportional to the w/o ratio in the internal phase (for TGFβ1 a slight effect of internal ratio was found after only 6 days). A more compact microsphere (lower w/o internal ratio) leads to an improved protection of the growth factor.

4.8. Microspheres Degradation

From the microspheres SEM pictures (Fig. 7), it can be seen that the spherical shape of the microspheres is deformed with time and surface irregularities appear with some pores on the surface and even some exploded microspheres can be detected.

Microspheres surface morphology observed by AFM microscopy after 30 days in water reveals a soft microsphere with a rough and irregular surface and confirms the loss of surface smoothness (Fig. 8).

SEC measurements (Fig. 9) show a slight decrease in the PLGA molecular weight during the contact with water.

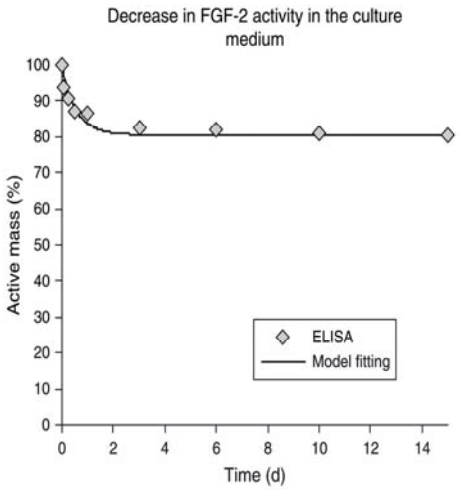

Fig. 4. Decrease of the activity of FGF-2 in culture medium at room temperature. The protein degradation rate coefficient represents the aggression of the medium. This coefficient was fitted using the ELISA data to be $k = 6.6e-4\ ng^{-1} \cdot h^{-1}$.

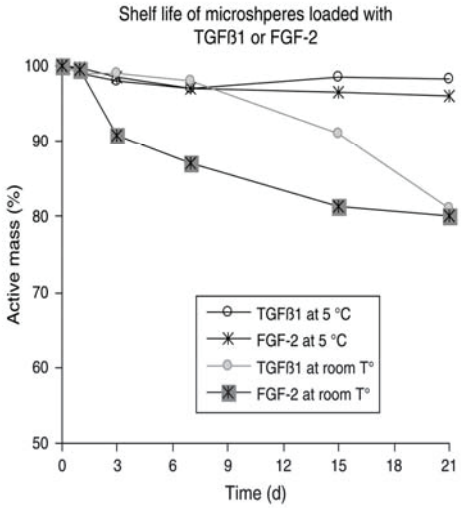

Fig. 5. Shelf life of FGF-2-loaded microspheres as a function of the particle size and temperature (w/o internal ratio = 1/5). The loading decreases more rapidly at higher temperature. The shelf life of big microspheres loaded with FGF-2 is shorter than smaller microspheres.

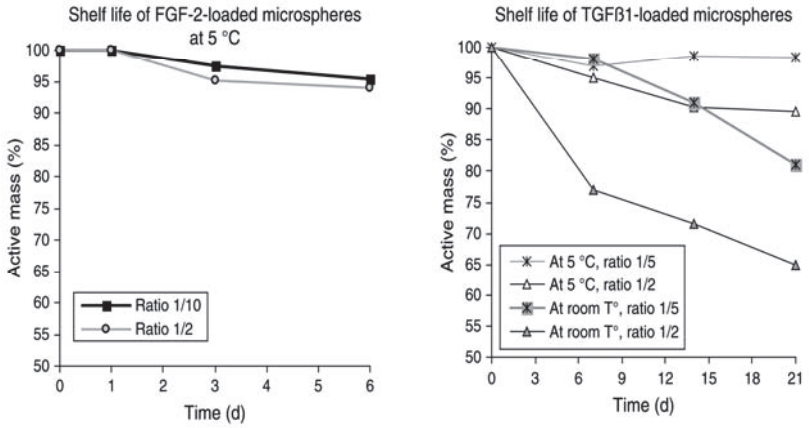

Fig. 6. Shelf life of small microspheres as a function of the internal w/o ratio for both growth factors. A very slight effect was found in the first 6 days as seen for TGFβ1. A significant effect of internal ratio was found after 21 days as seen for FGF-2.

This decrease is (partly) responsible of the polymeric matrix erosion and should affect the growth factors diffusion in the matrix and therefore the release rate.

4.9. *In Vitro* Release Study

The release kinetics showed the existence of a phase of rapid release during the first 24 hours in which about 30–56% of the drug is released (Figs. 10–11). This phenomenon is described in the literature as the burst effect and can be beneficial in order to ensure the therapeutic dose (see for instance Ref. [4]). This phenomenon can be due to the non homogeneity of the matrix that contains big and small cavities. Bigger cavities might be formed preferably close to the microspheres surface due to violent solvent extraction. Diffusion out of big cavities is rapid and therefore comes quickly to end while diffusion through very small cavities is lower and continue for a longer period of time.

Figure 10 compares the amount of FGF-2 released with time as a function of the microspheres size. It can be seen that almost the same amount is released from both small and big microspheres. Since small microspheres have a higher contact surface area with the release medium, then it can be concluded that the diffusion coefficient of small microspheres is lower than bigger ones.

The effect of the internal w/o ratio on the diffusion rate can be observed on Figure 10. It can be seen that the released amount of drug is proportional to the internal w/o ratio. Since the internal w/o ratio had no effect on the particle size as reported previously by our team,[15] then it can be concluded that a higher diffusion coefficient is obtained for higher internal w/o ratio.

4.10. Estimation of the Diffusion Coefficient

The second Fick's law of diffusion[32] was used to estimate the diffusion coefficient. In spherical particles, an analytical solution of this law can be derived assuming homogeneous dispersion of the drug in the sphere, constant diffusion coefficient on the particle radius (r) perfect

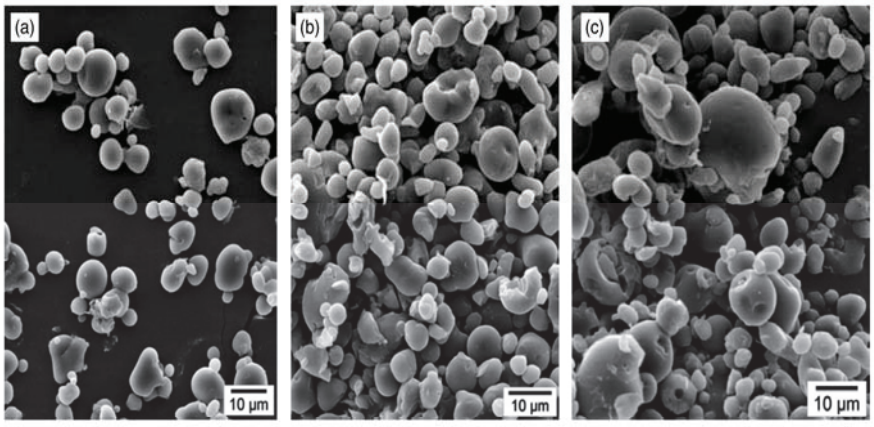

Fig. 7. SEM pictures showing the degradation of the microsphere after (a) 7 days, (b) 14 days and (c) 30 days of suspension in PBS.

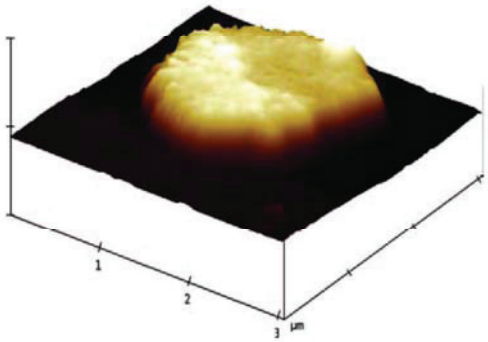

Fig. 8. Microspheres surface morphology by AFM after 30 days in water.

sink conditions and a drug loading that is lower than the solubility of the drug inside the polymer matrix:

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(\frac{-Dn^2\pi^2 t}{r^2}\right) \qquad (1)$$

where M_t and M_∞ are the cumulative absolute amounts of the drug released at time t and at infinite time respectively, $r(\mu m)$ is the average microspheres size and $D(\mu m^2/s)$ is the apparent diffusion coefficient.

In order to take in account the stability of released growth factors in the culture medium, we consider data given in Figure 4. It can be seen that the decrease in the growth factor activity is rapid at the beginning but almost stops after few days. Protein degradation is a complex domain that involves different chemical and physical pathways. The decrease in the protein activity in the release medium due to interactions with this medium (depending on the type of solvent, temperature, pH, presence of protective excipients…) is represented by superficial amount of the protein (X). The reduction in the growth factors activity in water and in the culture medium that can mathematically be represented by the following system:

$$\begin{cases} \dfrac{dM_t}{dt} = \underbrace{\Delta M_{t_released}}_{\text{Input due to release}} - \underbrace{k\,M_t\,X}_{\text{Output due to degradation}} \\ \dfrac{dX}{dt} = -k\,M_t\,X \end{cases} \qquad (2)$$

Where M_t is the protein mass and k is the protein degradation rate coefficient (Fig. 4). This unique coefficient represents the aggression of the considered medium. This coefficient was fitted using the ELISA data to be $k = 6.6e-4\ ng^{-1}\cdot h^{-1}$. In this model, the initial value of X(ng) is the amount of M_t to be denatured in the considered medium. When X is totally consumed, M_t becomes stable. In Eq. (2), $\Delta M_{t_released}$ = the released amount of drug during the sampling period.

In order to estimate the diffusion coefficient, one has to take in consideration the diffusion rate of the drug and the reduction in its activity simultaneously (Eqs. (1) and (2)). An optimization example is shown on Figure 11. The figure shows the released and residual amounts of growth factor obtained with the optimized diffusion coefficient. It can be seen when comparing the curves referred to as 'Released' and 'Residual' that an important amount of the drug is degraded during the release study. In these curves, a time-constant diffusion coefficient is considered (Table III) which assumes that the matrix porosity is homogeneous. However, the occurrence of a burst effect reveals some heterogeneity in the matrix (presence of small and big cavities) which leads to a variation in D with time. Therefore, the same optimization method explained above was applied by authorizing D to vary with time. Actually, optimization is done over 3 data measurements at a time. Then, the optimization recedes by ignoring the oldest data point and adding a new point at the right hand side of the figure and so on. The released and residual curves obtained by the receding horizon optimization are shown on Figure 11 and referred to as 'adaptive'. It can be seen that a more precise fitting is obtained in this case. The time-varying estimated diffusion coefficients as obtained by the adaptive method are shown on Figure 11. It can be seen that D decreases with time. It is important to remind that the molecular weight of polymer slightly decreased with time which could increase the diffusion rate, but this was not the case, therefore it was not necessary to incorporate degradation of the polymer molecular weight in the model.

The time-constant diffusion coefficients estimated in these experiments are shown in Table III. The table confirms our expectations regarding the size effect and the internal w/o ratio on the diffusion coefficient. It can be seen that the diffusion coefficient increases with increasing the internal w/o ratio which increases the microspheres

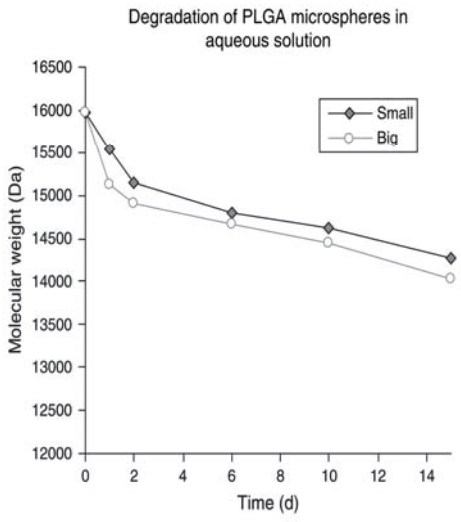

Fig. 9. Degradation of the polymer matrix measured by SEC.

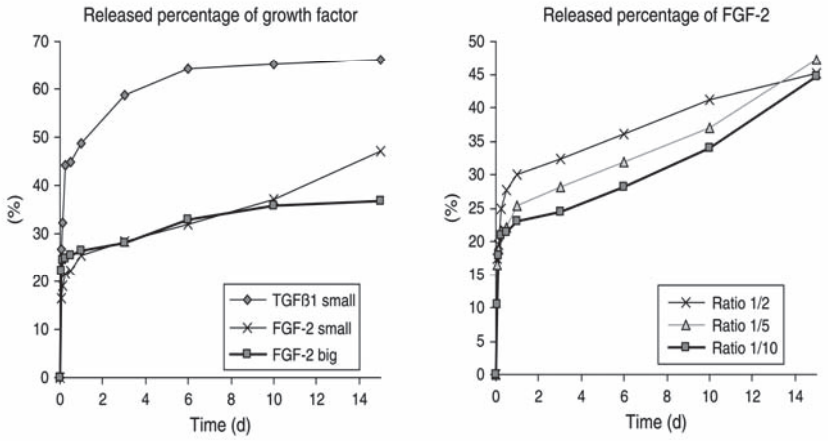

Fig. 10. Release profile of FGF-2- and TGFβ1-charged microspheres as a function of the microspheres size and w/o ratio in the internal phase.

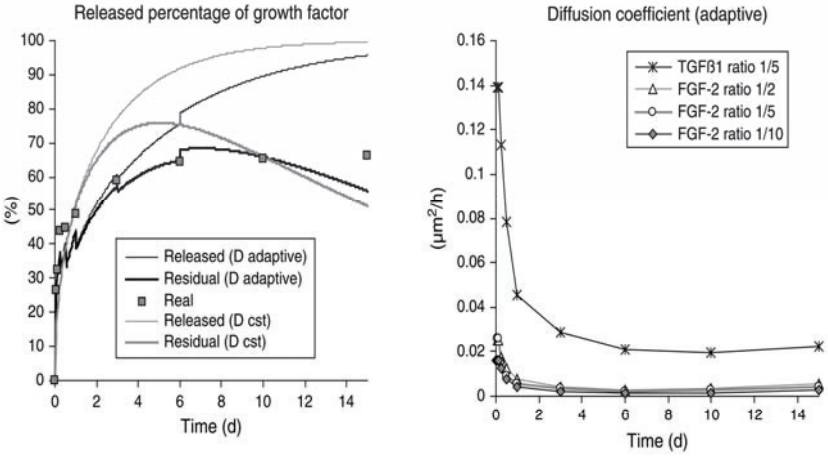

Fig. 11. At left, identification of D in experiment 2b. At right: Estimation of D by using receding horizon optimization for small microspheres (D of experiment 4a, bigger microspheres, goes from 6 to 0.3 ng^2/h). Adaptive refers to a receding horizon optimization (varying D), otherwise D is constant.

porosity. It can also be seen that the diffusion coefficient of small particles is much lower than that of bigger ones. Finally, the diffusion coefficient of TGFβ1 (25 KDa) (microspheres size $\approx$ 137 μm) is higher than that of FGF-2 (17 KDa) (microspheres size $\approx$ 93 μm). The release rate difference should mainly be due to differences in the molecular nature between these growth factors, since differences in the size are negligible when comparing to the diffusion coefficient of bovine serum albumin (BSA) (fraction V, 60 kDa) estimated previously,[15] it could be seen that the diffusion coefficient was 1000 times higher for BSA than FGF-2 for both big and small particles.

Table III. Estimation of the diffusion coefficient.

Experimental conditions (a stands for FGF-2 and b for TGFβ1)	Average diffusion coefficient (μm^2/h)
1a (Small microspheres, ratio 1/2)	0.0053
2a (Small microspheres, ratio 1/5)	0.0039
3a (Small microspheres, ratio 1/10)	0.0027
4a (Big microspheres, ratio 1/5)	0.5469
2b (Small microspheres, ratio 1/5)	0.0424

5. DISCUSSION

Spherical microspheres with a smooth surface were formed by the proposed method. Dependence of the microsphere size on the stirring energy was confirmed. Concerning the microspheres biocompatibility, it was evaluated by measuring the residual solvent and surfactant concentrations. Indeed, DCM is an organic solvent that can be dangerous for humans when inhaled at a high concentration and was found to cause cancer in humans exposed to vapours in the workplace.[24] studies of Serota et al. recommends that exposure of children to DCM be limited to less than 5 mg/L of drinking water for 1 day.[24,25] In the produced microspheres, DCM concentration was about

3 ppm per mg of microspheres, which allows the administration of several milligrams of microspheres without any risk. The PVA concentration was about 0.5% by weight. This concentration is also lower than the potential toxic dose.[26] The obtained microspheres loading (2.9 ng/mg for FGF-2 and 1 ng/mg for TGFß1) is appropriate for local applications since the therapeutic doses of growth factors are usually between 10 and 20 mg of microspheres.[27–31] The produced microspheres are therefore adequate for human use.

It can be confirmed that the polymeric membrane enhances the stability of the growth factor. The shelf life of the growth factor-loaded microspheres is much higher than solutions of these growth factors. At ambient temperature, more than 80% of growth factor in water solution was lost in 15 days. Only 20% of encapsulated growth factor was lost after 15 days at ambient temperature. In the other side, storing microspheres in freezer keep them entirely actives.

Since the produced microspheres are designated for dentin-pulp complex regeneration, the release study was done in a culture medium that is usually used for *in vitro* tests on cultured cells. Even though confocal microscopic images show a homogeneous distribution of the FITC–BSA in the microspheres and a gradual diffusion of FITC–BSA through the microsphere with time, a burst effect took place in this system which should be due to the presence of different sizes of cavities in the microspheres as previously shown by SEM.[15] The effect of alterations in the microspheres morphology and degradation of the polymer molecular weight on the protein diffusion coefficient can be supposed to be negligible during the considered period of time as supported by mathematical modelling and SEM and SEC analysis.

It is observed that the production of smaller microspheres has several beneficial effects. First of all, the encapsulation efficiency is higher in smaller microspheres. Second, the shelf life of small microspheres loaded with FGF-2 is somewhat longer than bigger microspheres. Third, the polymer molecular weight of microspheres suspended in aqueous solution decreases less rapidly in smaller microspheres even though their surface area is bigger. Fourth, the diffusion rate coefficient is lower in smaller microspheres. The explanation for these observations can be the following: during the formulation process, for small microspheres the polymeric precipitation is done more rapidly protecting thereby a higher amount of growth factor than bigger ones. It seems also that a higher compact polymer matrix is formed if polymeric precipitation is rapid which prevents explosion of the primary cavities. By this way, the protein diffusion coefficient out of the microspheres is lower in smaller microspheres. Water diffusion into the microspheres should also be slower and therefore the degradation of the polymer molecular weight is slower in smaller microspheres. Finally, the stability of the protein in smaller microspheres is enhanced due to the higher permeability ensured by the compact matrix.

6. CONCLUSIONS

FGF-2 and TGFβ1-loaded microspheres were prepared, characterized and their release profile was examined. The developed method was found to be adapted to growth factors encapsulation and leaded to an encapsulation efficiency of about 35%. Microspheres' loading is adequate for therapeutic applications. The obtained microspheres have no potential toxicity regarding the PVA and solvent content. The microspheres enhance the stability of the growth factors and ensure controlled release. The activity of the encapsulated growth factor was conserved and the microspheres were found to be biocompatible and adapted for tissue engineering.

The internal morphology of the microspheres was found to be related to the precipitation time of the polymer. Smaller microspheres are supposed to precipitate faster due to their higher surface area. It was found that smaller microspheres have a higher compact matrix. Indeed, the drug stability is enhanced in smaller microspheres. The degradation rate of the polymer molecular weight of smaller microspheres is lower than bigger ones and the diffusion coefficient of smaller particles is much lower than bigger ones. Note that this last comment does not mean that diffusion rate out of smaller microspheres is lower than bigger ones since their surface area is higher. Added to all these advantages of smaller microspheres, their encapsulation efficiency is also higher than bigger ones. Therefore, fabrication of small microspheres is recommended to have optimized microspheres characterizations such as low release rate and high drug stability.

It is amazing to see that combination of some characterization and process modelling could give information about the internal morphology of the microspheres while small microspheres could not be easily cut into parts to be observed by microscopy as done for bigger microspheres.[15]

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Travaux expérimentaux

Chapitre 4

Publication 5

Etude de cytotoxicité et vérification de l'activité biologique *in vitro* des facteurs de croissance TGF-β1 et FGF-2 après leur encapsulation dans des microparticules de PLGA

European cells and materials

Articles soumis : juillet 2010

Ce travail s'inscrit dans la continuité du chapitre précédent. Le but est de réaliser une étude biologique *in vitro* sur des cellules pulpaires (fibroblastes pulpaires humains) qui sont les cellules cibles de ces travaux (avec notamment comme objectif, la régénération de complexe pulpodentinaire). Nous cherchons à vérifier que les microparticules ne sont pas cytotoxiques et que les facteurs de croissance gardent leur activité biologique spécifique (prolifération ou différenciation cellulaire) après leur encapsulation.

Dans un premier temps, la vérification du contenu de microparticules en agent potentiellement toxique utilisé dans les procédés d'encapsulation tel que PVA et DCM a été effectuée. Ensuite, le test MTT a été utilisé pour étudier la toxicité des microparticules non chargées et pour mesurer l'activité biologique des facteurs de croissance (FGF-2 et du TGF-ß1) encapsulés et libérés au contact des cellules pulpaires (fibroblastes pulpaires humains).

Deux paramètres sont étudiés pour vérifier l'activité biologique :

1. la concentration des facteurs de croissance (quantité de microparticules utilisée)
2. la quantité libérée à plusieurs intervalles de temps (profil de libération)

Le profil de libération montre une phase de libération rapide pendant les premières 24 heures appelée *«burst effect»* (30 à 40% de la quantité encapsulé pour FGF-2 et du TGF-ß1 respectivement) suivie par un ralentissement de libération avec une libération totale de (45 à 65 % de la quantité encapsulée

pour FGF-2 et du TGF-ß1 respectivement) au bout de 15 jours.

Les résultats des tests MTT ne montrent aucun effet toxique des particules sur les fibroblastes pulpaires. Des observations *in vitro* au microscope optique montrent que les fibroblastes pulpaires humains ne semblent pas altérés par la présence de microparticules. De plus, la présence de cristaux de bleu formosane prouve l'existence d'une prolifération cellulaire en contact avec les microparticules par la méthode MTT.

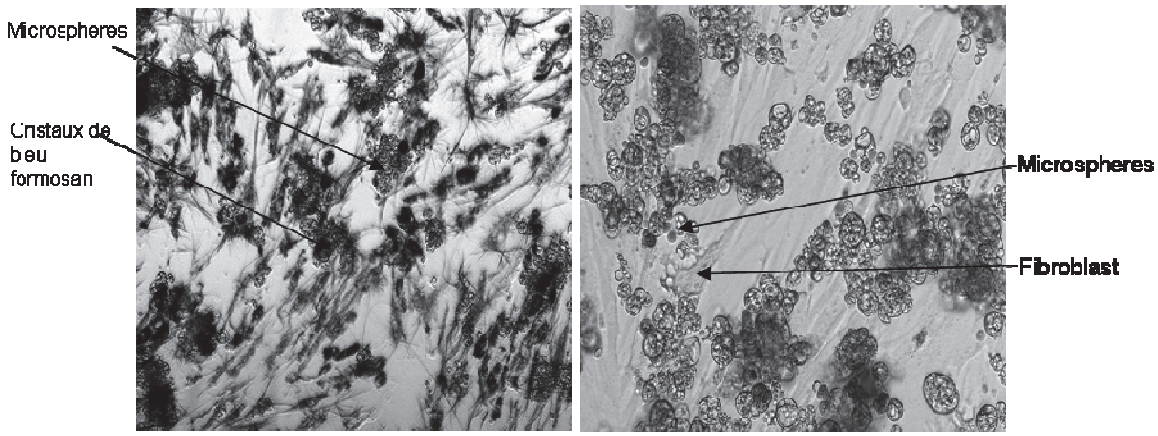

Figure (4.1) : microparticules en contact avec les cellules pulpaires, la prolifération est présentée par la formation des cristaux de bleu formosane (méthode MTT)

Les facteurs de croissance ont gardé leur activité biologique spécifique. L'augmentation de la quantité des microparticules chargées en FGF-2 ou l'augmentation de temps de libération de ce dernier augmente de façon significative la prolifération des cellules pulpaires ($p < 0,05$ selon le test statistique de Student), inversement, l'augmentation de la quantité des microparticules chargés en TGF-ß1 ou l'augmentation de temps de libération de ces microparticules diminuent légèrement la prolifération des cellules pulpaires.

A physicochemical approach for the controlled release of FGF-2 and TGFß1 for a potential use in the dentin-pulp complex regeneration

Running title: Controlled release of FGF-2 and TGFß1 for dentin-pulp complex regeneration

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Keywords

Growth factors, controlled release, double emulsion, microspheres, dentin regeneration

Abstract

The application of exogenous growth factors was extensively studied as a potential mean for stimulating the regeneration of the dentin-pulp complex. A physicochemical approach (microencapsulation) by the double emulsion method was used to separately encapsulate Basic Fibroblast Growth Factor (FGF-2) and Transforming Growth Factor-β 1 (TGFß1) in biodegradable poly(lactide-co-glycolide) (PLGA).

Microcapsules morphology, «loading», shelf life, potential toxicity and Growth factors release kinetics were studied. Then we examined the proliferation of dental pulp cells in contact with microspheres to verify the released growth factors biological activity.

Smooth and regular microspheres were produced. Growth factor «loading» was 1-2.9 ng per mg of microspheres. Shelf life test showed that the growth factors activity was protected in the polymeric matrix. The release kinetics showed a phase of significant release during the first 24 hours followed by a slower release phase leading to an overall liberation of 45% of FGF-2 and 65% of TGFß1 in 15 days. Solvent residuals quantifications and MTT test performed on dental pulp cells showed that the microspheres were non-cytotoxic. The proliferation rate showed that the encapsulated growth factor specific biological activity was

maintained. Increasing FGF-2-charged microspheres quantity or extending their incubation time with the culture medium significantly increased the dental pulp cells proliferation rate ($p < 0.05$) and conversely. Increasing TGFß1-charged microspheres quantity or extending their incubation time with the culture medium slightly decreased dental pulp cell proliferation rate. These findings provide baseline data for potential uses of FGF-2 and TGFß1-charged microspheres in dentin-pulp complex regeneration.

Introduction

Many growth factors and extracellular matrix proteins normally expressed and secreted during primary and secondary dentinogenesis are assumed to play a role in dental repair and dentin regeneration (Tziafas *et al*. 2000, Iohara *et al*. 2004, Unda *et al*. 2001). Among theses factors, Vascular endothelial growth factors (VEGF), Platelet-derived growth factors (PDGF), Basic Fibroblast Growth Factor (FGF-2) and Transforming Growth Factor-β 1 (TGFß1) are secreted and sequestered in the dentin (Sloan and Smith 1999, Goldberg *et al* 2006). When carious lesions occur, theses factors can be released and reach the pulp to stimulate tertiary dentine synthesis. Recent studies showed that they can also be released by the injured pulp fibroblasts suggesting a role in the reparative events after traumatic injury (Tran-hung *et al* 2008). The underlying signals regulating the sequence of events leading to reparative dentinogenesis have been the subject of intense investigation using a variety of *in vitro* and *in vivo* approaches (Tziafas et al, 2000; Smith and Lesot, 2001; About et al, 2000). Exogenous TGF-βs, FGFs and bone morphogenetic proteins BMPs were applied onto the exposed pulp *in vitro* (Six *et al*. 2002, Sloan and Smith 1999) or *in vivo* (Nakashima 1994, Lesot *et al*. 2001, Unda *et al*. 2001) and have demonstrated their potential in signalling reparative dentinogenic events.

It is important to take into consideration that the application of such exogenous growth factors requires the use of a suitable delivery system for different reasons:

- Growth factors are sensitive and their biological activity needs to be protected as they have a short half-life (Nakashima *et al* 2005). For example, the half-life of TGF-β1 in its free form is of a few minutes during which it is capable of signalling regenerative events (Smith 2003).
- The direct application of growth factors would give unsatisfactory results since the applied dose would be very high shortly after its application and might have major unpredictable effects.

- The use of a delivery system allows incorporating a reduced amount of growth factors which is crucial due to their high price.
- Growth factors must be present for an extended period in order to be effective, which can be ensured by sustained release systems.

Bioactive molecules delivery systems have long been investigated. Chemical approaches like vinyl polymerization, polycondensation, interfacial polycondensation and suspension crosslinking were used to fabricate polymeric microspheres (Arshady *et al* 1990; Al khoury *et al* 1986). Other techniques incorporate growth factors in a hydrogel or a polymer such as dextran-GMA (Chen *et al* 2007) or chitosan (Niu *et al* 2008) followed by polymerisation or crosslinking. Although these processes could ensure high encapsulation efficiency, the chemical and the exothermic reactions lead to growth factors degradation and loss of their biological activity.

Alternatively, a physical approach is usually used to deliver bioactive molecules because of its simplicity. With this approach, no chemical reactions are required and only physical interactions take place between growth factors and the carrier. The incorporation is usually done by impregnating agarose beads (Sloan and Smith 1999), collagen sponges (Six *et al* 2002), hydrogel microspheres (kikushi *et al* 2007) or polymeric (dextran or chitosan) microspheres (Chen *et al*. 2007; Niu *et al*. 2008) with a solution of growth factors. This approach was used in the study of dentin regeneration by investigating the effects of TGF ß 1,2, 3 (Sloan and Smith 1999); and BMP-7 release from agarose beads (Sloan *et al* 2000) but the effects of these factors were studied *in vitro* without any quantification of the «loading» or the release profil. Similarly, a collagen matrix was used to deliver BMP-7 but no information was provided on its insertion method or release profil (Six *et al* 2002). Other studies reported the fabrication of dextran-derived hydrogels loaded with BMP for bone healing enhancement. The release kinetics showed that such hydrogels had a very rapid burst release and retained BMP-2 for a few days only (Maire *et al* 2005; Chen *et al*. 2005 and 2007). In a recent work, gelatine hydrogel microspheres were prepared by impregnating prefabricated microspheres with FGF-2 solution but the «loading» and release kinetics were not investigated (Kikushi *et al,* 2007).

Preparation of biodegradable polymeric microspheres by a physicochemical approach can be used to deliver growth factors. The advantage with this approach is the possibilities of microspheres characterization such as size, morphology, «loading» and release profil measurement which allow a reproducible microparticuleproduction and well-known «loading» and release kinetics.

Although growth factors delivery by physical and chemical approaches provided valuable information regarding the dentinogenic capacity of dental pulp progenitor cells, they only allowed the investigation of growth factors short-term effects (Sloan and smith 1999; Six et al, 2002).

However, the growth factors prolonged release appears as a basic requirement for long term studies of dentin pulp healing and dentin regeneration and provides a better simulation of the situation *in vivo* where growth factors are released during the whole pulp healing and regeneration process.

Additionally, there is little information about the dentin regeneration initial steps, particularly the activation and migration of progenitor cells that precede reparative dentine synthesis. Thus, the controlled release of growth factors may provide precious information on the mechanisms involved in these unexplored processes.

Several studies Investigated microbeads as scaffolds alone (Kim et al. 2001) or containing microspheres (Kim et al. 2003, DeFail et al. 2006 and Kikuchi et al. 2007). The combination of both microspheres and scaffolds could ensure distinct functionalities during the same treatment (mechanical properties, permeability, release, protection of the drug). However, this combination has the disadvantage that the release from the microspheres has to be re-evaluated as it may be altered by the physicochemical characteristics of the scaffolds. In this study, the aim was to evaluate the microspheres alone in form of suspension in water as a material for dental tissue engineering.

In this work, a physicochemical approach based on solvent evaporation or solvent extraction was used to encapsulate growth factors. This physicochemical double-emulsion water/oil/water (w/o/w) solvent extraction/evaporation technique is one of the most usual methods to encapsulate hydrophilic drugs specially proteins and peptides (Jeffery et al, 1993; Ogawa *et al*. 1988, Kalaji *et al*. 2009, Deloge *et al*. 2009). In pharmaceutical industry, this process is widely used in the vectorization of drugs or vaccines to ensure drug delivery and controlled release. It allows an accurate control and a reliable reproducibility of all physicochemical properties including microspheres size, morphology, «loading» and release profil.

A biodegradable polymer of lactide and glycolide (PLGA) was used as a carrier to form the microspheres matrix. These microspheres were then used to separately encapsulate FGF-2 and TGFβ1. Before their application on living cells, the microspheres physicochemical characteristics were investigated. The microspheres morphology and «loading» efficiency were studied. The growth factor stability in the polymeric matrix was verified by studying the

shelf life. Residuals of the solvent and stabilising agents in the microspheres were controlled and the overall toxicity evaluated. The growth factors release profils were analyzed. Finally, the cell proliferation was investigated to demonstrate that the growth factors bioactivity was conserved after the encapsulation into the microspheres.

Materials and Methods

Materials

PLGA is RESOMER® RG 502H with a copolymer lactide-glycolide ratio of 48:52 to 52:48 was purchased from Boehringer Ingelheim. Recombinant Human Transforming Growth Factor-beta 1 (TGFß1) (25 KDa) was purchased from AbCys, France. Recombinant Human Fibroblast Growth factor FGF-2 (17 KDa) was kindly provided by Whenzou Medical College China. Bovine Serum Albumin was purchased from Sigma Chemical Company. Poly(vinyl alcohol) (PVA) was obtained from Fluka and dichloromethane (DCM) from Carlo Erba Reagents. Human TGFß1 and FGF-2 enzyme-linked immunosorbent assay (ELISA) Kit was purchased from BioCat, Germany. Cell culture medium used for stability and release studies was DMEM (Gibco, Cergy Pontoise, France) supplemented with micokill and ciprofloxacin (Bayer SAS, Puteaux, France).

Microspheres Preparation

The microspheres were prepared by a modified water-in-oil-in water (w/o/w) double-emulsion with solvent extraction/evaporation method. Briefly, 500mg of PLGA polymer were dissolved in 2ml of DCM. This oil phase was then emulsified for 1 min using a high speed mixing apparatus (Ultrathurrax®, T25 basic, IKA® Werke) (13000 rpm) with an internal aqueous phase phosphate saline buffer (PBS) solution containing 0.5mg of BSA and 1.5µg of TGFß1 (Peter *et al*. 2000, Jaklenec *et al*. 2008, and DeFail *et al*. 2006) or 5µg of FGF-2 (Kimoto *et al*. 1998 and Kikuchi *et al*, 2007). The resulting emulsion was then added to a 50ml of second water phase containing a stabilizer, polyvinylalcohol, PVA 0.1% (w/v) and emulsified with Ultrathurrax® apparatus stirring (6500 rpm) for 1 min in order to produce the double w/o/w emulsion. All preparations were performed at room temperature. The double emulsion was then poured into a large volume of water (100 mL) under magnetic stirring for 2.5 hours to allow organic solvent removal. Finally, the resulting microspheres were collected on a filter, washed twice with 50mL of deionized water, dried and stored at -20°C. All preparations were performed at ambient temperature.

3. Microspheres Characterization

Microspheres morphology

The surface morphology of the microspheres was investigated using scanning electron microscopy (SEM) (FEG Hitachi® S 800). The microspheres were mounted onto metal stubs using a double sided adhesive tape. Subsequently, they were vacuum-dried, contacted with silver paint, sputter-coated with a thin layer of gold (10-150A) and imaged with the SEM at 15kV or 10 kV. The size distribution was determined with a laser diffraction technique using a Coulter® counter multisizer (Beckman Coulter LS 230) after dispersion of the microspheres in deionised water.

«loading» and encapsulation efficiency

Two methods can be used to measure the encapsulation efficiency. The first one consists of measuring the amount of growth factor entrapped in the microspheres. This method requires an adequate extraction protocol of the growth factors from the microsphere. In the extraction protocol 20 mg of microspheres were dissolved in 1ml of Dimethyl sulfoxide (DMSO), then the solution was diluted by adding 9ml of culture medium.

In the second method, the quantity of growth factors in the solution (or in the supernatant) at the end of the preparation (after collection and filtration) is determined. The encapsulation efficiency can be calculated by deducting this quantity from the total amount of growth factor introduced in the recipe. In this work, both methods were used to measure the encapsulation efficiency.

The encapsulation efficiency and microspheres drug «loading» (DL) are calculated as follows:

Encapsulation efficiency (%) = (Entrapped fraction x 100) / Total drug (1)

Encapsulation efficiency (%) = Total drug (%) – Drug lost in the supernatant (%) (2)

Drug «loading» (DL) = (Entrapped fraction x 100) / Amount of microspheres recovered

The concentration of TGFß1 and FGF-2 was analysed by Enzyme-Linked ImmunoSorbent Assay (ELISA).

The maximum possible encapsulation efficiency is calculated as follow:

1.5µg TGFß1 /500mg microspheres= 3ng/mg microspheres

5µg FGF-2 /500mg microspheres= 10ng/mg microspheres

This calculation means that the maximum content of growth factors in the 20 mg reference quantity (used in all experiments and characterisation) is 60ng for TGFß1 and 200ng for FGF-2

ELISA protocol

Growth factors concentration was analyzed by enzyme-linked immunosorbent assay (ELISA) using a human ELISA kit specific for each growth factor. Briefly, 100 µL of each sample solution were added into appropriate wells of the kit microtiter plate. After incubation for 2.5 hours at room temperature, the solution was discarded. The plate was rinsed several times with the kit buffer solution, and then a prepared biotinyled antibody solution was added into each well, and incubated for 1 hour at room temperature. The solution was then discarded from the plate, rinsed several times. After washing away unbound biotinylated antibody, a prepared Streptavidine solution was added and after 45minuts the plates were finally washed five times and introduced in a luminescent plate reader (LabSystem, Helsinki, Finland). The light emission was recorded after the injection in each well of 150 µl of buffer containing 200 µM of luminol, 500 µM of hydrogen peroxide and 200 µM of p-iodophenol. The calibration curves were generated for each growth factor with the appropriate kit standard solutions ranging from 0 to 100 ng/ mL.

Growth factors Stability in the polymeric matrix

20 mg of microspheres were put in 2 mL test tubes at room temperature and at 4°C. The microspheres content was analysed at specific times for both FGF-2 and TGFß1-loaded microspheres by the ELISA after applying the extraction protocol explained earlier.

Residual solvent measurement

Gas chromatography was used to analyse the residual amount of DCM in the microspheres. Fifty mg of growth factor-loaded microspheres were dissolved in 2ml of DMSO (Boiling point (BP)=189°C), an appropriate amount of toluene (BP=111°C) was added as an internal standard. The calibration curve was made using a set of standard DCM (BP=40°C) concentrations in DMSO (500 – 31 ppm) with toluene (internal standard at a constant concentration of 100 ppm). Residual DCM levels in the microspheres were determined from the linear calibration curve constructed from the ratios of DCM standards to toluene (R2=0.9951). An Agilent Model 4890 gas chromatograph equipped with a split/splitless capillary injection port and flame ionization detector (FID) was used in this work. The separations were performed on a BONDED FSOT Capillary column (30m×0.53mm (i.d.); Superox-FA; polyethylene glycol ester; ALLTECH. The temperature conditions used are the following: injector temperature 250°C; detector temperature, 250°C; initial oven temperature 70°C, increased at a rate of 10°C/min to 220°C with a final stabilization at this temperature

for 2 min. The carrier gas was nitrogen at a flow rate of 13 ml/min. Injection was made in the split mode (split ratio, 1/40) with an injection volume of 0.1µl. The program Star Chromatography Workstation (version 4.0) was used for acquiring and processing the data.

Release study

In order to study the release kinetics, 20 mg of microspheres were dispersed into 2mL test tubes containing 1ml of a culture medium without adding bovine serum. The suspension was gently stirred at room temperature. At specific time points, the tubes were centrifuged (14000 rpm for 10 min) and analyzed by ELISA. Three replicates were done for each time point. The quantity of microspheres (20mg) has been chosen to ensure that the released growth factor will be in the effective range generally referred for both growth factors (Kimoto *et al*. 1998; Kikuchi *et al*, 2007; Peter *et al*. 2000; Jaklenec *et al*. 2008, and DeFail *et al*. 2006).

PVA titration

The residual amount of PVA in the microspheres was determined using an iodine-borate colorimetric method (Panyam *et al* 2003) with some modifications (Hamoudeh *et al*. 2007). The method involves the extraction of PVA from the polymer matrix into the aqueous phase, followed by the formation of a PVA–iodine–borate complex that can be detected by visible spectroscopy. The method consists of dissolving 10 mg of the microspheres into 2ml of 1 M NaOH for 30min at 90°C. The resulting solution was neutralized with 1 M HCl. Then, 3 ml of a boric acid solution (3.7% w/v) and 0.5ml of an iodine solution (1.66% KI + 1.27% I2 in distilled water) were added and the volume was completed to 10ml with distilled water. The samples were analyzed at 650 nm using a Cary 50 spectrophotometer (Varian) in triplicate. Known amounts of PVA added to 50 mg of PLGA were treated in the same way and used as standards. The correlation coefficient for the given standards of PVA was 0.9995. Samples were analyzed with and without double rinsing and before drying.

Human pulp cells preparation and culture

Human pulp cells were prepared from immature third molars as previously described by About *et al*. (2000). Briefly, the teeth were obtained from 16-year-old adolescents in compliance with the French legislation (informed patients and parents consent and Institutional Review Board approval of the protocol used). After the extraction process, the teeth were washed and their apical part was removed. The dental pulp was minced and the resulting small explants were cultured in 100-mm-diameter culture dishes. Confluent cultures

were collected by trypsinisation and sub cultured. Cells were cultured in 60-mm-diameter culture dishes in Minimum essential medium supplemented with 10% fetal bovine serum, 100 UI/ml penicillin, 100 µm streptomycin and 0.25 µg/ml amphotericin B (Fungizone).

Samples solutions preparation

In order to study the toxicity, non-charged microspheres (10 mg/ml) were suspended in 1mL culture medium without fetal calf serum for 24 hours. The same medium was used as a control but without the microspheres).

In order to study the biological effects of the released growth factors, two parameters were investigated:

1) The effect of the concentration of FGF-2 and TGFß1 charged microspheres: different concentrations (10, 20, 25 and 30 mg/ml) were suspended in 2mL of culture medium for 24 hours, then the suspension was centrifuged (at 14000 rpm for 15minutes) and the supernatant was added on the cell cultures for 48 hours to measure the cell proliferation of dental pulp cells.

2) The effect of the growth factors release kinetics from the microspheres: the same concentration (10 mg/ml) of FGF-2 and TGFß1-loaded microspheres was suspended in 2mLof culture medium for 1, 3 and 6 days. Then the suspension was centrifuged (at 14000 rpm for 15minutes) and the supernatant was added on the cell cultures for 48 hours to measure the cell proliferation of dental pulp cells.

The abovementioned concentrations were selected for two raisons:

1) To ensure a better microparticulesuspension in culture medium and to avoid the saturation or aggregation (this will improve their release).

2) To take into consideration the maximal «loading» of the microspheres calculated above in the «loading» section (3ng /mg microspheres for TGFß1and 10ng /mg microspheres for FGF-2).

The maximum released quantity will be in the effective range generally referred for such growth factors (Kimoto *et al*. 1998; Kikuchi *et al*, 2007; Peter *et al*. 2000; Jaklenec *et al*. 2008, and DeFail *et al*. 2006).

Microspheres toxicity

The toxicity non charged microspheres and the effect of FGF-2 and TGFß1 on dental pulp cell proliferation were estimated using the MTT colorimetric assay as described previously (Abou

Hashieh et al, 1998). Briefly, dental pulp cells were plated at 3000 cells/cm^2 (Falcon 3072, Becton Dickinson, Oxford, GB). The 96 well dishes were then placed into a humid incubator at 37◦, 5% CO_2, 95% air for 24h prior to use. Control fibroblasts were in the same culture medium but without Fetal calf serum and tested under the same experimental conditions.

After this period, the medium from the 96 well plates was removed and replaced by 100µL of the test medium of each growth factor. The 96 well plates were placed in an incubator again for 48h. After 48h, the MTT assay was performed on the dishes. The medium was removed and immediately replaced with 100 µL per well of a MTT solution (5mg/ml). After incubation for 2h at 37°C, the supernatant was discarded and the formed crystals were solubilised with dimethylsulfoxide (100µL per well). The absorbance of each 96-well dish was determined using an automatic microplate spectrophotometer (E960, Bioblock, Strasbourg, France) at 550 nm. Untreated cells were used as control (100% viability). Relative cell viability (%) was calculated by means of [abs]test/[abs]control ×100. All assays were performed in triplicates.

Effect of growth factors on cell proliferation:

The MTT assay was used as above except that the cell density was lower (1000cells/cm^2) to allow cell proliferation.

Statistical analysis

The student test was performed for each point. The threshold for statistical significance was set at $p<0.05$.

RESULTS

Microspheres morphology

Laser diffraction technique showed a large size distribution from 2 to 25µm with a mean diameter of 11µm for TGFβ1 (Figure 1a) and 8.5µm for FGF-2. Scanning electron microscopy showed spherical-shaped microspheres with a smooth and regular surface (Figure 1b). A large size distribution is usually obtained with the double emulsion method (Yang *et al*. 2000, Yang *et al*. 2001, Kalaji *et al*. 2009, Deloge *et al*. 2009). Recent results of our group have shown that microspheres prepared under the same conditions but with a bigger size had a honeycomb internal morphology (Dologe et al 2009).

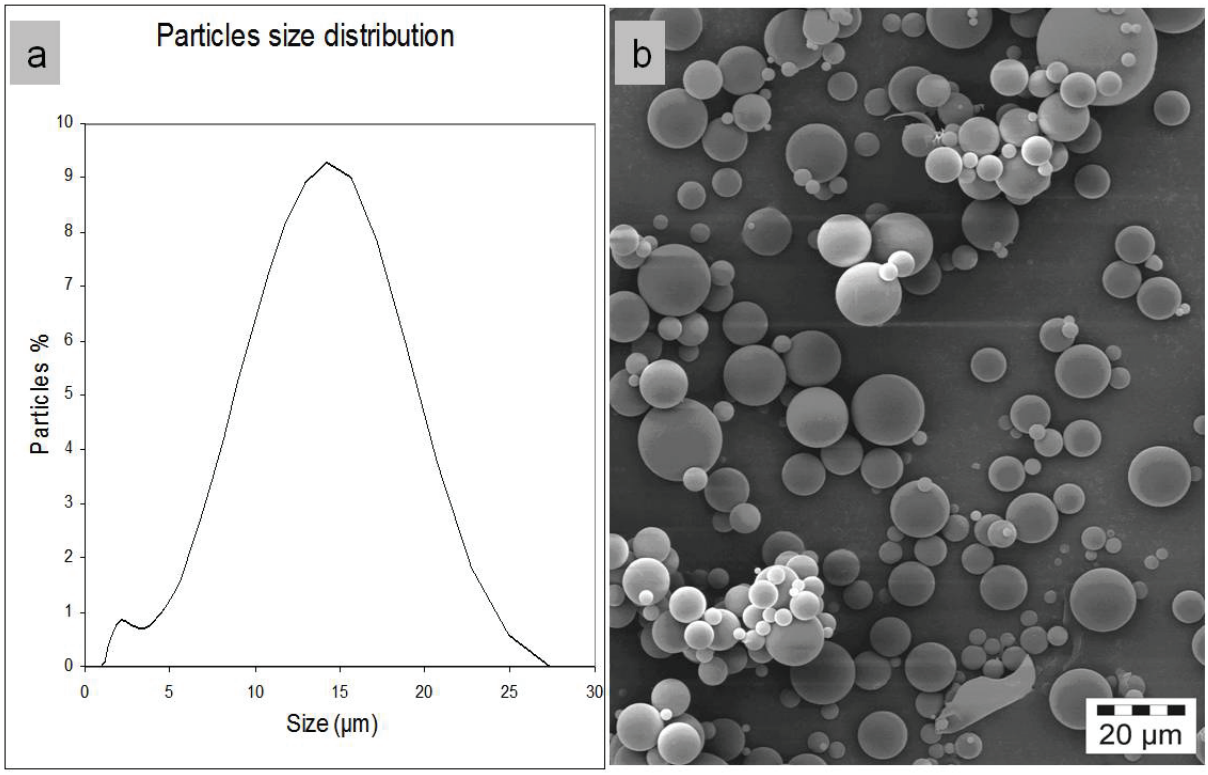

Figure 1: Size distribution and morphology of PLGA microspheres.

Laser diffraction method show size distribution of TGFβ1 (figure, 1a), and SEM picture showed that the produced microspheres have a spherical shape and a smooth surface (figure, 1b).

Encapsulation efficiency and «loading»

Using the extraction protocol, the encapsulation efficiency was 32%. By measuring the amount of growth factor lost in the supernatant, the encapsulation efficiency was between 35 % and 39%. The difference between both methods can be explained by the degradation of growth factors during the extraction protocol due to the contact with DMSO.

«loading» of microspheres with growth factors was calculated to be 2.9ng/mg for FGF-2 and 1ng/mg for TGFß1.

Growth factors stability in the microspheres

FGF-2 and TGFß1 stability in the microspheres was evaluated by measuring the growth factor content at specific time periods in a known amount of microspheres kept at room temperature or at 4°C. This test gives the shelf life and specifies the storage conditions of the microspheres.

As expected, it can be seen that the «loading» efficiency decreases more rapidly at ambient temperature. At 4°C, more than 95% of the growth factor was conserved after 21 days. On the

other hand, 82% of growth factor was conserved after 21 days at ambient temperature (figure 2).

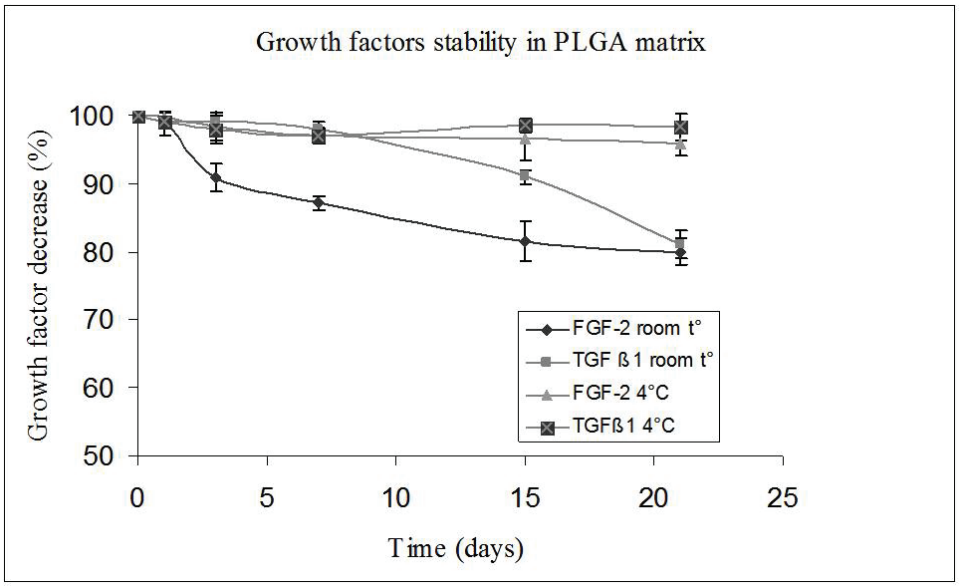

Figure 2: Stability of the encapsulated FGF-2 and TGFβ1 in function of the storage temperature. Dry quantities of each encapsulated growth factor were conserved for 21 days at room temperature or at 4°C, and then the Microspheres growth factors content was investigated by ELISA. Microspheres growth factors content slightly decreased to 82% after 21 days at room temperature but was conserved upto more than 95% at 4C° after the same delay.

Residual DCM content

DCM is an organic solvent which is widely used as an extraction agent in food processes such as the decaffeination of coffee. A limited amount of data on the chronic toxicity or carcinogenicity of DCM has been published in the literature. Rats exhibited an increased number of benign mammary tumours and an increased incidence of salivary gland sarcomas in males with DCM at levels of 500, 1500 and 3500 ppm was reported (Serota *et al* 1986). A safe dose of DCM in drinking water was recommended to be less than 10 mg/L per day or 2 mg/L in 10 days (Kanno *et al* 1993) and less than 5 mg/kg/day (Serota *et al* 1986).

The total amount of DCM residuals into the produced microspheres measured by GC was 3 ppm of DCM per mg of microspheres which is 30 ppm per 10mg of microspheres. It can be concluded that the residual quantity of solvents in microspheres is lower than the recommendation of EPA and that the produced microspheres can be applied safely on human tissues, in terms of the residual DCM content.

PVA titration

PVA is considered as a biocompatible stabilising agent (Ardashy et al, 1991, Jeffery et al., 1993). PVA toxic dose (15– 20 g/kg) is very high (DeMerlis *et al*. 2003). The colorimetric titration in this work was done to verify the residual amount of PVA in the microspheres. After microspheres rinsing, the PVA residual amount was 0.5% w/w. We consider that 10-20mg of microspheres will be used for in vivo applications. Consequently, the amount of PVA used in each application will be 0.05-0.1mg. This dose is lower than the potential toxic dose.

In vitro *release study*

The results show the existence of a phase of significant release during the first 24 hours, described in the literature as a «burst effect» (Lu *et al*. 2000). The released quantity after 24h is about 30-40% of the total released quantity for both growth factors. It can be noticed that the TGFβ1 is released faster than FGF-2. The smaller «loading» concentration of TGF□1 and its larger size (8.5µm for FGF-2, 11µm for TGF□1) are both factors that could justify its more rapid release. The difference in the release rates can be also due to the differences in the molecule nature and affinity to the polymer of these two growth factors. The release kinetics showed that 65% of TGF□1 was released after 15 days while 45% of FGF-2 was released after the same delay (Figure 3).

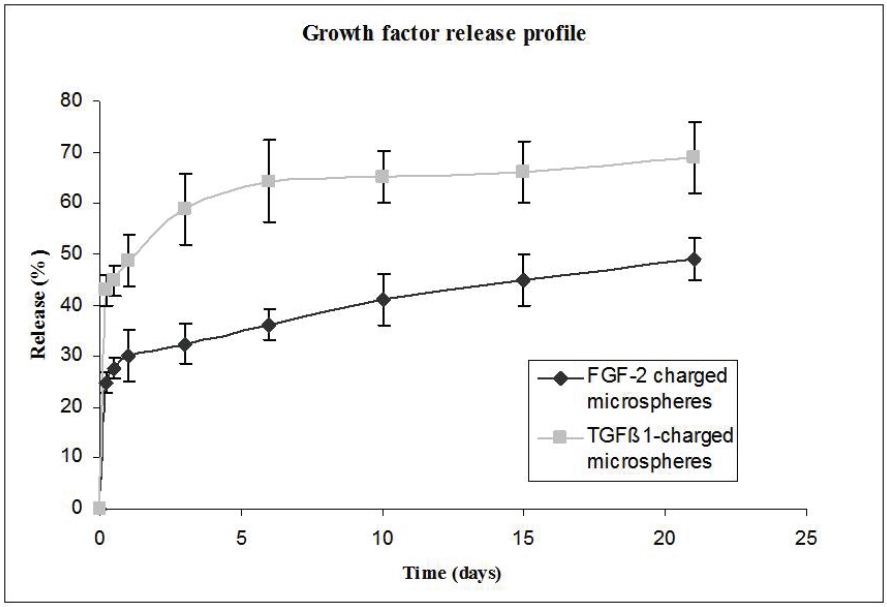

Figure 3: FGF-2 and TGFß1 release profil. Microspheres were incubated in an aqueous solution to investigate the growth factors release kinetics. The release kinetics showed an initial burst phase with a significant growth factors release during the first 24 hours followed

by a slower release phase. It also showed that 65% of TGFβ1 was released after 15 days while 45% of FGF-2 was released after the same delay. The released amount after 21 days was 50% for FGF-2 and 70% for TGFβ1.

Microspheres biocompatibility and growth factors biological activity study

The MTT method developed by Mosmann (1983), relies on the activity of the mitochondria to reduce a tetrazolium salt (3-(4,5- imethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to form a blue formazan product.

MTT test was performed after dental pulp cell contact with non-charged microspheres. Cell viability in contact with non charged microspheres was 98.37%. This relative cell viability was calculated in comparison with untreated control cells viability (considered as 100%). Dental pulp cells shows that cell viability was not affected by the empty matrix (figure 4). This indicates that no cell toxicity was observed due to the microspheres.

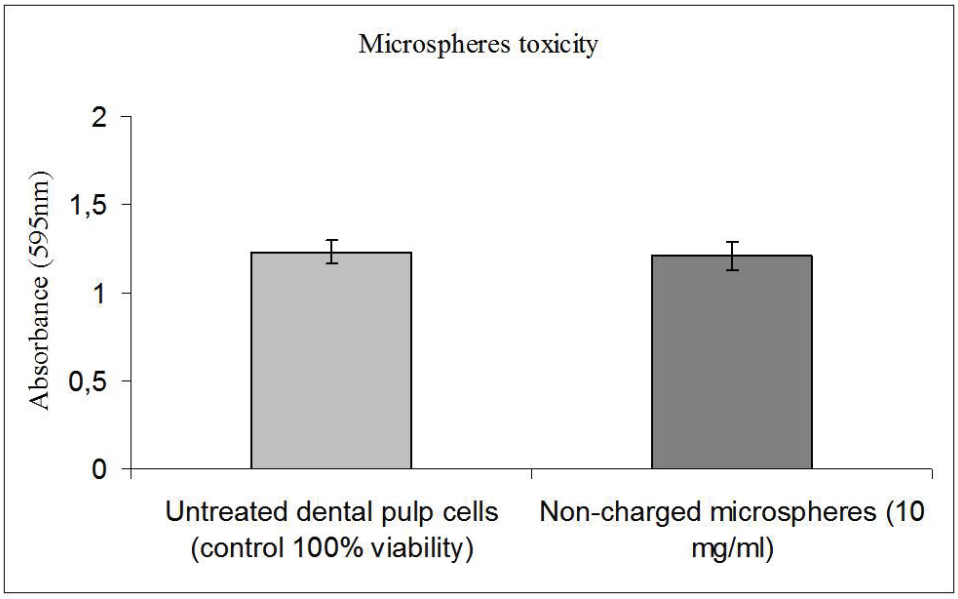

Figure 4: Toxicity of non charged microspheres. The toxicity was investigated with the MTT test on cultured human pulp fibroblasts. No toxicity of the non-charged microspheres was observed and the cell viability in contact with non charged microspheres was 98.37% (calculated as percentage of control cells.

In order to investigate the effect of growth factor-loaded microspheres on the proliferation of dental pulp cells, two factors were evaluated: microspheres concentration (growth factor dose effect) and growth factors release rate during 6 days. The results show that the specific biological activity of each growth factor was maintained after the encapsulation in the microspheres.

Increasing FGF-2-charged microspheres significantly increased ($p<0.05$) the dental pulp cells proliferation rate after incubation for 1day ($p<0.05$) for all FGF-2 concentrations (except for 10mg/ml $p>0.05$) in a dose dependent manner with the highest increase (133%) obtained with 30mg/mL FGF-2 charged microspheres. Increasing TGFß1-charged microspheres concentrations slightly decreased dental pulp cell proliferation rate. The decrease results was not significant as compared to the control cultures ($p>0.05$) (figure 5).

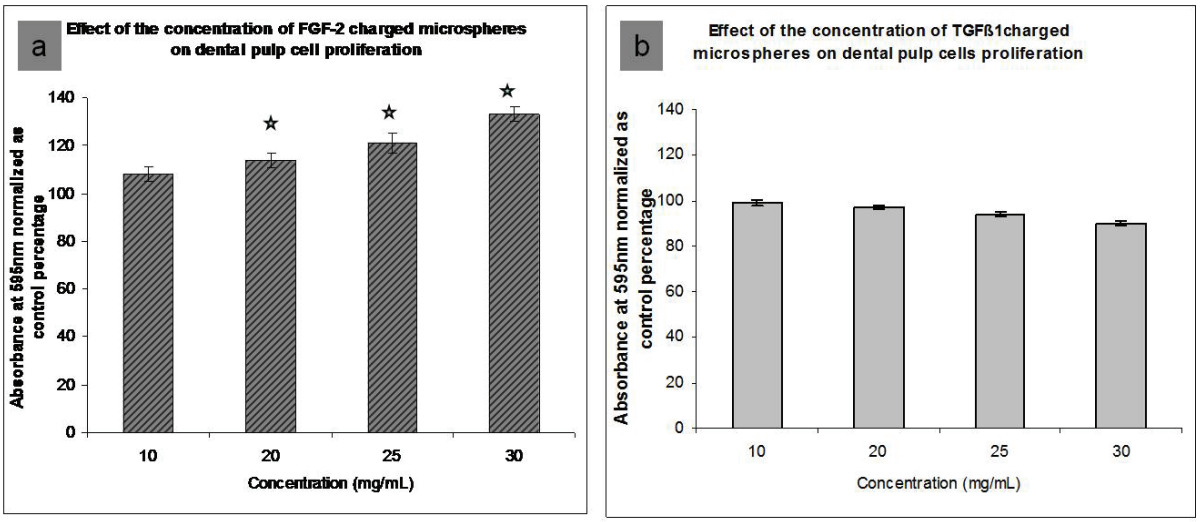

Figure 5: Microspheres growth factors «loading» effect on cell proliferation. Microspheres loaded with increasing growth factor concentrations were incubated with 2 mL culture medium for 24 hours. The obtained media were added onto cultured human pulp fibroblasts for 48 hours to investigate the cell proliferation with the MTT test. (a) Increasing the concentration of FGF-2-loaded microspheres significantly increased the dental pulp cells proliferation ($P<0.05$). (b) Increasing the concentration of TGFß1-loaded microspheres slightly decreased dental pulp cell proliferation. Stars refer to the significant results when compared with non charged microspheres.

Similarly, the sustained FGF-2 release rate significantly increased the dental pulp cell proliferation ($p<0.05$) after 3 (113% cell viability) and 6 days (123% cell viability) while that of TGFß1 slightly decreased the dental pulp cell proliferation ($p>0.05$) (Figure 6).

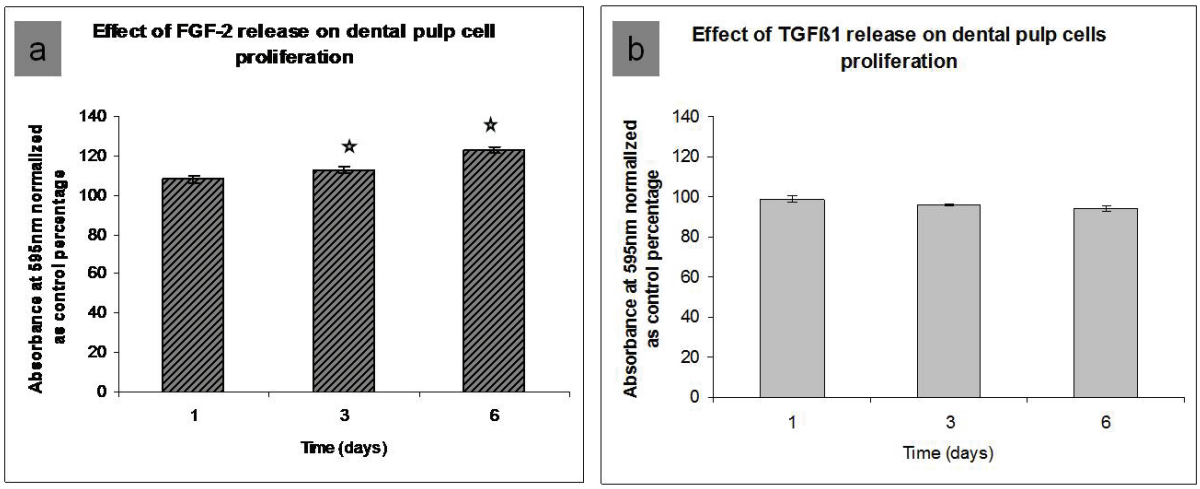

Figure 6: Effect of growth factors sustained released on the cell proliferation. Microspheres loaded with the same growth factor concentration (10mg/mL) were incubated with the culture medium for different periods (1, 3 and 6 days). The resulting media were incubated with human pulp fibroblasts for 48 hours and cell proliferation was investigated with the MTT test. (a) The sustained FGF-2 release increased the dental pulp cell proliferation significantly and in function of the release period ($P<0.05$). (b) The sustained TGFß1 release slightly decreased the dental pulp cell proliferation. Stars refer to the significant results when compared with non charged microspheres.

Discussion

The main result of this work is the elaboration of a growth factor biocompatible delivery system, its successful use for encapsulating FGF-2 and TGFß1 and the demonstration that these encapsulated factors have a long shelf life and keep their biological effects after proper storage conditions. The release kinetics of the encapsulated growth factors suggests that the delivery system represents a suitable sustained release carrier in the study of dentin regeneration.

In this study, the encapsulation processes was carried out by avoiding high temperature, pressure and prolonged contact with organic solvents during microspheres preparation. A biodegradable polymer of lactide and glycolide (PLGA) approved by the FDA was used as a carrier to form microspheres matrix (Jain *et al* 2000). The produced PLGA microspheres were used as colloidal carriers to encapsulate TGFß1 and FGF-2 for the controlled release of these growth factors for potential use in dentine regeneration and for future investigations of the early steps of this process particularly, the activation and migration of progenitor cells.

The produced microspheres were smooth and regular with a large size distribution (2-25µm) and could be dried and stored. The encapsulation efficiency immediately after preparation and after a 21 days storage period, measured by two complementary methods, showed that the growth factors were stable upon storage. While a slight decrease in the stability was observed at room temperature, ELISA test didn't reveal any significant differences in the growth factors stability when the microspheres were stored at 4°C. Indeed, the prepared microspheres protected the encapsulated growth factors for up to 82% at room temperature and up to more than 95% at 4° during the investigated period of time. These data suggest clearly that after re suspension in aqueous solutions, the encapsulated growth factors bioactivity is preserved. Thus, they can be used in the form of aggregated powder or colloidal suspension. Although we didn't investigate longer storage periods, it can be speculated from the preservation of more than 95% of the biological activity after 21 days that longer storage of encapsulated growth factors seems to be possible particularly at 4°C.

It was found important also to verify the quantities of solvents or formulation agents in the produced carriers since they may have toxic effects on the target tissues. It has to be pointed out that most of the organic solvents used to solubilise the polymer (chloroform, acetone, dichloromethane and acetic acid ….) are toxic even at very low concentrations. For example, Dichloromethane was found to be toxic and mutagenic after the administration of more than 10 ppm per day (Serota *et al* 1986). Some stabilising agents might also be toxic. In this study it was demonstrated by a calorimetric and chromatographic titrations that the quantities of these residuals were small and MTT test confirmed that they do not have cytotoxic effects.

Microspheres' «loading» was 2.9ng/mg for FGF-2 and 1ng/mg for TGFß1. The release kinetics show that the resulting quantity is 29-58 ng for FGF-2 and 10-20 ng for TGFß1 from 10-20 mg of microspheres. This value is in the range of FGF-2 application used for bone healing (10-100 ng) (Kimoto *et al*. 1998). A similar application reported FGF-2 incorporation into gelatine hydrogel microspheres in the same range (Kikuchi *et al*, 2007). TGFß1 was usually applied in the range of (1-40 ng) (Peter *et al*. 2000, Jaklenec *et al*. 2008, and DeFail *et al*. 2006). Based on these findings, the «loading» and release profils seem to be appropriate for the use of microspheres in local applications with 10-20 mg of microspheres.

It has been reported that tertiary dentinogenesis can be produced in 2 weeks (Tziafas 2004). Mioli *et al* (2006) studied the release of TGFß 3 from PLGA microspheres. They reported 10-30% release over a 40 days period. Lu *et al* (2000) reported also 10-20% release of TGFß1

from PLGA microspheres during 30 days. This release rate is rather low compared to our results and the release period is too long for dentin regeneration study.

The ratio of PLA/PGA used in our work was 50:50. This allowed a rapid overall release of 45% of the encapsulated FGF-2 and 65% of the encapsulated TGFß1 in 15 days. This PLA/PGA ratio effect on growth factors release is in agreement with another study upon investigating TGFß3 encapsulation and release rate where increasing PGA ratio from 25 to 50% in the polymer showed a significant acceleration in the release kinetics (Moioli et al, 2006). This ensures suitable growth factor quantities release for the aimed final dentin regeneration application.

Finally, the released FGF-2 and TGFß1 biological activity was examined by investigating dental pulp cells proliferation. Increasing the FGF-2 loaded microspheres quantity or extending their incubation time with the culture medium increased the cell proliferation rate in a dose and releasing period dependent manner. This statistically significant effect on cell proliferation at each FGF-2 concentration and at each release period is in agreement with previously published data on cultured rat incisor dental pulp cells proliferation. Indeed, while investigating the effect of different cytokines on dental pulp cells proliferation, FGF-2 even at 10ng/ml was shown to significantly induce cell proliferation (Nakao et al 2004). TGFß1-encapsulated microspheres quantity or extending their incubation time with the culture medium slightly decreased the dental pulp cells proliferation (non significant difference $p>0.05$). This could be related to the role of TGFß1 as a pulp cells differentiation factor. When mice dental papillae were cultured in agar containing TGFß1, an increase in the production of extracellular matrix was observed. Cells expressed collagen type I and fibronectin and the dental papillae cusp morphology was maintained (Bègue-Kirn et al 1992). Similarly, investigating the effect of TGFß1 on human dental pulp repair on cultured human tooth slices showed an upregulation of dentin matrix secretion and an induction of odontoblast-like cell differentiation with subsequent secretion of regular tubular dentin matrix on cut pulpal surfaces (Dobie et al .2002).

Taken together, these data clearly showed that the biological effects of the investigated growth factors are conserved.

Conclusion

The added value of this work is the demonstration that the microencapsulation allows reproducible microspheres characteristics, «loading» and release profil. Growth factors like FGF-2 and TGFß1 can be encapsulated in these PLGA microspheres and can also be released

in a controlled manner. The control of the microparticles fabrication parameters could allow the release kinetics adaptation to the growth factors final application. Investigating the microspheres cellular effects showed that they are non toxic and studying the released growth factors effects on dental pulp cell proliferation demonstrated that the growth factors were protected in the microspheres and their specific biological activity on dental pulp cells was conserved.

Based on theses findings, the produced microspheres seem to be suitable carriers for growth factor controlled release in the studies of dental pulp tissue regeneration. *Ex-vivo* studies of loaded microcapsules application on a model of entire human tooth culture are actually in progress.

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Travaux expérimentaux

Chapitre 5

Evaluation des effets des microparticules chargés en FGF-2 et du TGF-ß1 sur la régénération du complexe pulpodentinaire en utilisant un modèle de culture de dents entières *ex-vivo*

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1. Introduction

L'évaluation biologique des matériaux dentaire passe par plusieurs étapes incontournables. Elle commence par des tests de cytotoxicité sur culture cellulaire *in vitro*, ensuite des études *in vivo* sont réalisées chez l'animal puis elle se termine par des essais cliniques.

La Loi Huriet de 1988 modifiée en 2000 et 2004 impose un cadre législatif très strict pour la recherche biomédicale dans le but de réglementer les essais clinique et protéger ainsi l'être humain qui se prête à ce type d'expérimentation. La tendance actuelle est donc de privilégier les études sur des modèles expérimentaux *in vitro* ou *ex vivo* qui se rapprochent le mieux possible des conditions cliniques.

C'est dans ce but que l'équipe de recherche du laboratoire IMEB (faculté d'odontologie de Marseille) a développé depuis l'année 2008 un modèle de culture de dents entières qui permet de simuler des thérapeutiques dans des conditions *ex-vivo*. Ce modèle est utilisé dans cette partie de travail en collaboration avec l'équipe du professeur About à la faculté d'odontologie de Marseille. Nous utilisons donc ce modèle pour tester l'utilisation de nos microparticules en coiffage pulpaire direct et pour confirmer l'activité biologique des facteurs de croissance TGF β1 et FGF-2 *ex-vivo*.

Nous allons donc détailler ce modèle et aborder des études préliminaires réalisées sur celui-ci pour tester des microparticules chargées en facteurs de croissance.

2. Etudes préliminaires : l'utilisation des microparticules sur le modèle de culture de dents entières

Nous avons choisi comme première approche d'appliquer les microparticules sous la forme d'agrégats réhydratés. Pour éviter d'inclure les microparticules dans un ciment ou un gel, et afin d'évaluer les effets biologiques des microparticules sans aucune interférence, nous avons cherché à remplir totalement la cavité avec des microparticules.

Les obturations sont réalisées en plaçant les microparticules chargées de FGF-2, ou de TGF-ß1 directement sur l'effraction pulpaire de troisièmes molaires immatures afin d'évaluer après une période de 1, 3 ou 14 jours de culture, la prolifération cellulaire, la différentiation cellulaire et la régénération tissulaire en utilisant des colorations histologiques et des marquages immunohistochimiques.

3. Matériels et méthodes

3.1 Dents

Les dents utilisées pour l'expérimentation sont des troisièmes molaires extraites pour des raisons orthodontiques chez des adolescents. Toutes les avulsions ont été réalisées en bloc opératoire dans des conditions d'asepsie optimales (figure 5.1). Seules les dents immatures (racines constituées jusqu'au 2/3 de leur longueur environ, apex largement ouverts), indemnes de lésions carieuses ou iatrogène (lors de l'avulsion) sont conservées pour l'étude.

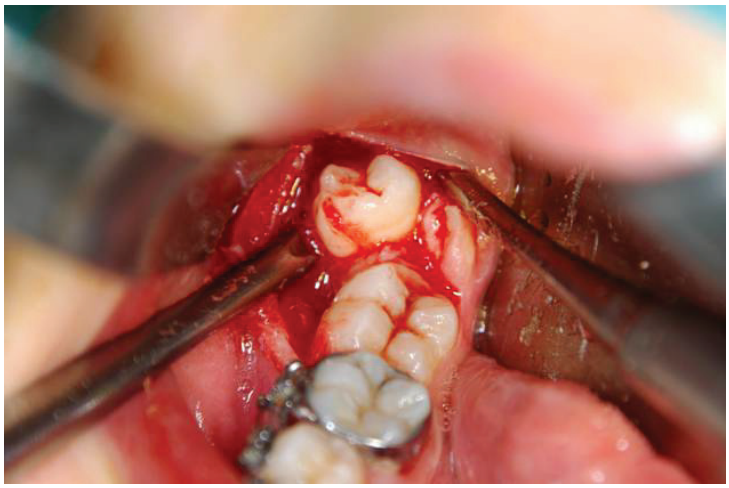

Figure 5.1 : Avulsion atraumatique du germe (3ème molaire mandibulaire) en position d'inclusion

Les dents fraîchement extraites sont conservées à 4°C dans un milieu de culture de stockage (MEM) additionné de pénicilline (300 UI/ml), de streptomycine (300 µg/ml) et d'amphotéricine B (0,75 µg/ml).

Au laboratoire, les dents sont nettoyées, débarrassées de leur débris et du sac péricoronaire à l'aide de curettes parodontales et de compresses stériles, puis disposées dans un nouveau « milieu de stockage » stérile.

Une cavité est ensuite réalisée sur chaque dent à l'aide d'une fraise diamanté tronconique (Réf. 878-2872.016, Maxima®) montée sur turbine sous irrigation constante au sérum physiologique, jusqu'à l'effraction pulpaire (minimale) (figure 5.2).

Figure 5.2 : Réalisation de la cavité jusqu'à l'effraction pulpaire *ex-vivo*

La cavité est alors rincée avec du sérum physiologique et séchée à l'aide de boulettes de coton stérile. Les microparticules sous forme de poudre (agrégats) sont réhydratées avec la solution de culture cellulaire, puis condensées délicatement à l'aide d'un fouloir afin de remplir la cavité (environ 20mg de particules).

3.2 Méthode de culture

Afin de favoriser les échanges entre le milieu de culture et l'organe pulpodentinaire, les dents sont immergées dans le liquide, en évitant tout contact direct entre le matériau et le milieu de culture.

Apres avoir bien séché la surface occlusale des dents, un fil d'acier stérile disposé sur la couronne dentaire est collé à l'aide d'un sealant (Embrace®, Pulpdent Co., USA) sur la surface occlusale pour permettre de « suspendre » les dents dans les puits contenant le milieu de culture (figure 5.3).

Ce dispositif facilite le changement quotidien des milieux de culture, il limite les risques de contaminations lors de ces manœuvres, et il permet de contrôler le niveau d'immersion de chaque dent dans son milieu (figure 5.3).

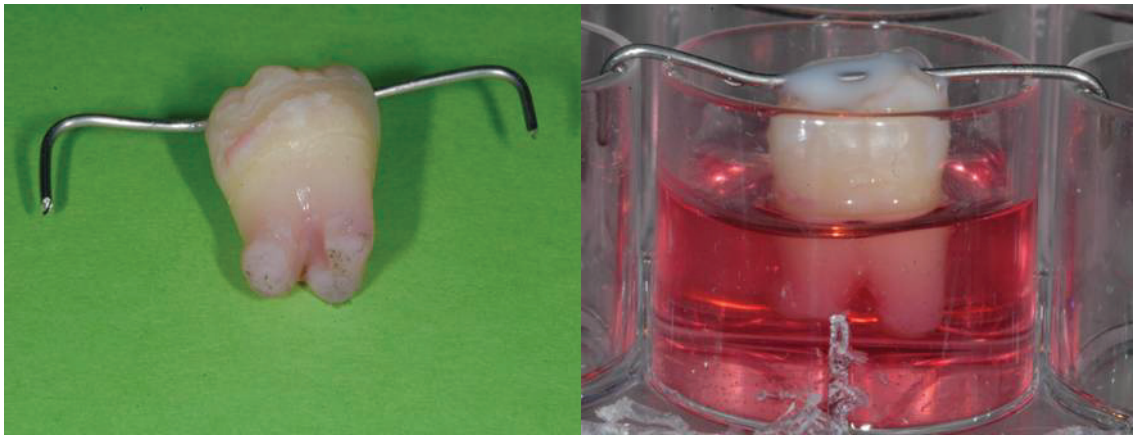

Figure 5.3 : Mise en place du dispositif de suspension, permettant d'immerger la dent dans le milieu de culture jusqu'au collet.

Les dents ainsi préparées, sont mise en culture dans des boites à 12 puits (Falcon®, Becton Dickinson, USA) (figure 5.4).

Figure 5.4 : Disposition des dents dans une boite de culture.

Le milieu de culture à 37°C est disposé dans les puits jusqu'au niveau du collet dentaire (jonction couronne-racine).

Les boites sont mises dans un incubateur à 37°C, en atmosphère humide enrichie en CO_2 (5%).

Le milieu de culture est renouvelé quotidiennement sous hotte à flux laminaire jusqu'au terme de l'expérimentation.

Des échantillons de chaque série seront prélevés du dispositif de culture, pour entrer dans la phase de traitement histologique, au bout de 1, 3 et 14 jours.

Trois groupes des microparticules sont utilisés pour obturer les dents : des microparticules chargées de FGF-2, des microparticules chargées de TGF-ß1 et des microparticules non chargées.

La répartition des dents et la distribution des échantillons sont présentés dans le tableau 1 :

	3j	14j
Microparticules non chargées	1	2
Microparticules chargés en FGF-2	3	5
Microparticules chargés en TGF-ß1	3	7

Tableau1 : Distribution des dents obturées avec chaque type de microparticules

3.3 Traitement histologique des dents avant les études histologiques

3.3.1 Fixation

Les dents sont immergées individuellement dans des tubes contenant du formol neutre à 4 % pendant 7 jours.

3.3.2 Déminéralisation

Les dents sont ensuite immergées dans une solution aqueuse composée de formiate de sodium à 3,4% et d'acide formique à 17%. Cette solution va dissoudre la partie minérale de la dent pour ne garder que la trame organique.

Le volume de déminéralisation est de 30 fois celui de la dent. Cette phase de décalcification dure 30 jours en moyenne à température ambiante, en renouvelant le liquide

déminéralisant chaque semaine. Les dents étant de tailles différentes, le contrôle de la déminéralisation est effectué radiologiquement.

3.3.3 Rinçage

Les pièces déminéralisées sont lavées à l'eau courante pendant 24 heures pour éliminer toute trace d'acide formique.

3.3.4 Déshydratation

La déshydratation des dents se fait par un passage dans des bains d'alcool de concentrations croissantes. Les dents sont immergées dans un bain d'alcool à 70% pendant 24 heures puis dans trois bains d'alcool à 95% de 4 heures chacun et enfin, dans un bain d'alcool à 100% pendant 12 heures.

L'éthanol n'étant pas miscible dans la paraffine, il est éliminé en plongeant les dents dans deux bains de 4 heures de xylène.

3.3.5 Imprégnation – Inclusion

L'imprégnation des dents par la paraffine permet d'obtenir une structure dent-paraffine homogène (duretés voisines). Trois bains de paraffine (point de fusion 56 à 58°C) de 12 heures chacun sont réalisés dans une étuve à 60°C.

La dent est ensuite mise en bloc dans un moule. Elle est disposée dans la paraffine avec une orientation bien déterminée afin d'obtenir des coupes selon le plus grand axe de la dent, permettant d'objectiver la cavité et la pulpe corono-radiculaire.

Des coupes sériées de 7µm d'épaisseur sont réalisées après un durcissement de 24 heures. Elles se font à l'aide d'un microtome type Minot Leitz 1512 (figure 5.5 photo A) à lame biseautée de type D (Leica® Microsystèmes, Rueil-Malmaison, France) avec un angle de 6° (figure 5.5, photo A). Les coupes sont déposées sur une lame parfaitement propre, silanisée (Superfrost plus®), à l'aide d'eau distillée. Elles sont étalées sur une plaque chauffante à gradient (figure 5.5, photo B). Les lames sont séchées dans un séchoir à lame pendant 20 minutes à 60°C (figure 5.5, photo C.D).

Figure 5.5 : A : microtome pour la réalisation de coupes, B : plaque chauffante pour l'étalement sur les lames, C et D : séchoir à lame pour la fixation des coupes

3.3.6 Colorations histologiques

Deux types de colorations ont été utilisés : l'Hématoxyline-Eosine et le Trichrome de Masson. Les coupes déshydratées doivent au préalable être déparaffinées et réhydratées car les colorants utilisés sont hydrophiles.

Le déparaffinage est réalisé en immergeant les lames dans des bains successifs d'histolemon et d'alcool : 3 bains d'histolemon de 5 minutes chacun puis 3 bains d'alcool à 95% de 5 minutes chacun.

Les coupes sont ensuite réhydratées dans de l'eau distillée pendant 5 minutes.

Hématoxyline Eosine :

Hématoxyline de Harris, 1 minute (coloration nucléaire)

Rinçage à l'eau courante, 5 minutes

Eosine à 1 %, 3 minutes (coloration de fond)

Rinçage à l'eau courante pour éliminer les excès d'éosine.

Trichrome de Masson :

Hématoxyline de Groat, 6 minutes (coloration nucléaire)

Rinçage à l'eau courante, 5 minutes

Ponceau fuschine, 4 minutes (coloration cytoplasmique)

Rinçage à l'eau courante et à l'eau acétique à 1 %, 5 minutes

Acide phosphomolybdique, 10 minutes (décoloration sélective des fibres de collagène colorées en rouge par le Ponceau)

Rinçage à l'eau courante et à l'eau acétique à 1 %, 5 minutes

Vert lumière 0,5 %, 6 minutes (coloration des fibres de collagène en vert)

Rinçage à l'eau acétique à 1 %.

Après la phase de coloration proprement dite, les lames sont déshydratées par des passages successifs de quelques secondes dans des bains d'alcool de concentrations croissantes : un bain d'alcool à 70%, 95% et trois bains à 100%. Enfin, les lames sont immergées pendant quelques secondes dans 2 bains de xylène.

Les préparations sont protégées par une lamelle couvre-objet avec un milieu de montage interposé (Lemonvitrex®, Laboratoires Carlo-Erba). (Figure 5.6)

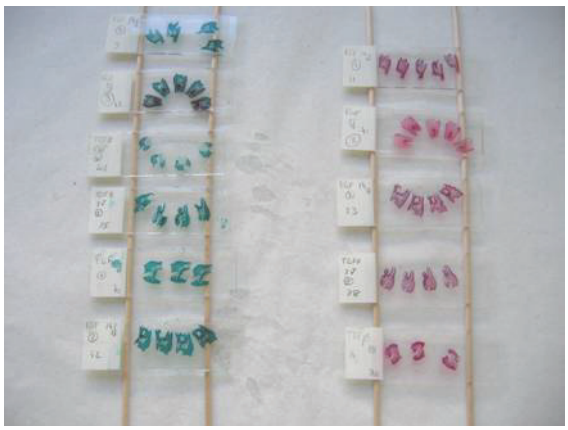

Figure 5.6 : Les coupes histologiques après le montage final

3.3.7 Immunohistochimie

Réactifs et anticorps

Réactifs :

- Histolemon
- Alcool
- PBS (avec Ca et Mg)
- H_2O_2 (à 30%)

Un kit de révélation LSAB2 (Dako Corporation, Carpinteria, CA, EU) a été utilisé pour le marquage immunohistochimique.

Anticorps :

- des anticorps monoclonaux contre le BrdU (Sigma Chemicals Co. St. Louis, MO)
- des anticorps monoclonaux contre la Nestine (Chemicon International Inc., CA, EU).
- des anticorps monoclonaux contre Le FGF-2 (R&D system, France)
- des anticorps polyclonaux contre la VEGF (R&D system, France)

Les marquages immunohistochimiques sont réalisés sur des coupes successives. Les coupes sont déparaffinées par immersion dans 3 bains d'histolemon de 5 minutes chacun. Elles sont réhydratées dans des bains d'alcool successifs : 1 bain d'alcool à 100% pendant 5 minutes, 1 bain d'alcool à 95% pendant 5 minutes et enfin 1 bain d'alcool à 70% pendant 5 minutes. La réhydratation des lames se termine par leur immersion dans de l'eau distillée pendant 5 minutes.

Après réhydratation, les coupes sont incubées dans un tampon phosphate (PBS) à pH 7,4. L'immunomarquage est réalisé dans une chambre humide.

Le blocage de l'activité de la péroxydase endogène est fait dans l'eau oxygénée à 3 % pendant 10 minutes. Les anticorps primaires ont été dilués avec du PBS contenant de l'albumine de sérum bovin (BSA) à 0,1%.

Après saturation dans une solution d'albumine à 0,5% pendant 45 minutes et après rinçage au PBS, les coupes sont incubées avec les anticorps primaires pendant une nuit à 4°C aux concentrations suivantes :

- Anti Nestine 1/200 : dans BSA 0,1%
- Anti BrdU 1/500 : dans BSA 0,1%

Après incubation avec les anticorps primaires et rinçage au PBS, les lames sont incubées avec les anticorps secondaires biotinylés pendant 30 minutes. Ensuite, elles sont rincées et incubées en présence de la streptavidine péroxydase pendant 30 minutes. La péroxydase est révélée par incubation avec le substrat 3,3' diaminobenzidine pendant 10 minutes.

Après rinçage à l'eau distillée, les lames sont montées en utilisant un milieu de montage aqueux, le Glycergel® (Dako Corporation, Carpinteria, CA, EU). Les contrôles sont réalisés par incubation sans anticorps primaire.

4. Résultats et discussion

4.1 Observation des coupes histologiques (effets au niveau tissulaire)

L'examen des coupes colorées à l'hématoxyline éosine et au Trichrome de Masson, révèle des structures tissulaires bien conservées jusqu'au terme de l'expérimentation (14 jours de culture *in vitro*).

La structure tubulaire de la dentine et la zone de prédentine sous jacente sont bien visibles. La couche odontoblastique est bien individualisée. Parfois, les odontoblastes paraissent décollés de la dentine, il s'agit là d'un artéfact lié à la technique de fixation des tissus comportant des zones minéralisées et non minéralisées (Mjör, 1983). Dans la plupart des dents observées, la pulpe semble normale, le tissus conjonctif est homogène, l'organisation et la répartition des éléments cellulaires, fibreux et vasculaires sont identiques à ceux rencontrés *in vivo* dans une pulpe « jeune » de dent immature. Une vasodilatation est fréquemment observée, mais semble peu spécifique.

La mise en évidence de la bonne conservation du tissu pulpaire étant faite, nous avons focalisé notre attention sur la région de la pulpe située à proximité de la cavité contenant les microparticules.

Pour les dents obturées par les microparticules chargées en FGF-2 et les microparticules chargées en TGFß1, aucune réaction pulpaire particulière n'a été observée en dehors d'une rétraction de la pulpe exposée. Aucune néoformation minéralisée n'a été observée avec ce matériau au cours du temps de cette étude (14 jours). Figure 5.7

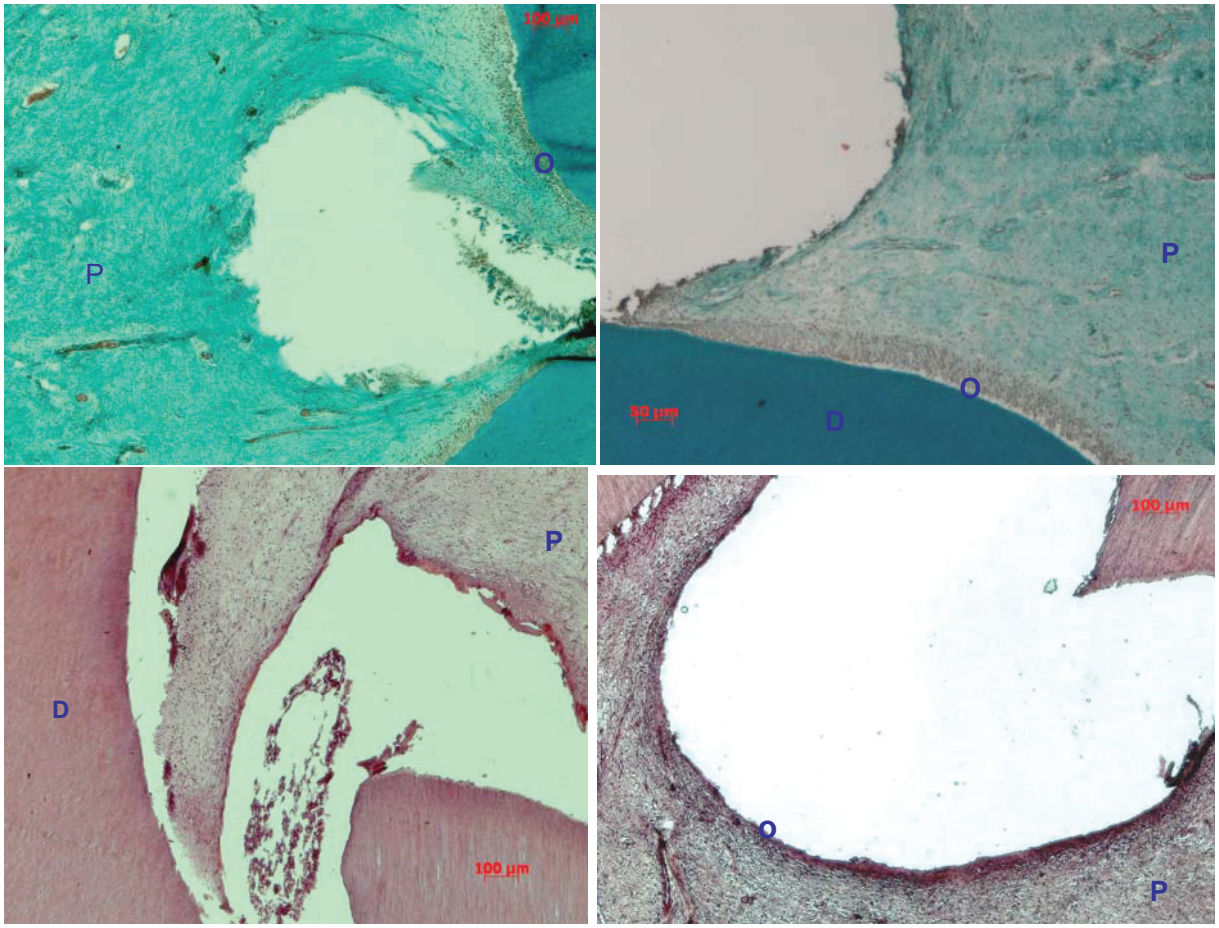

Figure 5.7 : les structures histologiques dentaires conservées avec la récession et aucune trace de minéralisation 14 jours après le coiffage. D : Dentine, O : Odontoblastes, P : Conjonctive pulpaire.

4.2 Observation après marquage immunhistochimique (effets au niveau moléculaire)

Suite à nos résultats histologiques préliminaires qui révèlent l'absence de foyers de minéralisation, les marquages immunohistochimiques ont été réalisés pour évaluer l'expression du BrdU, de Nestine, et de VEGF afin de mieux comprendre l'effet de microparticules sur les étapes précoces de la cicatrisation (avant la minéralisation).

Le bromodeoxyuridine (BrdU) est un analogue de la thymidine. Il est intégré dans l'ADN des cellules en division au cours de la synthèse de l'ADN et peut être détectée par immunocytochimie *in vitro* et *in vivo*, permettant d'identifier des cellules en division.

La Nestine est une protéine des filaments intermédiaires exprimée de façon prédominante dans les systèmes nerveux et musculaire en cours de développement (Lendahl et al., 1990). Dans les dents immatures, cette protéine est exprimée dans le corps cellulaire et dans le prolongement odontoblastique. Dans les dents adultes, matures, aucune expression de la Nestine n'est révélée. Une étude *in vivo* a montré une ré-expression de la Nestine à proximité de la dentine réparatrice formée, neuf semaines après la réalisation d'une cavité pulpaire coiffée avec de l'hydroxyde de calcium (About et al., 2000).

L'expression de cette protéine est donc considérée comme un marqueur spécifique des odontoblastes fonctionnels, en cours de différenciation et présentant une activité sécrétoire.

Le VEGF est considéré comme un facteur essentiel pour la différenciation du système vasculaire. L'expression de ce facteur de croissance pourrait permettre d'identifier une stimulation de l'angiogenèse ou de la néovascularisation.

Nous avons observé des zones de marquage de BrdU dans la région à coté de la cavité contenant les microparticules chargées en FGF-2 (figure 5.8 photos, A, C) et celles chargées en TGFß1 (figure 5.8 photos, B, D). Le marquage de BrdU permet d'identifier des cellules en phase de prolifération (Magavi and Macklis, 2008). Cette prolifération correspond à l'activation des cellules progénitrices cette région après la libération des facteurs de croissance.

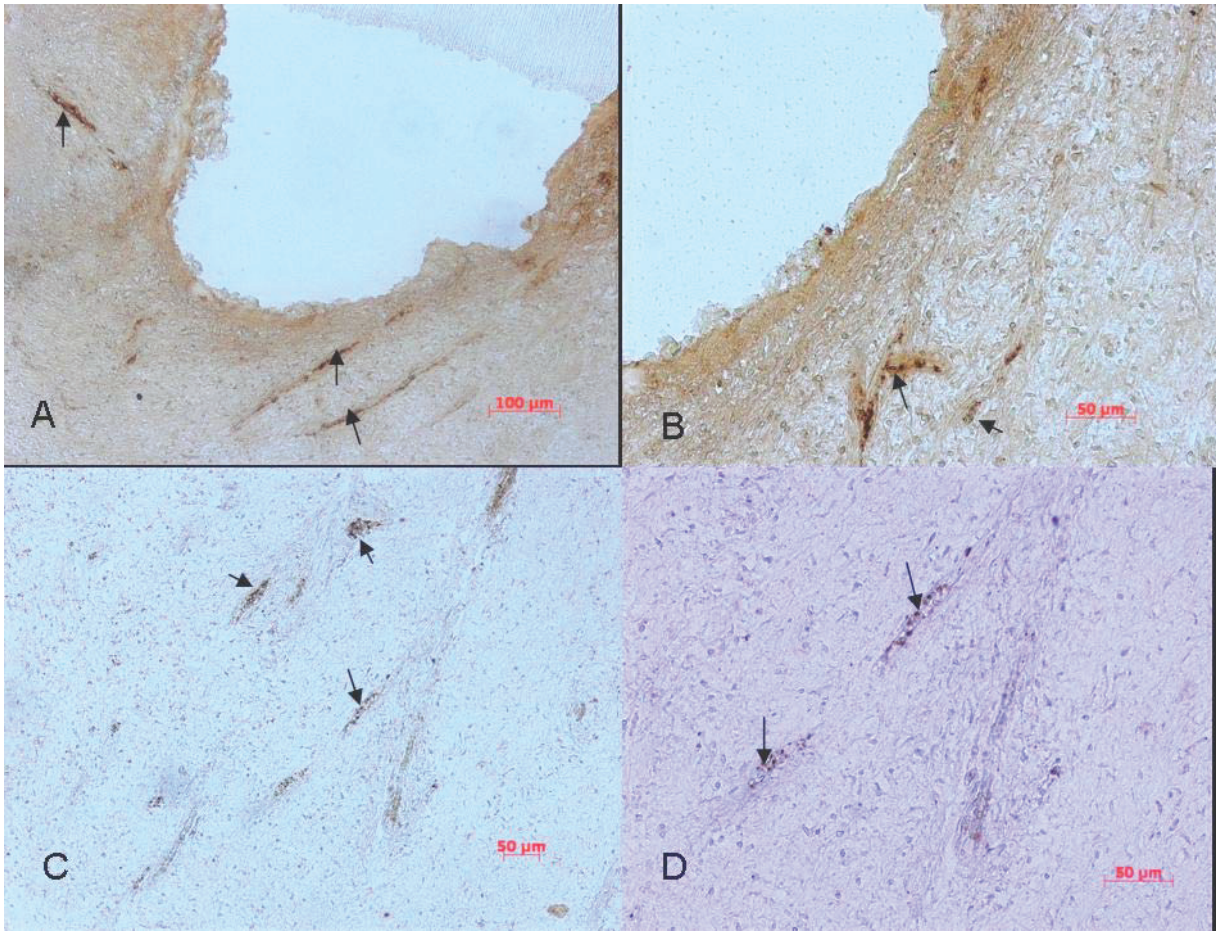

Figure 5.8 Marquages immunohistochimiques avec l'anticorps anti-BrdU (flèches), photo A, C suite à l'application des microparticules chargées en FGF-2, photo B, D suite à l'utilisation des microparticules chargées en TGFß1

L'étude préliminaire du marquage de la Nestine et le VEGF n'a pas donné des résultats significatifs. De nouvelles expérimentations de marquage sont en cours de préparation.

Dans cette étude préliminaire, l'absence de réaction de minéralisation sur les coupes nous a incités à réévaluer notre protocole d'utilisation des microparticules. Les raisons possibles de cette absence de minéralisation sont les suivantes :

- quantité de facteurs de croissance libérés insuffisante.

- étanchéité du coiffage pulpaire insuffisante : en l'absence de ciment, les agrégats de microparticules réhydratées pourrait se déshydrater et avoir une libération limitée des facteurs de croissance.

Le protocole d'utilisation des microparticules a donc été modifié. Une nouvelle série d'échantillons a été préparée en prenant en considération toutes les remarques précédentes. Les modifications du protocole sont :

1. Le *«loading»* des microparticules en facteur de croissance sera doublé
2. Le temps d'utilisation est augmenté à 21 jours
3. Nous avons prévu l'utilisation des microparticules en technique sandwich qui consiste à appliquer des microparticules dans la cavité en contact avec le tissu pulpaire et ensuite à assurer l'étanchéité de la cavité par une fine couche de ciment comme du MTA ou un substitut dentinaire (Biodentin®).

Les expérimentations en utilisant le protocole modifié sur le modèle de dent sont en cours de préparation.

5. Conclusion

Ces études préliminaires nous ont aidés d'abord à nous familiariser avec les techniques de culture cellulaire sur ce modèle original ainsi que les méthodes de préparation et coloration des coupes histologiques et immunhistochimiques.

L'intérêt important de cette étude réside dans les possibilités qu'offre ce modèle de culture de dent entière pour évaluer les biomatériaux de coiffage pulpaire direct, dans des conditions proches des conditions *in vivo*.

Le modèle de culture de dents entières développé dans le laboratoire IMEB permettra donc d'explorer les étapes précoces de la régénération dentinaire.

Il permettra aussi d'étudier la régénération dentinaire lors des coiffages directs, et la prédiction du comportement de nouveaux biomatériaux comme les microparticules colloïdales chargées en facteurs de croissance dans une situation proche de la situation clinique.

Discussion générale

Discussion générale

Après une effraction pulpaire d'origine (bactérienne, physique ou chimique), l'utilisation des matériaux de coiffage pulpaire ($CA(OH)_2$, MTA, …) est souvent le choix de traitement pour favoriser la régénération dentinaire. Ces matériaux ne sont pas en mesure de former de vrais nouveaux tissus. L'ingénierie tissulaire peut offrir une solution pour ce problème. Certains facteurs de croissance (VEGF, PDGF, FGF-2 et TGF β1) sont séquestrés dans la dentine et peuvent être libérés lors des lésions carieuses. Il a été démontré récemment qu'ils peuvent également être libérés par les fibroblastes pulpaires lésés (Tran-Hung et al 2008). L'utilisation exogène de ces facteurs de croissance sur la pulpe exposée *in vitro* ou *in vivo* a démontré leur potentiel dans la signalisation de réparation dentinaire (Sloan et Smith 1999, Unda et al 2001). Un système de libération (comme des microparticules biodégradables) est indispensable pour assurer l'efficacité clinique et la régénération tissulaire au site d'utilisation.

L'objectif essentiel de cette thèse est l'élaboration d'un système biocompatible pour délivrer les facteurs de croissance FGF-2 ou TGF-β1 et pour assurer leur protection et leur libération prolongée au contact de la pulpe dentaire pour favoriser la régénération dentinaire.

Les systèmes de délivrance de molécules bioactives ont été longtemps étudiés. Dans la littérature, trois méthodes de préparation sont rapportées : Les méthodes chimiques, physiques et physico-chimiques.

Les méthodes chimiques comme la polymérisation et la polycondensation ont été utilisés pour fabriquer des microparticules polymérique (Arshady et al 1990, Chen et al 2007). Bien que ces processus puissent assurer une efficacité d'encapsulation élevée, les produits chimiques et les réactions exothermiques peuvent provoquer la dégradation des facteurs de croissance et la perte de leur activité biologique.

Les méthodes physiques sont généralement utilisées pour véhiculer des molécules bioactives en raison de leur simplicité. Avec cette approche, il n'y a pas de réactions chimiques, il n'y a que des interactions physiques entre les facteurs de croissance et leur véhicule. L'incorporation des facteurs de croissance se fait généralement en imprégnant des

billes d'agarose (Sloan et Smith 1999), des éponges de collagène (Six et al 2002), des hydrogels (Kikushi et al 2007) ou des microparticules polymériques (dextran ou du chitosane) (Chen et al. 2007; Niu et al. 2008) avec une solution de facteurs de croissance. Dans ces dernières études, les effets des facteurs ont été étudiés sans aucune quantification de la charge*«loading»* ou le profil de libération.

La délivrance des facteurs de croissance par des méthodes physique a fourni des informations précieuses sur la capacité dentinogenic des cellules souches de la pulpe dentaire, mais elle a seulement permis l'investigation sur l'effet des facteurs de croissance à court terme (Sloan et Smith 1999; Six et al, 2002). Cependant, la libération prolongée des facteurs de croissance apparaît indispensable pour les études à long terme de la réparation et la régénération de complexe pulpo-dentinairee et fournit une meilleure simulation de la situation *in vivo* où les facteurs de croissance sont libérés au cours de l'ensemble du processus de réparation dentinaire.

L'avantage des méthodes physico-chimiques pour la préparation de microparticules de polymères biodégradables est la possibilité de caractériser les microparticules par des mesures de la taille, de la morphologie, du *«loading»* et du profil de libération. La maitrise de ces caractéristiques permet une production reproductible des microparticules.

Le travail expérimental de cette thèse nous a permis d'optimiser une méthode physico-chimique de fabrication de microparticules encapsulant des protéines (facteurs de croissance). Cette méthode est basée sur la réalisation d'une émulsion multiple W/O/W suivie de l'extraction du solvant. Dans ce travail, le procédé d'encapsulation a été réalisé en évitant la température élevée, la pression et le contact prolongé avec des solvants organiques au cours de la préparation des microparticules. Un polymère biodégradable, le PLGA approuvé par la FDA a été utilisé comme matrice pour former des microparticules (Jain et al 2000).

L'optimisation du procédé d'encapsulation consiste en l'étude de l'influence des différents paramètres de formulation (la vitesse d'agitation mécanique, les concentrations et les rapports de concentration des réactifs utilisés) sur les caractéristiques des microparticules. Les résultats montrent que plus que la vitesse d'agitation pendant la deuxième émulsification est élevée, plus que la taille des particules est petite. De même, plus que la concentration du stabilisant dans la phase aqueuse externe est grande, plus que la taille des particules est petite.

Cependant, la diminution du rapport principe actif-eau/polymère conduit à une structure interne moins poreuse.

La modélisation mathématique montre que les paramètres de formulation qui diminuent la taille des particules (vitesse d'agitation et concentration de PVA) sont supposés augmenter la vitesse de libération. L'augmentation de ratio eau/polymère dans l'émulsion première augmente la porosité interne des microparticules et augmente ainsi la vitesse de libération.

La production de petites microparticules (5-15µm) représente plusieurs avantages. Tout d'abord, l'efficacité d'encapsulation est plus élevée dans les petites microparticules. Deuxièmement, la stabilité de FGF-2 dans les petites microparticules est un peu plus élevée que dans les grandes microparticules. Troisièmement, le poids moléculaire du polymère des microparticules en suspension dans une solution aqueuse diminue moins rapidement dans les petites microparticules même si leur rapport surface/masse est plus grand (la dégradation est plus lente). Quatrièmement, le coefficient de diffusion est plus faible dans les petites microparticules.

Les explications de ces observations peuvent être les suivantes: au cours du procédé de formulation et pendant l'étape de la solidification, pour les petites microparticules, la précipitation de polymère se fait plus rapidement conduisant à la protection d'un ratio plus élevé du facteur de croissance. Il semble également qu'une matrice de polymère plus compacte est formée si les précipitations de polymère est rapide ce qui empêche la formation de grosses cavités (pores interne). De cette manière, le coefficient de diffusion des protéines à travers les microparticules est plus faible dans les petites microparticules. La diffusion de l'eau dans les microparticules devrait également être plus lente et donc la dégradation du polymère est plus lente dans les petites microparticules. Enfin, la stabilité de la protéine dans les petites microparticules est renforcée en raison de la matrice compacte.

Les microparticules pourraient être séchées et stockées sous forme de poudre et redispersées dans l'eau avant leur utilisation. Les caractéristiques physico-chimiques des microparticules avant et après séchage et redispersion dans l'eau ont été étudiées. Une étude de la stabilité colloïdale a été effectuée en fonction du pH et de la salinité. Nous pouvons déduire que la stabilité colloïdale des microparticules est essentiellement gouvernée par

l'interaction électrostatique répulsive due à la présence de groupes carboxyliques dans le PLGA. La quantité utilisée de PVA est faible mais suffisante pour assurer la stabilité de microparticules avant et après le processus de séchage. Le procédé de séchage n'a donc aucun effet sur les propriétés de surface et la stabilité colloïdale des microparticules préparées.

Le taux d'encapsulation « *loading* » a été mesuré après la préparation des microparticules et après 21 jours de leur stockage sous forme de poudre, ces mesures ont montré que les facteurs de croissance sont stables pendant cette période de stockage. Si une légère diminution de la stabilité a été observée à la température ambiante, le test ELISA n'a pas révélé une différence significative lorsque les microparticules ont été conservées à 4°C. En effet, 82% des facteurs de croissance encapsulés ont été conservés à température ambiante et jusqu'à plus de 95% à 4°C. Ces données suggèrent que la matrice polymérique protège les facteurs de croissance encapsulés pendant leur stockage jusqu'à leur resuspension dans l'eau pour leur utilisation. Même si nous n'avons pas prolongé les mesures sur des périodes de stockage plus longue, on peut supposer de la préservation de plus de 95% après 21 jours que le stockage de facteurs de croissance encapsulé semble être possible en particulier à 4° C. cela constitue une avantage car les lyophilisats de facteur de croissance se conservent à -20° C.

Il est aussi très important de vérifier les quantités des résidus des solvants organiques ou des agents chimiques utilisés pendant la formulation au sein des microparticules car ils peuvent avoir des effets toxiques sur les tissus cibles. Il doit être souligné que la plupart des solvants organiques utilisés pour dissoudre les polymères (chloroforme, l'acétone, le dichlorométhane et d'acide acétique ....) sont toxiques même à très faibles concentrations. Par exemple, le dichlorométhane est toxique et mutagène après l'administration de plus de 600 ppm (Serota et al 1986). Certains agents de stabilisation pourraient aussi être toxiques. Dans cette étude, dans les microparticules produites, la concentration de DCM était d'environ 3 ppm par mg de microparticules. Cela permet l'administration de plusieurs milligrammes de microparticules sans aucun risque. La concentration de PVA était environ 0,5% en poids. Cette concentration est largement inférieure à la quantité toxique (15– 20 g/kg) (DeMerlis et al, 2003).

Le *«loading»* de microparticules est 2,9 ng/mg pour FGF-2 et 1 ng/mg pour TGFß1. cela signifie que la quantité maximum encapsulée dans 10 à 20 mg de microparticules est de 29 à 58 ng pour FGF-2 et 10-20 ng pour TGFß1. Ces valeurs sont similaires aux valeurs de FGF-2

(10-100 ng) utilisées par Kimoto et al. 1998. Kikuchi et al, 2007 qui est du et celles de TGFß1 (1-40 ng) utilisées par (Peter et al. 2000, Jaklenec et al. 2008, et DeFail et al. 2006). Basé sur ces donnés, le *«loading»* des microparticules semble être approprié pour des utilisations locales avec une quantité de10-20 mg de microparticules.

Le profil de la libération est caractérisé par un effet *burst* dans les premières 24h où 30 à 45% de la quantité encapsulée est libérée, ensuite la libération tend à se ralentir conduisant à une libération de 45% du FGF-2 et 65% des TGFβ1 au bout de 15 jours. Ce profil de libération est adapté avec la durée de la réparation dentinaire qui peut être produits en 2 semaines (Tziafas et al 2004).

Mioli et al (2006) ont étudié la libération de TGFß-3 en utilisant des microparticules de PLGA. Leurs résultats montrent une libération de 10-30% sur une période de 40 jours. Lu et al (2000) ont fabriqué des microparticules chargées en TGFß1, ils ont trouvé une libération de 10-20% pendant 30 jours. Dans ces deux études, les quantités libérées de facteur de croissance sont faibles par rapport à nos résultats et la période de libération est trop longue pour étudier la régénération de la dentine.

Le ratio de PLA / PGA utilisé dans notre travail a été 50:50. Cela a permis d'avoir une libération rapide. Cet effet du rapport PLA / PGA sur la liberation des facteurs de croissance est en accord avec une autre étude sur l'encapsulation de TGFß-3 dans laquelle l'augmentation de taux de PGA de 25 à 50% a provoqué une accélération significative de la cinétique de libération (Moioli et al, 2006).

Une phase de libération rapide (effet *burst)* a eu lieu dans ce système, cette phase (d'environ 24h) pourrait être due à la présence de différentes tailles de pore dans la matrice des microparticules comme indiqué par SEM dans la partie expérimentale, chapitre 1. La libération pendant cette période a lieu exclusivement par diffusion à travers la matrice polymérique ou par le principe actif présent à la surface. L'effet des modifications de la morphologie des microparticules et de la dégradation du polymère est donc supposé négligeable au cours de la période de libération et n'est pas considéré dans le calcul du coefficient de diffusion des protéines.

L’étude de libération a été faite dans un milieu de culture qui est habituellement utilisé pour les tests *in vitro* sur des cultures cellulaires. Ce choix a été fait pour garder le même milieu de libération lors des études biologiques qui seront réalisées par la suite.

Enfin, l’activité biologique des facteurs de croissance (FGF-2 et TGFß1) libérés a été vérifiée en utilisant des tests de prolifération des fibroblastes pulpaires humains. L'augmentation de la quantité de microparticules chargées en FGF-2 ou la prolongation de leur temps d'incubation avec le milieu de culture augmente significativement ($p<0.05$) la prolifération cellulaire. Cet effet statistiquement significatif sur la prolifération cellulaire est en accord avec les données publiées antérieurement sur la prolifération des cellules de la pulpe dentaire de l'incisive de rat (Nakao et al 2004).

L'augmentation de la quantité de microparticules chargées en TGFß1 ou la prolongation de leur temps d'incubation avec le milieu de culture diminue légèrement le taux de prolifération cellulaire des cellules de la pulpe dentaire (différence non significative $p> 0,05$). Cela pourrait être lié au rôle de TGFß1 comme un facteur de différenciation des cellules de la pulpe dentaire (Bègue-Kirn et al 1992 et Dobie et al. 2002).

Les résultats des tests de la prolifération cellulaire montrent clairement que les activités biologiques spécifiques des facteurs de croissance encapsulés sont conservées

La question de fabriquer des nanoparticules a été abordé au début de ce travail, mais puisque l’utilisation locale dans une cavité dentaire ne nécessite pas de particules très petites et vu la difficulté de sécher les nanoparticules (passage obligatoire par plusieurs étapes de filtration ou centrifugation), nous avons privilégié le choix de fabriquer des petites microparticules.

Récemment, nous avons participé au travail de recherche au sein de notre laboratoire basé sur l’encapsulation des molécules bioactives hydrophiles pour le traitement des cancers. Ce travail utilise la méthode de l’émulsion multiple avec un protocole similaire du celui réalisé dans cette thèse pour fabriquer des nanoparticules. (Publication soumit dans *journal of biomédicale nanotechnology*) (Annexes).

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Conclusion générale et perspective

Conclusion générale et perspective

Le but de ce travail est d'élaborer des microparticules chargées en FGF-2 et du TGF-β1 pour assurer leur protection et leur libération prolongée et d'évaluer l'effet des ces microparticules sur l'ingénierie tissulaire de la pulpe dentaire *in vitro* et *ex vivo*

Ce travail utilise et relie deux disciplines très importantes dans le milieu de recherche biomédicale, pharmacotechnie et l'ingénierie tissulaire pour une application en odontologie conservatrice..

La méthode utilisée pour fabriquer les microparticules est basée sur la réalisation d'une émulsion multiple W/O/W suivie de l'extraction du solvant.

Ce travail montre que la microencapsulation permet de fabriquer des microparticules avec des caractéristiques reproductibles et maitrisées. Les facteurs de croissance comme le FGF-2 et TGFß1 peuvent être encapsulés dans ces microparticules et peuvent aussi être libérés de façon contrôlée. Le contrôle des paramètres de fabrication de microparticules pourrait permettre l'adaptation de la taille des microparticules (nanoparticule ou microparticules) et la cinétique de libération des facteurs de croissance (libération pendant plusieurs heures, jours ou mois) selon l'utilisation finale.

La fabrication de petites microparticules est recommandée pour fabriquer des microparticules avec une libération des facteurs de croissance plus lente et une stabilité facteurs de croissance plus élevée.

Le procédé de séchage est une étape indispensable pour éviter la libération pendant la période de stockage. Les résultats présentés dans ce travail montrent que ce précédé n'a aucun effet sur les propriétés de surface et la stabilité colloïdale des microparticules préparées.

Le «loading» de microparticules est suffisant et adapté à l'utilisation thérapeutique choisie. Les microparticules améliorent la stabilité des facteurs de croissance et assurent une libération contrôlée pendant 15 jours, période qui correspond à la période de la réparation dentinaire. Les microparticules obtenues ne sont pas toxiques en terme de teneur en résidus de

solvants ou de stabilisant. L'activité biologique du facteur de croissance encapsulé a été conservée et les microparticules sont biocompatibles.

Les microparticules chargées en FGF-2 et TGFß1 sont donc prometteuses dans les études des étapes précoces de la régénération dentinaire, l'activation et la migration des cellules progénitrices de la pulpe dentaire.

Ce sujet de recherche a ouvert la porte à des perspectives à court et à moyen terme :

A court terme :

L'utilisation de microparticules comme un matériau de coiffage dentaire direct afin de confirmer leur activité biologique *ex-vivo* est actuellement en cours réalisation. Un modèle de culture de dent entière humaine sera utilisé. Ce modèle est validé récemment par l'équipe du Professeur About de la faculté d'odontologie de Marseille et nous avons présenté une étude préliminaire sur ce modèle dans le cinquième chapitre de la partie expérimentale de cette thèse.

A moyen terme :

Les microparticules chargées en FGF-2 et TGFß1 peuvent être incluses au sein des systèmes d'ingénierie tissulaire destinés à régénérer des tissus ou des organes (scafold avec des cellules souches).

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Références hors publications

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ANNEXES

Nanoencapsulation of hydrophilic drug via double emulsion technique

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Abstract:

Preparation of biodegradable nanoparticles containing active molecule is now taking much attention of researchers. The aim of the present work is to achieve the nanosize particles using for the first time double emulsion ($W_1/O/W_2$) evaporation method to encapsulate hydrophilic substance using high performance stirring apparatus. A fluorescent stable hydrophilic agent (Stilbene derivative) was used as a model drug to be encapsulated. For this purpose, PCL (polycaprolactone) was chosen as polymer in this study. Several kinds of surfactants [triton-405, tween 80, poloxamer, PVP (polyvinylpyrrolidone), PEG (poly ethylene glycol) & PVA (poly vinyl alcohol)] were investigated and it was found that the PVA (0.5% concentration) showed most stable double emulsion with the particle size in nano range. Different parameters affecting the size of particles have been studied such as stirring time (for 1^{st} & 2^{nd} emulsion), speed of stirring (for 1^{st} & 2^{nd} emulsion), polymer and surfactant concentrations etc. After duration of one month, the encapsulation efficiency of particles prepared was estimated using U.V. analysis. Transmission Electron Microscopy (TEM) showed that the prepared particles were spherical in shape. The size and size distribution were found to be submicron and ranging from 150 to 400 nm.

Key words:

Double emulsion, hydrophilic substance, encapsulation, polymer concentration, nanoparticles.

Introduction:

In the pharmaceutical field, several advantages of drug delivery systems with nanosize range have been shown including increase solubility, enhancing dissolution rate and improving bioavailability. The main purpose of using nanotechnology in the pharmaceutical field is the design of nanosazied drug carrier systems to achieve adequate stability, improved absorption, controlled release, quantitative transfer and therefore the expected pharmacodynamic activity [1] but this may also leads to toxicological responses [2] which will not be discussed here because its beyond the study. An important requirement for the nanoparticles is biodegradability. Non-biodegradable matrixes are ideal for mechanistic study but not for therapeutic use [3]. Over the past few decades, there has been considerable interest in developing biodegradable micro and nanocapsules as

effective drug delivery device for targeting, tracking, imaging and sensing.[4,56].Various polymers have been used in drug delivery research, as they can effectively deliver the drug to a target site and thus increase the therapeutic benefit, while minimizing the side effects [7]. Biocompatible and biodegradable polymers such as poly (ε-caprolactone) (PCL) [8], poly (D, L-lactide-co-glycolides) (PLGA) [9,10] and Ammonio Methacrylate copolymers (Eudragits RL and RS) are widely used in medicines for the preparation of micro and nanoparticles. The nanospheres of biodegradable polymer are preferred because surgical removal of spent device, as in the case of implants, is not required. Further, there is minimal possibility of toxicological problems, their release rates can be tailored and they degrade in biological fluids to produce biocompatible or non-toxic products in the body, which are removed by normal physiological pathways. [11]

There are different pharmaceutical methods which are being utilized by researchers to encapsulate various types of drugs e.g. single emulsion process [12], double emulsion, organic phase separation, super critical fluids, spray drying techniques [13,14] miniemulsion polymerization [15], nanoprecipitation [ref], solvent diffusion [ref] etc but the choice of a technique is determined by the nature of the drug[16]. Colloidal drug carriers are interesting in the field of drug delivery systems because of their small size allowing them to permeate through biological barriers [17]. The basic purpose of all these techniques was to enhance bioavailability of poorly soluble drugs [18]. Among various colloidal drug delivery systems, nanoparticles (NP) potentially represent one of the most promising approaches to this goal [19] and the double emulsion is one of the methods to prepare these nanoparticles. Many authors [20,21] have previously shown that the double emulsion technique was the most appropriate method to encapsulate hydrophilic drugs and proteins within microparticles. This technique has been later applied to nanoparticles [22,23,24] by using sonication or ultra-turrax. However, in all cases the size of the particles obtained was above 1 μm.

The double emulsions can be defined as complex, heterodisperse systems called “emulsions of emulsion” that can be classified into two major types; water in oil in water emulsion (W/O/W) and oil in water in oil emulsion (O/W/O) [25]. Thus the dispersed phase is itself an emulsion and the inner dispersed globules/droplets are separated from the outer liquid phase by a layer of another phase. Double emulsions are usually prepared in a two step emulsification process using two surfactants, a hydrophobic one designed to stabilize the interface of the W/O internal emulsion and a hydrophilic one to stabilize the external interface of the oil globules for W/O/W emulsions [26].

Another technique of microencapsulation of the active substances is the combination of membrane emulsification to the double emulsion. It was first proposed by Nakashima et al [27] to prepare uniform O/W emulsion, and later developed by Omi and Ma et al [28,29,30,31,32] to prepare uniform microspheres by polymerizing monomer droplets. However, it is little bit difficult to prepare uniform double emulsion as compared to that of single emulsion because of the following reasons: i) complexity of double emulsions, there exists more factors influencing the size distribution of double emulsion obtained, and ii) the double emulsion is prepared by pressing W_1/O primary emulsion through the membrane pores into the external phase (W_2),so the primary emulsion should be stable during the emulsification process which had never been encountered in the preparation of O/W or W/O emulsion [33].

The aim of this work is to study the different parameters affecting the size of the nanoparticles obtained by double emulsion technique such as stirring speed (1^{st} & 2^{nd} emulsions), stirring time (1^{st} & 2^{nd} emulsions), polymer concentration and surfactant concentration. The size of particle obtained was measured by light scattering and TEM and the release of the water-soluble stilbene derivative as a model drug was investigated after one month using U.V.Spectrophotometer.

Materials & Methods:

Materials:

Water soluble Stilbene (CBS-X) was kindly provided by Digast (Lille, France), The polyvinyl alcohol (PVA) (Mowiol® 4-88. Mw = 31,000 g/mol), polycaprolactone (PCL) (Mw = 14,000 g/mol), dichloromethane (DCM), Triton X-405 (70% solution) were purchased from Aldrich Germany while poloxamer-188 (Lutrol F-68) and tween 80 (Mw = 79,000 g/mol) was purchased from Ludwigshafen Germany. Polyethylene Glycol (PEG) (Mw = 200 g/mol) was obtained from Sigma Aldrich, Germany. Polyvinylpyrrolidone (PVP) K 90 (Mw = 360.000 g/mol) was purchased from Fluka analytical Sigma-aldrich, USA and

The particle size and size distribution were examined using Zetasizer nanoseries "zs" from Malvern instruments (England). The Ultra-turrax T-25 basic IKA® -WERKE was used for stirring purpose. Buchi rotavapor R-144 was used for the evaporation. Ultracentrifugation was done using Optima Max-XP ultracentrifuge, Beckman Coulter made in USA. The CARY 50 probe U.V. Visible spectrophotometer from Australia was used for the analysis of release. The transmission electron microscope (TEM) Phillips CM120 electron microscope (CMEABG,University of Claude Bernard Lyon1) was used for measuring encapsulated nanoparticles.

Nanoparticle preparation:

The double emulsion-evaporation method for the preparation of nanoparticles was chosen by focusing on the solubility of the active molecule. The experiment was carried out using 20mg of active molecule and dissolved it in 1ml distilled water, and then 0.5ml of this solution was poured into organic phase containing 1g of PCL dissolved in 4ml of DCM along with stirring using ultra-turrax for 15 minutes at a speed of 16,000 rpm. This 1^{st} emulsion (W_1/O) was then transferred to the aqueous phase (a mixture of 25ml distilled water and 25ml of 1% PVA resulting in 0.5% PVA solution) along with stirring with ultra-turrax at speed of 21,500 rpm for 15 minutes. The obtained double emulsion ($W_1/O/W_2$) was subjected to evaporation under vacuum using rotavap until the whole organic solvent has been removed. To ensure the complete removal of organic solvent, we measured the volume of emulsion after evaporation. Figure 1 shows a diagrammatic representation of the double emulsion evaporation process.

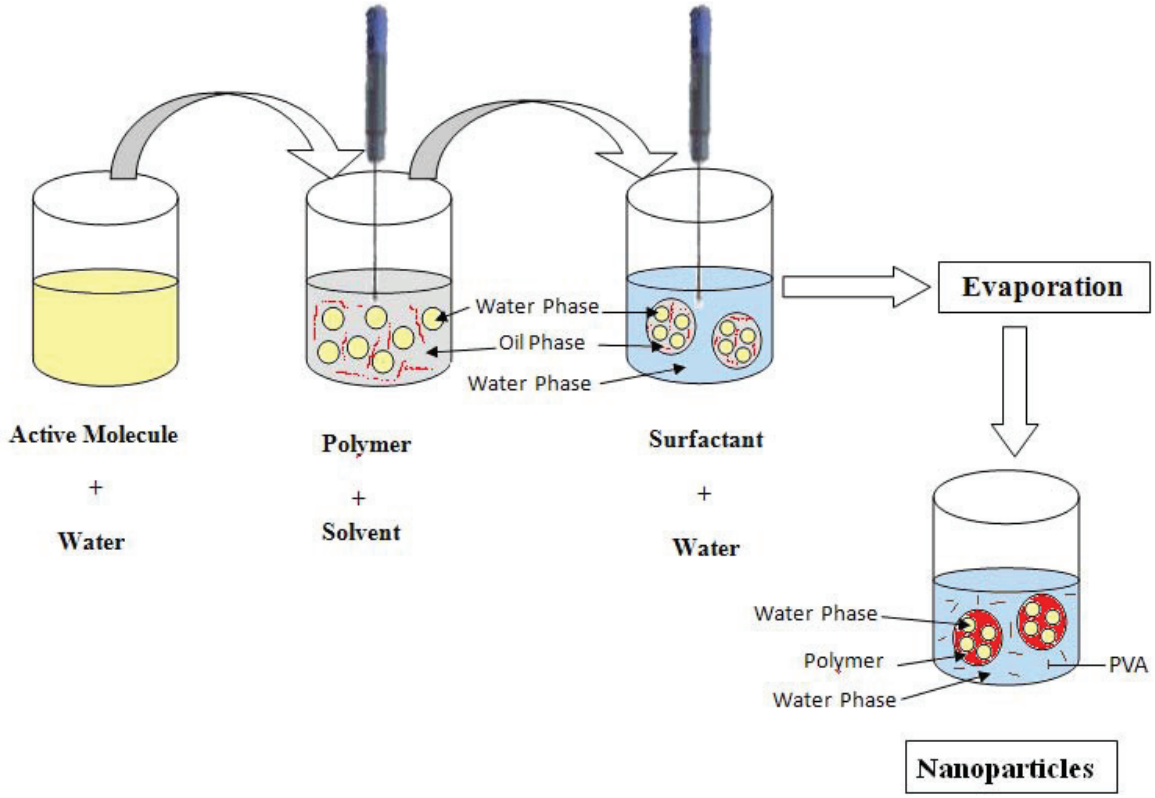

Figure 1: Diagrammatic representation of the Double Emulsion-Evaporation method

Methods:

Release Measurement:

The release of the active molecule in the final emulsion was analyzed after one month of preparation using U.V. spectrophotometer. Before analyzing in U.V. Spectrophotometer, the samples were centrifuged twice at a speed of 40,000 rpm at 5 °C (firstly for 1h and secondly for another 30 minutes). Then the supernatant was taken for the measurement by U.V. spectrophotometer. For measurement, five standards (0.1, 1, 2, 4, 8 µg/ml) were prepared in 0.5 % PVA for the calibration curve and all measurements were done at the wavelength of 350nm.

Size Measurement:

The hydrodynamic size of the prepared particles was measured in 1 mM solution of NaCl (the dispersion was diluted using 1mM NaCl solution), using Zetasizer nanoseries "zs" (Malvern, England) at 25°C where each measurement was performed at less 5 times and then the average hydrodynamic diameter was taken.

Transmission Electron Microscopy (TEM):

The transmission electron microscopy (TEM) of the drug encapsulated nanoparticles were done by a Phillips CM 120 Transmission electron microscope (CMEABG,University of Claude Bernard Lyon1) at an electron accelerating voltage of 80 KV. A drop of sample was deposited on a copper grid covered with a 200 mesh formvar-carbon membrane and dried at room temperature. It should be noted that no staining agent was used.

Results and Discussion

The aim of this work was to attain the submicron size of the particles by the double emulsion (W/O/W) evaporation technique. Double emulsion was prepared by a two-step emulsification process using only one stabilizing agent (polymer or surfactant) specifically in the second step. The preparation of double emulsion evaporation method was started by using different stabilizers such as triton-405, tween 80, poloxamer, PVP (Polyvinylpyrrolidone), PEG (poly ethylene glycol) and PVA (poly vinyl alcohol), where stable emulsion was achieved by using PVA at a concentration of 0.5 %. It should also be noted that there was no emulsion formation and two phases separated when PVA (0.1%) was used. The table 1 shows the results of different stabilizers examined for emulsion preparation which indicates PVA (0.5 %) a good choice for the technique used here to produce nanoparticles using double emulsion evaporation method.

Table 1; Study of various surfactants used for double emulsion preparation.

Surfactant with concentration	Results
Span 80 (0.5%)	Not soluble in water
Tween 80 (0.5%)	Sedimentation
Triton-405 (0.5%)	Phase separation
PVA (0.1%)	Phase separation
PVA (0.5%)	Good and stable emulsion
PVP (0.5%)	Sedimentation
PEG (0.5%)	Phase separation
Poloxamer -188 (0.5%)	Phase separation

As a general tendency, the stirring time for both the 1st emulsion (W_1/O) and the 2nd emulsion ($W_1/O/W_2$) was 1 min, which resulted in production of particles in micro size (generally above 5 μm). By increasing the stirring time to 5 min for both emulsions (primary and secondary emulsion), the particles size was reduced in order to reach nanosize. The established process and recipe was found to be reproducible. The reproducibility was also investigated by examining the effect of scale-up on the final properties of the prepared dispersion. This has been performed by increasing the volumes and stirring times twice, triple and six times. For the twice and the triplet scale-up, the results were totally reproducible since the particles size was found to be submicron and in the same range. For the six times scale-up, the volume become larger and the resulted particles were found to be again in micron range.

In basic experiment performed using the above described process and recipe, the submicron particles size (ranging from 250 to 300 nm) was reached as expected from this well controlled double emulsion technique. Along this, a study of parameters effect was done to have the idea about importance of changing the conditions/parameters in this double emulsion based method.

It is interesting to notice that in some samples (i.e. batches), in addition to the representative peak of the mean particles size, a second small peak showing the particle size in micro range was also observed. This was assumed to be resulted from a long solvent evaporation process under pressure resulting in the formation of some aggregated particles. The observed second peak was easily removed via simple dispersion filtration using glass wool fiber only.

Effect of Parameters on the formulation;

1) Effect of Time of Stirring

In order enhance the dispersion of any liquid phase in another non-miscible liquid phase, an energy contribution is necessary. This can be performed by high stirring, ultrasound system, high shearing or membrane based technology. In this study, the effect of stirring and time of stirring are investigated using ultraturax system.

Above 5 minutes, there is no significant effect of increase in the stirring time for the 1^{st} emulsion on the particle size keeping all other parameters constant. The study of hydrodynamic particles size versus stirring time for 1^{st} emulsion preparation as reported in figure 2 shows that by changing the time of stirring for 1^{st} emulsion, keeping other parameters (including the parameters for 2^{nd} emulsion preparation) constant, the particle size haven't been affected except a small reduction in particle size for 15 minutes that gives the particle size less than 300 nm proving it a suitable time. The observed tendency demonstrate that the stirring process of the second emulsion is the driving parameter.

The time of stirring during la preparation of the 2^{nd} emulsion can be a significant factor because if the second emulsification is not sufficiently dispersed via the stirring process, large particles, highly polydisperse dispersion and the aggregation of formed particles can occur and generally resulting in larger particle size. Different experiments were performed by changing the stirring time of 2^{nd} emulsion keeping other parameters (including the parameters for 1^{st} emulsion preparation) constant in order to point out which step is the driving process. The obtained results are reported in figure 2 for the 2^{nd} emulsion preparation and indicated that 10 to 20 minutes as stirring time are suitable for 2^{nd} emulsification. This result proves that the selection of 15 minutes is well appropriate for performing basic experiment. The results show that less than 10 minutes are not sufficient for this process because it may be unable for the good fragmentation/dispersion of the particles and on the other hand a long time (e.g. more than 20 minutes) is not suitable also that may be again because of aggregation.

It interesting to notice that a very exciting fact can be concluded from this diagram that the 15 minutes stirring period is a suitable time for preparation of both (1^{st} & 2^{nd}) emulsions. Both curves are well superimposed at 15 minutes hence justifying the selection of 15 minutes for basic experiment.

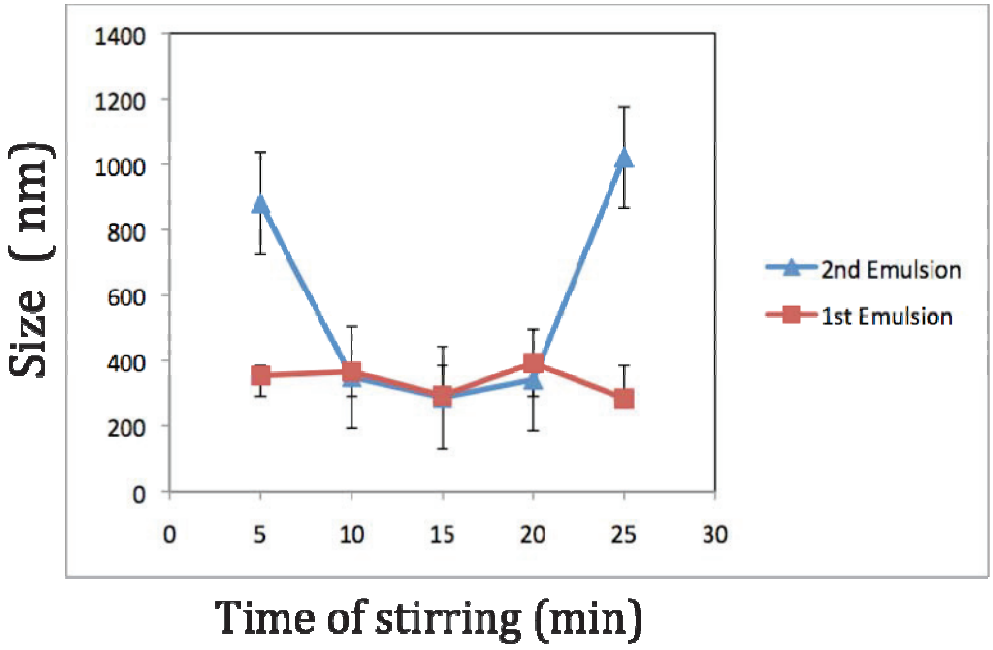

Figure 2: Effect of Stirring time on the size of drug loaded particles prepared at 0.5% PVA & 25 % polymer concentrations and at a speed of 16000 rpm and 21500 rpm for 1st & 2nd emulsion respectively.

2) Effect of Stirring Speed

The stirring speed for the preparation of emulsion is a dominating factor to determine the particle size because it provides the needed energy to adequately disperse the oil phase in water phase. According to Jeffery et al [34] and Yang et al [35], by increasing the stirring speed of 1st emulsion the particle size should reduced but they also mention that by using very high speed can break the particles. It should be noted that the particle size obtained by these researchers were in micro range, so it is assumed that no such destruction is possible in our case since the particle size is in nanorange. Figure 3 represents that by changing the speed of stirring for the first emulsion, keeping other parameters (including the parameters for 2nd emulsion preparation) constant, at lower speed 13,000 rpm, the particles become larger (356 nm) but by increasing the speed to 16,000 rpm the particles become smaller and within the range of nanosize (297nm) which is in accordance with the literature survey.

Increasing the speed beyond 16,000 rpm has same effect but not so prominent and approximately remain constant even by increasing more the speed (like 19,000, 22,000 and 24,000 rpm). Here, 16,000 rpm speed can be selected as a suitable one because higher the speed, higher will be the temperature of the emulsion that can cause the evaporation of dichloromethane (DCM) and hence no formation of second emulsion because of the unavailability of oily phase (O) to encapsulate the water phase (W_1). Consequently, this is what was done in basic experiment that proves 16000 rpm as a good selection.

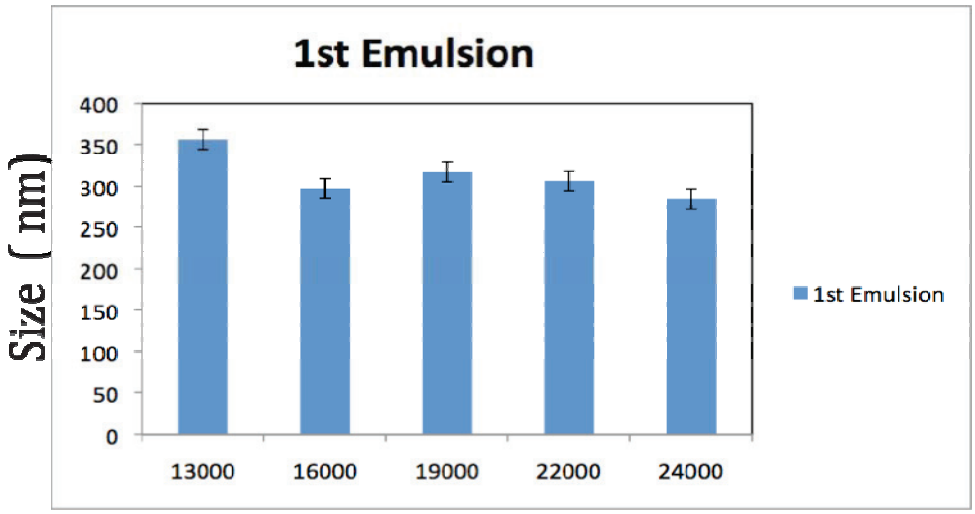

Figure 3 : Effect of speed of stirring (1st emulsion) on size of drug loaded particles prepared at 0.5% PVA & 25 % polymer concentrations and at a speed of 21500 rpm for 2nd emulsion for 15 minutes.

As shown in figure 4, by changing the speed of stirring and keeping other parameters (including the parameters for 1st emulsion preparation) constant in second emulsification step, at lower speed the particle size is increased and by increasing the speed, the particle size started to decrease and become within range of nanosize. It means that a suitable stirring speed can provide good results than by using either a very high or a very low speed.

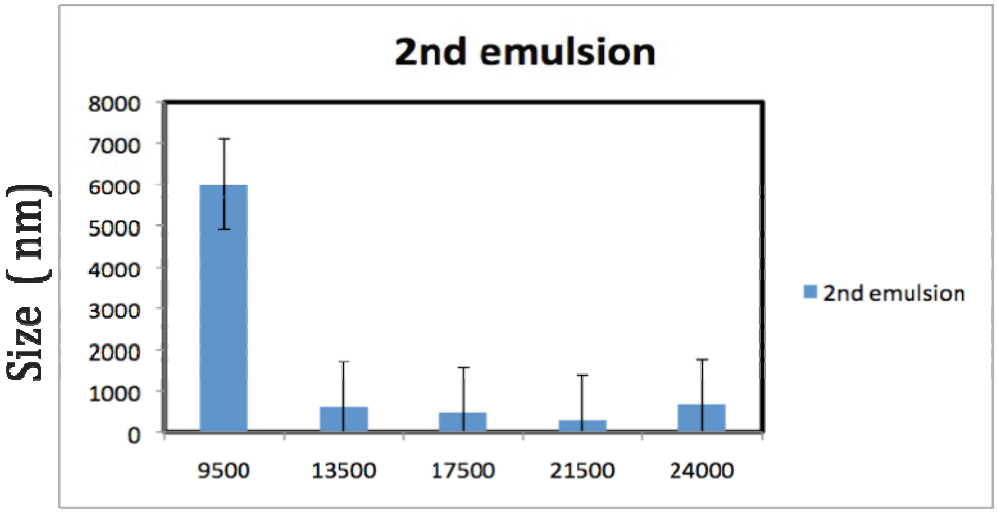

Figure 4: Effect of speed of stirring (2nd emulsion) on size of drug loaded particles prepared at 0.5% PVA & 25 % polymer concentrations and at a speed of 16000 rpm for 1st emulsion for 15 minutes.

3) Polymer Concentration

The polymer concentration can be a critical factor influencing the characteristics of the final particles and it may affect also the encapsulation efficiency. In this work, Polycaprolactone was selected as polymer to be used and keeping all other parameters constant, the effect of change of its concentration was studied. The results were shown in a form of curve in figure 5, implies that at lower concentration of polymer, there is no big effect on the size of resulting particles. But increasing the concentration of poly caprolactone beyond the 3 gram (25 %) 4 & 5 grams (for 33% & 41%), the particle size increased from 266-431 nm.

This shows that polymer has no significant effect until a specific percentage and after that by increasing its concentration; it increases the particle size and these results are in accordance with those obtained and reported by Lamprecht et al [36]. If we assume that the stirring process controls the particles number, then by increasing the concentration of the polymer should leads to large particles size and also to highly polydisperse system.

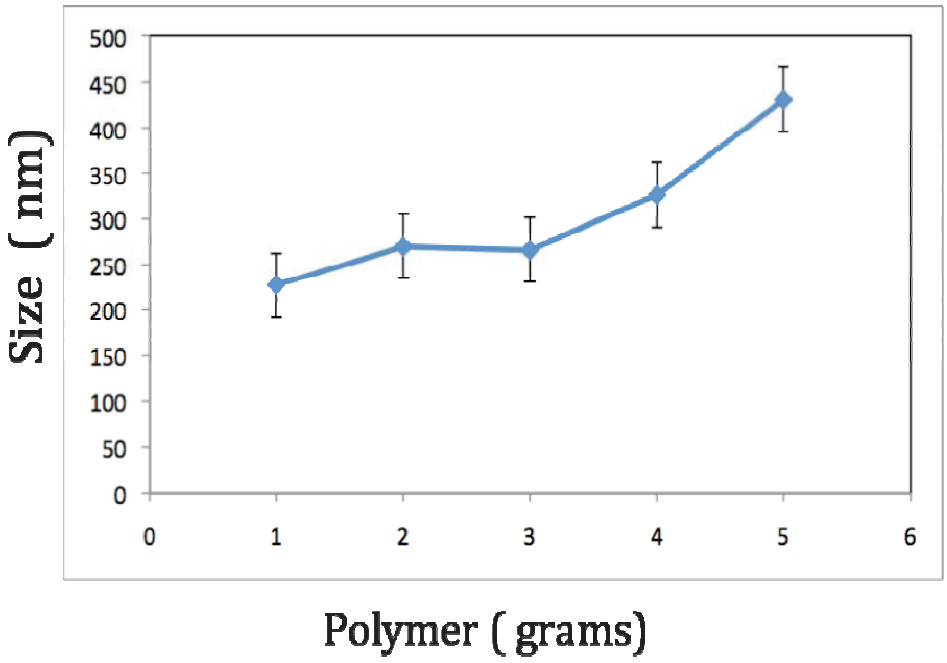

Figure 5: Polymer concentration (in grams) effect on size of drug loaded particles prepared at 0.5% PVA concentration and at a speed of 16000 rpm and 21500 rpm for 1st and 2nd emulsion respectively for 15 minutes.

4) Effect of Surfactant Concentration

The type and concentration of surfactants affect the formulation process as well as the long-term stability of double emulsions. The colloidal stability of the emulsion is important because during the solvent evaporation, there can be a gradual decrease in the volume and subsequent increase in the viscosity of dispersed phase and the solid content. This can affect the droplet size equilibrium, involving the coalescence and the agglomeration of the formed droplets during the early step of the solvent removal [37]. The different surfactants were studied for their suitability to prepare stable emulsion by this method where PVA was found to be the most suitable surfactant.

PVA is the most commonly used stabilizer mainly due to its low toxicity, good solubility in water and its availability in range of molecular weights [38]. The concentration of surfactant can be considered as one of the important factors influencing the particle size and their colloidal stability because the presence of the surfactant in the external aqueous phase cause the stabilization of emulsion droplets/particles against coalescence and its suitable concentration can be effective in determining the size.

The PVA concentration in the external aqueous phase has a relatively negative influence but however less clear than the stirring speed's one. Indeed, the PVA induced particles size reduction has been frequently mentioned in literature [39,40]and it was explained by the fact that the increase in PVA concentrations would render the external aqueous phase mores viscous, which intensifies the shear forces impact on the oily phase and allows obtaining smaller emulsion droplets.

In this work, high concentration of emulsifier leads to an increased size of the nanoparticles produced. This phenomenon can be expected from the stabilizing function of an emulsifier. It is easy to understand that an insufficient amount of emulsifier would fail in stabilizing all the nanoparticles and thus some of them would tend to aggregate. As a result, nanoparticles with larger size would be produced, these findings were found also by Feng and Huang. [41]

A suitable PVA concentration in external aqueous phase and the effect of change of concentration on the method, keeping the other parameters constant as in the basic experiment, were investigated. The results obtained are shown in figure 6 proved that the 0.5 % PVA concentration was the most suitable for this method in which PCL was used as polymer.

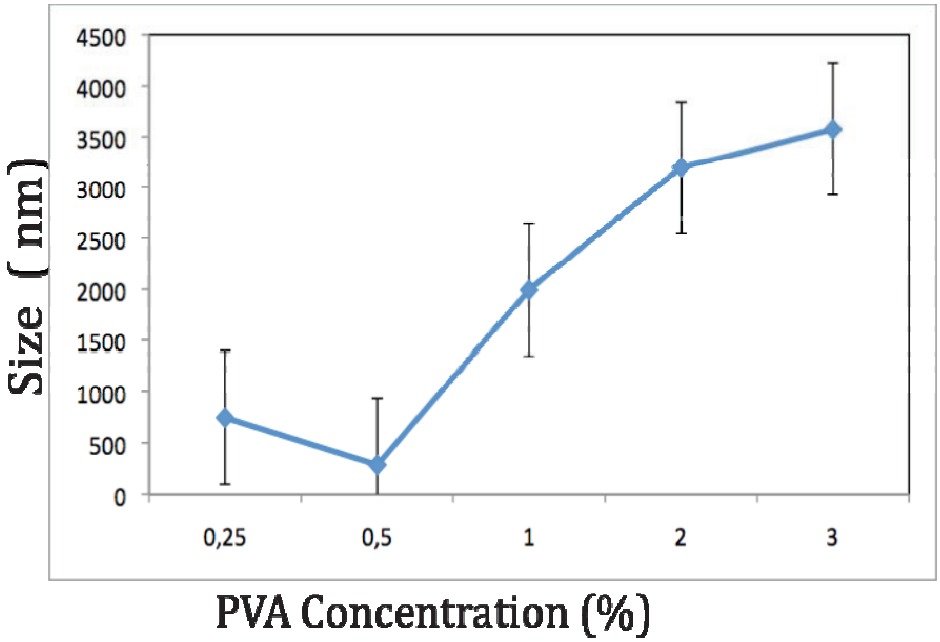

Figure 6: Effect of PVA concentration (%) on size of drug loaded particles prepared at 25% polymer concentration and at a speed of 16000 rpm and 21500 rpm for 1st and 2nd emulsion respectively for 15 minutes.

As shown in Figure 6, by increasing the percentage of PVA in external phase, the particle size increases from 250 nm to reach 4000nm. This result revealed that PVA in such process is not acting as stabilizing agent only but also contribute to particle size control and modification. To understand exactly the driven role of PVA is such system and process, the partition of PVA between continuous phase and particles is of paramount importance.

Here in all experiments designed to study the effect of different parameters, there is a very interesting coherence of the results in the experiments performed on the basis of same conditions. Under the conditions are the 16,000 rpm speed for 15 minutes for 1st emulsion and 21,500 rpm speed for 15 minutes for 2nd emulsion preparation with 25 % & 0.5 % concentration for polymer and surfactant respectively, it can be seen that the average particle size ranges from 265-297 nm which proves the choice of conditions of different parameters for basic experiment as a good selection.

Release Study

The release of loaded drug was measured after nearly one month. The absorbance of the substance was at 350nm using U.V. spectrophotometer. Twelve representative samples of nanosized particles were selected to measure the release at wave length 350nm. The percent release was calculated from the following equation,

$$Percent\ release = \frac{Total\ amount\ of\ drug\ released}{Total\ amount\ of\ drug\ loaded} \times 100$$

The average of drug released was 30 μg/ml against 202 μg/ml of drug loaded initially. The results showed that there was release less than 15% release of active molecule after one month storage at room temperature for the whole samples. This explains that 85 % active molecule is still encapsulated in the particles.

Transmission Electron Microscopy (TEM);

The transmission electron microscopy (TEM) of the drug encapsulated nanoparticles were done to observe the diameter, which gave the satisfactory results. A typical micrograph taken for emulsion prepared using specific parameters (16000 rpm for 15 minutes for 1st emulsion and 21500 rpm for 15 minutes for 2nd emulsion) are shown in figures 7 & 8. These figures show that the particle size ranging from 175nm to 350 nm are relatively spherical and unifrom in size with no aggregation. One can also see that the drug is distributed evenly within the interior of the nanoparticles.

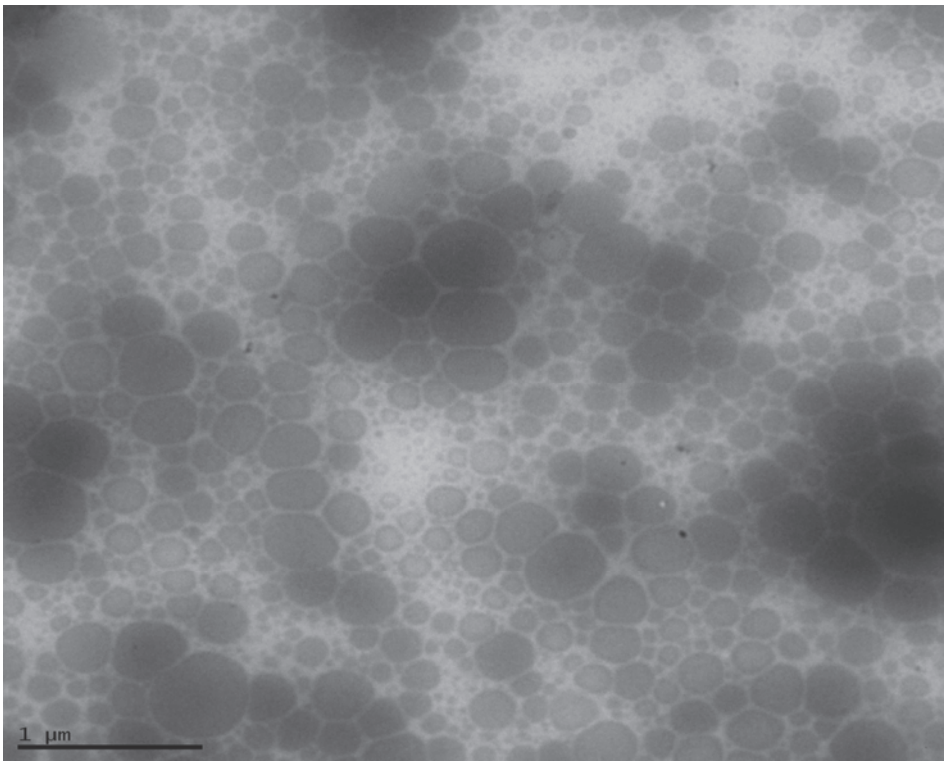

Figure 7; TEM micrograph at 1 µm showing the drug loaded particle distribution of sample 3 prepared by double emulsion evaporation method

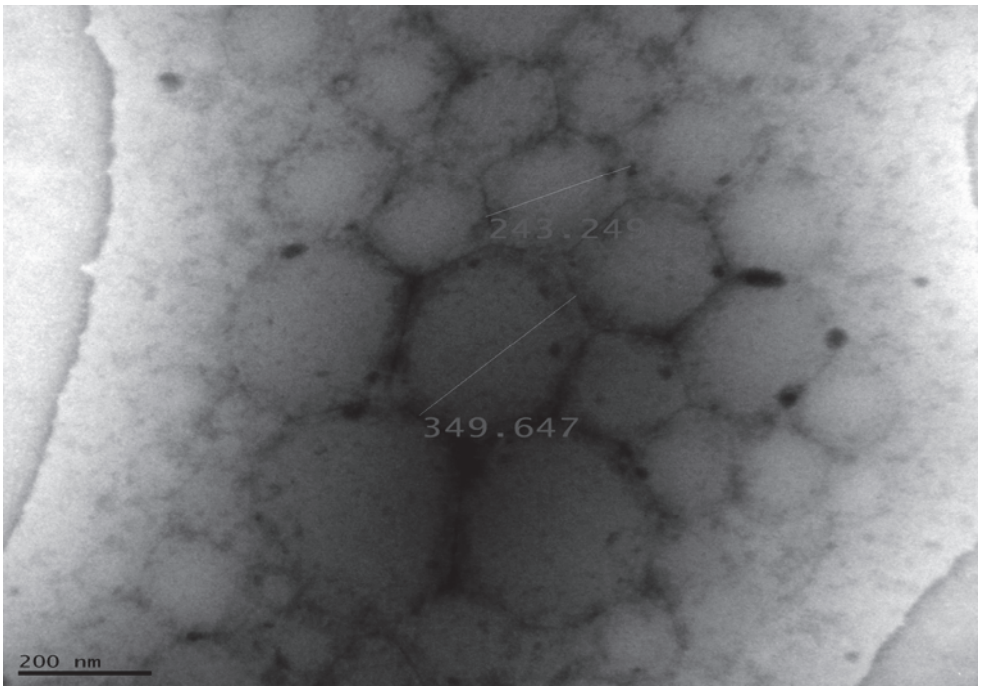

Figure 8; TEM micrograph of the drug loaded particles showing the size of particles ≤ 350 nm

The presence of drug molecules encapsulated in polymer matrix was examined using transmission electron microscopy by analyzing nanocapsules prepared and stored five months. The obtained image is shown in figure 8 in which we can easily see the presence of black dots dispersed

in the particles. The viewed black dots correspond to active molecules stain adequately encapsulated in the polymer particles.

Conclusion:

Nanoparticulate drug delivery system is a promising strategy for the biopharmaceutical industry as it has advantages over conventional drug delivery systems because of showing improvement in solubility, bioavailability and permeability of the drugs encapsulated inside these particles. Nanoparticles containing a fluorescent stable hydrophilic agent as a model drug were prepared. The technique employed was double emulsion ($W_1/O/W_2$) evaporation using poly caprolactone as polymer. Poly vinyl alcohol among different surfactants (e.g. triton-405, tween 80, poloxamer, PVP, PEG etc) was chosen as a suitable one for this preparation method. The results proved that the double emulsion evaporation method is a good choice for the encapsulation purpose, PCl as polymer and PVA as surfactant. Also several factors influencing the size of particles were investigated and impact of change of every parameter was studied rendering five experiments of different ranges to each. The stirring time, stirring speed, concentrations of polycaprolactone and PVA were found to be considered important for nanoparticles preparation using this method. The active molecule release in the prepared emulsion after a time of one month storage was measured using U.V. spectrophotometer and the residual encapsulated amount was found to be more than 85%. The obtained nanocapsules are submicron in size as examined by transmission electron microscopy analysis.

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RESUME
Ce travail a été mené afin d'étudier l'effet du FGF 2 et du TGF-β1 sur les étapes précoces de la régénération dentinaire en utilisant la micro-encapsulation de ces facteurs dans une matrice pour les protéger et contrôler leur libération et ensuite l'application des microparticules obtenues en coiffage pulpaire direct dans un modèle de culture de dents entières.
Ce travail consiste d'abord à l'optimisation des moyens techniques mis en œuvre pour réaliser l'encapsulation du TGFß1, FGF-2 à l'aide de l'acide poly (lactique-glycolique) PLGA. Les études de la caractérisation colloïdal et physico chimique des microparticules montre que les microparticules gardent leurs caractéristiques physicochimiques après séchage et re-suspension dans l'eau. La procèdes optimisé a été ensuite utilisé pour encapsuler les facteurs de croissance. L'encapsulation de FGF-2 et TGF-β1 a été obtenue avec une taille, une efficacité d'encapsulation et une profile de libération adaptés au type d'application choisi. Les études biologiques ne montrent aucun effet toxique des particules sur les fibroblastes pulpaires. Les facteurs de croissance ont gardé leur activité biologique spécifique. Un modèle de culture de dent entier humain a été utilisé pour réaliser l'application de nos microparticules comme un matériau de coiffage dentaire pour confirmer leurs activités biologiques ex-vivo.
Ces microparticules peuvent être utiles dans les études des étapes précoces de la régénération dentinaire, l'activation et la migration des cellules progénitrices de la pulpe dentaire.

TITLE
Controlled release carriers of FGF-2 and TGFß1 for a potential use in conservative dentistry and endodontics.

SUMMARY
The purpose of this work was to investigate the effect of FGF 2 and TGF-β1 on the early steps of dentin regeneration using microencapsulation of theses factors into a microparticles matrix to ensure growth factors protection and to provide bioactive sustained release in contact with dental pulp cells and then the application of the obtained microparticles in direct pulp capping using a culture model of entire tooth.
This work involves the optimization of technical means used to achieve encapsulation of TGFß1, FGF-2 using the poly (lactic-glycolic acid) PLGA. Physicochemical and colloidal characterization of microspheres shows that the microparticles retain their physicochemical characteristics after drying and re-suspended in water. The double emulsion method was used to separately encapsulate (FGF-2) and (TGFß1). Microparticles morphology, loading, shelf life, potential toxicity and release kinetics were studied. Then the proliferation of dental pulp cells was examined in contact with microparticles. Biological studies show no toxic effect of particles on pulp fibroblasts. Growth factors have kept their specific biological activity. A culture model of human entire tooth was used to achieve the application of microparticles as a dental direct capping material to confirm their biological activities ex vivo. These microparticles can be useful in studies of early steps of dentin regeneration, activation and migration of progenitor cells in dental pulp.

DISCIPLINE : Odontologie conservatrice, pharmacotechnie et nanotechnologie biomédicale

MOTS-CLES: Ingénierie tissulaire, coiffage pulpaire, régénération dentinaire, FGF-2, TGF-β1, microparticules, nanoparticules, émulsion multiple, libération contrôlée

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