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Naveed Ahmed

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Naveed Ahmed. Elaboration of nanoparticles for theranostic applications : In vivo imaging and drug delivery. Human health and pathology. Université Claude Bernard - Lyon I, 2012. English. NNT : 2012LYO10128 . tel-00980587

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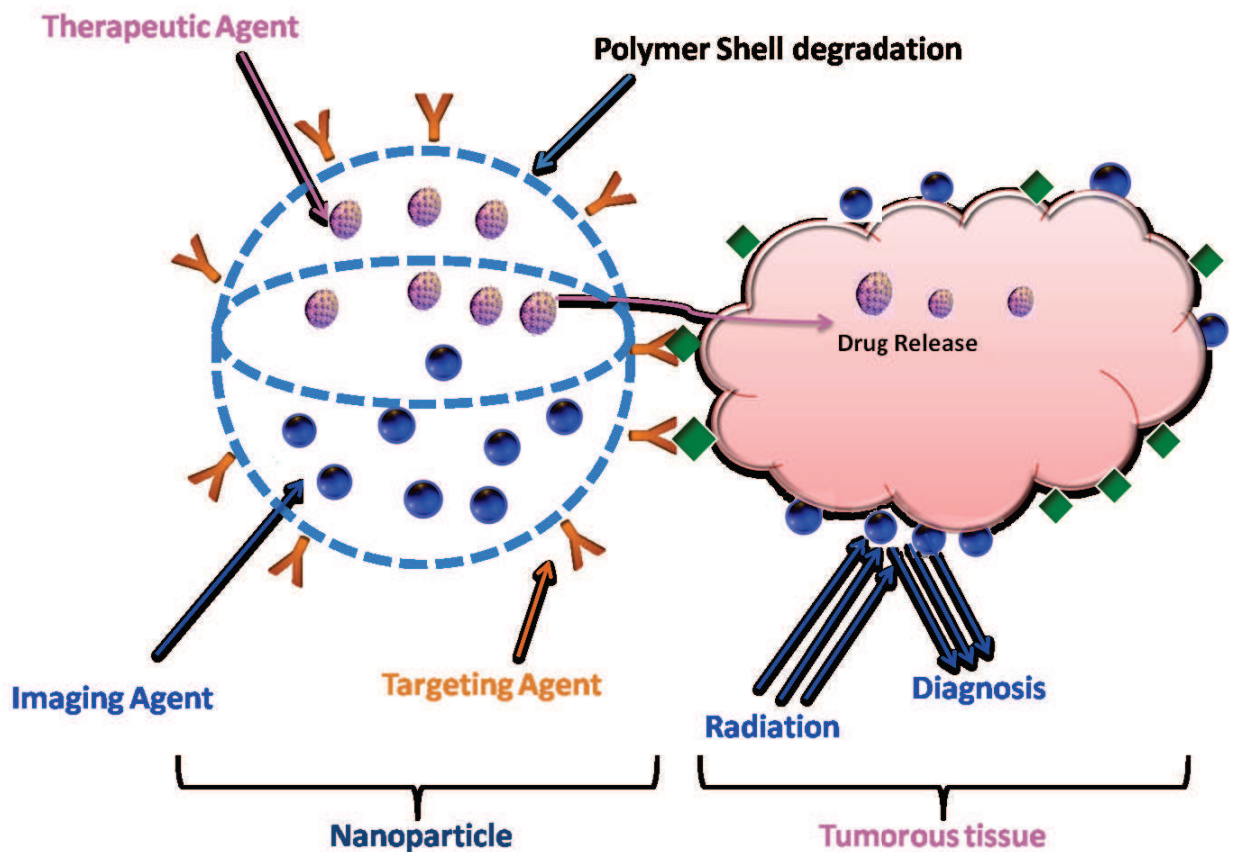
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Elaboration of nanoparticles for theranostic applications: In vivo imaging and drug delivery



Naveed Ahmed

Claude Bernard University Lyon 1



THESE

Présentée devant

L'UNIVERSITE CLAUDE BERNARD – Lyon 1

Pour l'obtention du Diplôme de DOCTORAT
Spécialité Pharmacotechnie

Par

Naveed AHMED

Soutenue publiquement le 20 septembre 2012

« Elaboration de nanoparticules pour application théranostics: Imagerie *in vivo* et vectorisation de médicaments »

Jury :

Salima Saidi	Professeur	Rapporteur (université d'Oran)
Christine Vauthier	DR-CNRS	Rapporteur (Université Paris Sud)
Nasir M. Ahmad	Professeur	Examineur (NUST, Pakistan)
Philippe Chaumont	Professeur	Examineur (Université Lyon-1)
Abdelhamid Elaissari	DR-CNRS	Directeur de thèse (CNRS)
Hatem Fessi	Professeur	Co-directeur de thèse (Université Lyon-1)

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*Sincerity to everything is key to success.....
Prayer to God can change a lot in life.....*

My Beloved Parents

What is next?.....

Abdelhamid Elaissari

It's research, Everytime opens a door.....

Hatem Fessi

Remerciements

Ce travail de recherche a été effectué au Laboratoire d'Automatique et de Génie des Procédés (LAGEP) dont le directeur est Monsieur le Professeur Hatem Fessi, qui m'a fait l'honneur de m'accueillir au sein de son laboratoire pendant ces trois merveilleuses années de thèse que je remercie infiniment.

Que Monsieur Abdelhamid Elaissari, mon directeur de thèse, reçoit toute l'expression de ma reconnaissance pour m'avoir proposé ce sujet de recherche, et pour tout son dynamisme et ses compétences scientifiques qui m'ont permis de mener à bien cette étude.

Je remercie tous particulièrement Madame Christine Vauthier, Directeur de recherche au CNRS à l'Université de Paris Sud, (Laboratoire Physico-chimie, Pharmacotechnie, Biopharmacie, UMR CNRS 8612), et Madame Salima Saidi, Professeur à l'Université d'Oran Es-Senia, (Laboratoire de Synthèse Organique Appliquée LSOA), qui ont accepté de juger ce travail et d'en être les rapporteurs.

Je suis très sensible à la présence dans le jury de Monsieur Philippe Chaumont, Professeur à l'Université Claude Bernard-Lyon 1 (Laboratoire de l'Ingénierie des Matériaux Polymères, UMR INSA/CNRS 5223) qu'il trouve ici mes remerciements.

Je tiens également à remercier Monsieur Nasir M. Ahmad, Professeur à National University of Science and Technology (NUST) [Department of Materials Engineering, School of Chemical and Materials Engineering (SCME)], d'avoir accepté de participer au jury de cette thèse et pour son excellente collaboration.

Je veux adresser tous mes remerciements à mes collègues et en particulier Muhammad, Millan, Mahbub, Valentina, Chiraz, Nader, Ahmad Bitar, M.Eissa, Rachid, Audrey, Hector, Sofiane, Zoaoui et Claudia pour l'aide apportée tout au long de ces travaux de thèse.

Merci également à tous mes collègues Nadia, Jean Pierre et Olivier pour leur aide inestimable. Je tiens également à remercier mes amis Pakistanais en particulier Usman et Naveed pour leur accueil et l'aide apportée lors de mon séjour à Lyon.

Enfin, un grand merci à ma famille ; mes parents, ma femme, mon frère Zaheer et sa famille, et mon cousin Ehsan de m'avoir aidé durant mon voyage et mes études en France.

Résumé

L'objectif de ce travail a été la préparation de nanoparticules pour l'administration intratumorale. La double fonctionnalisation de ces nanoparticules est rapidement approuvée comme extrêmement intéressante, car elles peuvent être utilisées pour le diagnostic *in vivo* et la thérapie (théranostics). Pour réaliser ce type de nanoparticules, l'oxyde de fer a été choisi comme agent de contraste pour une utilisation en imagerie par résonance magnétique (IRM) pour le diagnostic de cellules cancéreuses. Les nanoparticules d'oxyde de fer ont été préparées dans des milieux aqueux et organiques. La méthode d'émulsification multiple suivie de l'évaporation de solvant a été utilisée pour l'encapsulation concomitante d'un principe actif et des nanoparticules de l'oxyde de fer. Tous les paramètres affectant la taille des nanoparticules pendant le procédé ont été étudiés en utilisant une molécule active hydrophile modèle (une dérivée de la Stilbene) et le protocole standard a ensuite été évalué. Dans une seconde étape, les particules d'oxyde de fer ont été encapsulées par la même méthode d'émulsion évaporation et entièrement caractérisées en termes de morphologie, taille, magnétisation, et composition chimique. La visualisation *in vitro* des particules modèles a été réalisée par IRM et comparée à un produit commercial à base de Gadolinium (Gd). D'autre part, la quantité nécessaire d'oxyde de fer permettant une bonne visualisation par IRM a été déterminée par une étude *in vivo* menée sur des souris. Enfin la double encapsulation d'oxyde de fer avec une molécule anticancéreuse a été effectuée par la méthode développée, la formulation obtenue a été entièrement caractérisée.

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PART I

GENERAL INTRODUCTION

Résumé

Les nanotechnologies en oncologie sont utilisées pour le développement du diagnostic et le traitement de divers types de cancers. Le concept theranostic est d'avoir un avantage double en utilisant une seule action ou un seul produit. L'agent theranostics est un motif qui fournit le diagnostic et la thérapie simultanément. Ce travail de recherche est basé sur la préparation de nanoparticules à des fins théranostics.

Dans cette introduction générale, les bases générales sur le cancer, ses étapes et les différentes modalités de diagnostic seront présentées et discutées. Les divers problèmes rencontrés par les professionnels de la santé seront mentionnés et l'application des nanomatériaux sera prise en compte. Enfin, l'objectif du travail sera brièvement exposé.

Introduction

The nanotechnology application in biomedical field is praiseworthy leading to find the solutions for many diseases. This development can be in terms of rapid diagnosis, increased absorption, enhanced bioavailability, rapid detection of causes and reduced side effects, *etc.* An important point is the earlier application of nanotechnology for improvement in diagnosis and treatment of major diseases like cancer, HIV, diabetes, *etc.* which caused many deaths and final curable work is still to discover. Nanotechnology is primarily and vastly applied in cancer because of the severity of disease. Cancer is a malignant disease and its treatment with an anticancer drug is only to give palliation, induce remission but still there is little possibility of cure.

In a simple way, cancer occurs when the normal cells have been transformed into neoplastic cells that can happen through alteration of their genetic material or by the abnormal expression of certain genes. The neoplastic cells can be distinguished by their abnormalities at chromosome level and by loss of their differentiated properties. Such types of changes result in an uncontrolled growth and cell division leading to the invasion of previously unaffected organs, and the whole process termed as metastasis. If this uncontrolled spread is not controlled, it can result in death. American Cancer society report 2009 explains that cancer is caused by both external (tobacco, infectious organisms, radiations and chemicals) and internal factors (inherited mutations, immune conditions, hormones, and mutations that occur from metabolism). These factors may either act together or in sequence to initiate or promote carcinogenesis [1]. The cancer caused by external factors can be prevented however, in case of external factors it takes a long time to develop in an individual e.g. in some cases it takes about ten years from exposure to detectable cancer.

It will be useful here to differentiate between cancer and tumor because these both terms are used frequently and create ambiguity. A tumor is a mass of tissues that is abnormal and can be benign or malignant while cancer is a disease characterized by abnormal cell development which may or may not be from a tumor. In other words, there are two types of tumors, benign and malignant and a malignant tumor is cancer. Hence it could be concluded “not all tumors are cancer, not all cancers form tumors” or one can have tumor without cancer and cancer without tumors.

There are problems in handling cancer starting from diagnosis to the treatment. Diagnosis and declaration of cancer is not so easy because of the complexity of disease. Similarly, techniques or materials used for diagnosis and treatment, can be hazardous for the normal human cells which are the main problem for treatment of cancer and hence they reduce the overall efficiency of the therapeutic tools. A large number of mortalities occur due to the late diagnosis of the disease as cancer is a malignant disease. Declaration of cancer can be made on the basis of different screening tests and physical examination. The complications occur for various types of cancer and the location of the cancerous tissues. However, with research and development in diagnosis and therapy, things are improving. Various newer diagnostic techniques have been developed. Similarly newer therapeutic modalities are in development process for the cure of disease. Modern imaging techniques including X-rays, ultrasounds, magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET) and near infrared fluorescence (NIRF) are in practice. However, improvement in these existing diagnostic protocols is in process for rapid and accurate diagnosis of cancerous tissues. There are other complementary or confirmatory assays or tests that are required for the declaration of cancer but an accurate, fast and economical diagnostic method/technique capable of early cancer detection is still waiting. Similar complex issues are known for the treatment of cancer as well. Many therapeutic methods have been tried and others are in progress for trials. Chemotherapy, immunotherapy, hormonal therapy and various other methods are in practice with various drugs/chemical agents/hormones from both animal and plant origin are in practice and researchers/physicians are also in search of newer ones.

Various problems faced by physicians/researchers/surgeons start from the diagnosis till treatment. These problems include: late detection of cancerous tissues depending upon the site and type of cancer, complications after diagnosis, failure of the therapeutic agents to reach the cancerous tissues, *etc.* The failure of the therapeutic agents can be due to drug resistance including both cellular and non-cellular resistance, lower bioavailability. It can also be due to pharmacokinetic and pharmacodynamic complexities of the active ingredient. However, the most important is the effect to the normal cells/tissues that cause the most damage for the receiving organism. Chemotherapeutic agents cause more damage to the neighboring tissues than the cure. Various approaches were tried to solve this problem and with the boom of nanotechnology, it was applied in oncology to get advantages especially for the reduction of side effects along with increased absorption of the active ingredients.

However, the important aspect to be considered in the application of nanomaterials in medical field especially in biological organisms is biocompatibility. To apply nanotechnology to biology and medicine, several conditions must be considered;

- Nanomaterial must be designed to interact with proteins and cells without interfering with their biological activities.
- Nanomaterial must maintain their physical properties after surface modifications.
- Nanomaterials must be non toxic [2].

With arrival of biomedical nanotechnology and application of newer preparation techniques of the chemotherapeutic agents, the improvement arrived but the problem of time lapse between diagnosis and therapy still persists. Hence, with the expertise in biomedical nanotechnology and newer ideas both resulted in the formulation of multipurpose moieties especially in case of diagnosis.

This work has been dedicated to similar domain that is application of multiple approaches for the cancer cells. This multiple approach consists of diagnosis and therapy at same time with same moieties, giving rise to a new term; theranostic borrowing thera- from therapy and -nostic from diagnostics. In this work, it has been targeted to prepare the nanoparticles that can be used for the diagnosis and therapy. One of the diagnostic mechanisms has been explored for the diagnostic purposes and an active ingredient especially the chemotherapeutic agent has been involved for the therapeutic purposes. MRI was chosen as the diagnostic technique for the detection of cancerous tissues while an anticancer agent was used for the therapeutic purposes. A contrast agent to be used in MRI was prepared, characterized and encapsulated with one of the pharmaceutical techniques. Then the final particles were analyzed for *in vitro* diagnosis. On other hand, the active ingredient was encapsulated using the same pharmaceutical technique (double emulsion based process) and has been characterized before *in vivo* evaluation at the end.

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PART II
BIBLIOGRAPHY

Résumé

Le cancer étant une maladie maligne et pour la compréhension détaillée des causes, des mécanismes et des phénomènes liées, une enquête bibliographique sur le sujet est indispensable. L'objectif de ce travail de recherche est la préparation de nanoparticules pour le traitement du cancer mais également le diagnostic *in vivo* des cellules cancéreuses. Par conséquent, cette partie introduit la physiopathologie d'un cancer, les avancées en matière de diagnostic et de thérapie, réalisées ou en cours de développement. Les différents moyens de traitement de cancer sont les agents chimio-thérapeutiques, l'immunothérapie, la thérapie hormonale, les thérapies naturelles, *etc.* Ainsi, un aperçu de ces traitements est discuté dans ce chapitre pour une meilleure compréhension du choix et du type d'actifs à sélectionner.

Cette étude inclut également le choix et la préparation de l'agent de marquage qui sera utilisé comme agent de contraste pour le diagnostic. Diverses méthodes ont été discutées et différents outils du diagnostic tel que des agents de contraste ont également été examinés. La recherche dans le domaine théranostics et les travaux de diagnostic déjà réalisés avec les différents mécanismes de diagnostics en utilisant les différents ingrédients actifs ont été pris en compte. Toutefois, un accent particulier est mis sur l'imagerie utilisée pour le diagnostic de tissus cancéreux en utilisant l'IRM.

L'objectif principal de cette revue de la littérature est de présenter l'état de l'art des techniques d'encapsulation ou des techniques de préparation des nanoparticules contenant des molécules actives. Diverses techniques pharmaceutiques ont été examinées et une attention particulière est dédiée à l'encapsulation de principes actifs hydrophobes. La nanoprecipitation, techniques d'émulsification (y compris d'émulsion diffusion, émulsion coacervation, émulsion évaporation et les émulsions multiple), la préparation des polymersome, technologies des fluides supercritiques et couches adsorbées de polymères (layer by layer) ont été discutées. En outre les paramètres affectant l'efficacité d'encapsulation et la libération du médicament *in vitro* et *in vivo* ont été discutés.

Cette partie nous a permis la compréhension relativement complète des types de cancer, le diagnostic et les mécanismes thérapeutiques utilisés pour le traitement. Divers agents de contraste ont été étudiés et discutés en fonction du diagnostic cible. Une étude détaillée des techniques de préparation nous a permis de choisir une technique appropriée pour la réalisation de notre étude de recherche par la suite. Pour la préparation de nanoparticules utilisées simultanément pour le diagnostic et la thérapie. Cette étude a également contribué à comprendre les limites dans divers cancers en fonction de

l'emplacement, le niveau et le type de cancer. Elle nous a également donné un aperçu général sur le diagnostic *in vivo* et ces limites. Ceci nous a permis de présenter une solution potentielle pour un meilleur traitement du cancer.

Summary

Cancer being malignant disease, the detailed understanding of causes, mechanisms and other related phenomenon, a bibliographic survey was inevitable. According to our target of nanoparticles preparation for the treatment of cancer and *in vivo* diagnosis of cancerous cells at the same time, this part primarily deals with introduction of cancer, developments in diagnosis and therapy, which has been done or in process although the diagnosis portion mainly deals with diagnosis *in vivo*. Various treatment options for cancer have been classified in broad categories like chemotherapeutics, immunotherapy, hormonal therapy, natural agents involved for the treatment, *etc.* Further classification of these classes has been given for the well understanding of the type of the active ingredient to be selected.

Secondly, it includes the choice and preparation of the labeling agent which will be used as a helping agent for diagnostic mechanism. Various diagnostic modalities have been discussed with new research developments. Different materials helpful for diagnosis as contrast/ labeling agents has been also studied with their specific modalities. Research in theranostics and the already done works with different diagnostic mechanism and using different active ingredients have been brought in the account. However, the focus is on the imaging mechanism being used for diagnosis of cancerous tissue and especially on MRI.

The main focus of this literature survey will be on the encapsulation techniques or it can be said as the preparation techniques for the nanoparticles containing active ingredient. Various pharmaceutical techniques have been discussed especially focusing on the encapsulation of hydrophobic active ingredients. These may include nanoprecipitation, emulsification techniques (including emulsion diffusion, emulsion coacervation, emulsion evaporation and multiple emulsion), polymersome preparation, supercritical fluid and layer-by-layer technologies has been discussed. Further the parameters affecting the efficiency of these methods and *in vitro* and *in vivo* drug release has been elaborated in a short but comprehensive way.

This part helps the complete understanding of the cancer phenomena, diagnosis and therapeutic mechanisms used to deal with cancer accompanied by recent developments. Various contrast agents has been studied for their combination with various diagnostic mechanisms. On the other hand, detailed study of all preparation techniques permitted us to choose a suitable technique for the realization of our theme which is unique approach within specific limits including the preparation of nanoparticles targeted to be used for diagnosis and therapy simultaneously. It also helped to understand the limitations in various cancers

depending upon the site, stage, and type of cancer. In a simple way, it provided a deep understanding to diagnose the problem with its limitations and enables to present a solution that can be useful to deal with problem first and then for better treatment of the cancer for mutual benefit of the mankind.

CHAPTER II.1

CANCER DIAGNOSIS AND TREATMENT: NANOTECHNOLOGY APPLICATIONS

Naveed Ahmed, Ahmed Bitar, Hatem Fessi and Abdelhamid Elaissari

(In preparation for submission as a book chapter in ‘Control release systems: Advances in nanobottles and active nanoparticles’ will be published by Pan Stanford Publishing, Singapore)

Résumé

Le traitement de cancer pose un problème majeur aux niveaux de la thérapie et l'effet secondaire indésirables. De nombreuses possibilités ont été proposées, mais un mécanisme de traitement sûr n'est pas encore établi. Le problème commence à partir de la détection du cancer, le choix des médicaments appropriés pour le traitement, la durée du traitement, et enfin les effets secondaires. D'autres méthodes basées sur la mort des cellules cancéreuses ont également été appliquées, par exemple la radiothérapie, mais le succès complet est encore à découvrir. Applications récentes de la nanotechnologie en oncologie a créé un nouveau espoir pour le traitement de cette maladie mortelle. Le cahier des charges pour un diagnostic plus précoce est de bien comprendre les mécanismes du cancer, le choix approprié de l'actif ou de la méthode de traitement, l'amélioration des mécanismes de libération de médicaments, la réduction des effets secondaires, *etc.* Il existe d'autres approches thérapeutiques comme l'hyperthermie, les approches magnétiques et les radiations, *etc.*

Ce chapitre porte sur le diagnostic de cancer en utilisant les différentes méthodes d'imagerie et en mettant l'accent sur le diagnostic *in vivo* et l'IRM en utilisant l'oxyde de fer comme un agent de contraste. Une classification des agents anticancéreux a été réalisée pour la compréhension du choix d'actif nécessaire. Différents types de traitements ont également été reportés, mais l'accent reste mis sur la thérapie par des nanoparticules comme outil nanotechnologique et différents exemples ont été décrits. Divers nanomatériaux organiques et inorganiques utilisés en oncologie ont été également discutés. Enfin, un aperçu sur la toxicité des nanomatériaux a également été présenté.

Summary

Cancer is among diseases which are major challenge for the physicians and researchers to treat. Many possibilities have been proposed but a fully trusted treatment mechanism is yet to be arrived. The problem starts from detection of cancer, choice of the suitable drugs for treatment, treatment duration and reaches reducing the side effects. Other methods for the death of cancerous cells have also been applied e.g. radiotherapy but complete success is still to be discovered. Recent nanotechnology applications in oncology again created a hope for the treatment of this fatal illness. This application is for earlier diagnosis, well understanding of the cancer mechanisms, suitable choice of drug or method of treatment, improvement in drug delivery mechanisms, reduction in side effects, *etc.* along with approaching other treatment options like hyperthermia, magnetic approaches, *etc.*

This chapter deals with the diagnosis of cancer using different imaging mechanisms with emphasis on *in vivo* diagnosis. However, the focus will be on MRI and iron oxide as contrast agents to be used for MRI. Further classification of anticancer agents has been made for the understanding and easy choice of active ingredient to be used. Different types of therapies have also been touched but the focus remains on the nanoparticles therapy along with the different nanotechnology tools with their existing examples have been described. Various organic and inorganic nanomaterials being used in oncology have also been brought in the account. Finally a short account of toxicities due to the nanomaterials has also been discussed.

Introduction

Biomedical nanotechnology has given a variety of opportunities to fight many diseases. Drug and gene delivery, Protein and peptide delivery and recent advancements of theranostics are the important applications along with other applications. Diseases which are in dire need of complete solution to save the humanity include diabetes mellitus, cancer, neurodegenerative and cardiovascular problems. Every year millions of people lost their struggle for life against these well known illnesses. Cancer is important and well investigated for the cure but still there is no valuable success.

Along with other branches like nano-nephrology, advancements in proteomics and genomics, nanotechnology is also emerged as one of the most fruitful application in oncology and can be understand as a definite medical boon for diagnosis, treatment and prevention of cancer disease. In last decade, the application of nanotechnologies for drug delivery in cancer has been extensively explored hoping to improve the efficacy and to reduce side effects of chemotherapy [1]. It might be applied in cancer through molecular tumor imaging, early detection (like high-throughput nanosensor devices for detecting the biological signatures of cancer), molecular diagnosis, targeted therapy and cancer bioinformatics. As our preference is intratumoral administration, the various methods of direct introduction of anticancer drugs should kept in mind including injection of drugs directly into the tumor, tumor necrosis therapy, injection into the arterial blood supply of cancerous tissues, local injection into tumor for radiopotential, localized delivery of anticancer drugs by electroporation (electrochemotherapy) and local delivery by anticancer drug implants. Further specifications may include heat activated targeted drug delivery, tissue selective drug delivery, using vascular targeting agents, using a carrier or by selective permeation of the anticancer agent into the tumor, *etc.* the choice of a method depends upon the location of tumor, drug specifications, and the importance of treatment, *etc.* In this review, it has been tried to shed some light on the cancer nomenclature, diagnosis, treatment, classification of the materials used at present for the treatment with a special focus on the nanoparticles used for both diagnosis and treatment purposes.

Cancer Diagnosis

Before starting the treatment of any disease a good diagnosis is necessary and in case of cancer, the importance increases due to complexity of the disease, having similarity of symptoms with other diseases, different types of cancer and sometimes because of different

protocols for the diagnosis. Cancer can be declared on the basis of different facts, the most important of which are physical examination and the results of screening tests. Generally X-ray examinations for any other purpose e.g. an injury, showing some abnormalities which can result in cancer, could be considered as basis of diagnosis. For the confirmation purpose, further diagnostic tests are required. After the diagnosis, next step is staging, that is finding how advance is cancer or in other words staging can be understand as how big the tumor is or if it is spread to other neighboring tissues or not. A good and timely diagnosis can be helpful for successful cancer therapy and improves the survival rates [2, 3]. A similar term to diagnosis used in medical field is prognosis that means the prediction of outcome of a disease. In cancer, it's the disease duration, chances of complications, possible outcomes, recovery aspects, recovery period, death rates, survival rates, and other possible results. At present prognosis of cancer has improved because of the modern early detection techniques and due to the public awareness.

The basis of cancer diagnosis is the difference in the morphological changes of a normal tissue to that of a cancerous tissue e.g. in terms of structure, cancer cells develop a different DNA or gene structure or acquire abnormal numbers of chromosomes as compared to the normal cells. Another such useful characteristic of cancerous cells (although not seen in all cancer tissues) is the 'Warburg effect' [4,5] an observation that most cancer cells predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria like most normal cells [6]. The diagnosis of cancer can be done by various assays and tests. For the simplification of understanding all these assays and tests are divided in two broad categories; *in vivo* diagnosis and *in vitro* diagnosis but here *in vivo* diagnosis will be reviewed shortly.

***In vivo* diagnosis**

Tumors possess many characteristics and can arise and develop in different ways in various body parts, thus a single method cannot be sufficient for accurate diagnoses of tumors. Hence, for declaring that a person has cancer, a time taking series of tests is required [7]. Various modern techniques can be used for localization and diagnosis of tumors on the basis of characteristics of the malignant tissue. These techniques may include computed tomography (CT), positron emission tomography (PET), single-photon emission computed tomography, near infrared fluorescence (NIRF), ultrasound, X-rays and magnetic resonance

imaging (MRI). Different morphology of tumor tissues as compared to the normal tissues provides a basis for their detection e.g. in case of CT, Ultrasound, X-rays and magnetic resonance imaging (MRI), contrast agents are used [8] to detect this variation in morphology. The location of tumor in body might be a deciding factor in choice of techniques but till now mostly imaging techniques used for *in vivo* detection have a problem of limited tumor: background ratio in conventional tumor targeting [9]. Here, focus will be on the imaging techniques used for diagnosis. General imaging studies include dynamic or static imaging and *in vivo* functional tests. Dynamic imaging provides data about biological turnover of radioisotopes in different body compartments and organs [10]. Imaging might be Optical imaging, magnetic resonance imaging (MRI), radionuclide based imaging, computed tomography, ultrasound, *etc.* In case of radionuclide imaging, signals might be obtained at low background signals and require less signal amplification. Magnetic nanoparticles are being used as contrast agents for the reticulo-endothelial system (RES). Two generally divided techniques of radionuclide imaging are positron emission tomography (PET) and single photon emission computed tomography (SPECT). However the basis for MRI signal is the precession of water hydrogen nuclei in an applied magnetic field. After application of radiofrequency pulses, the relaxation process through which the nuclei return to the original aligned state can be exploited to produce an image [11]. With the progress in research, numerous materials are devised to be used in imaging techniques especially as contrast agent. Gadolinium, Manganese and Iron oxide nanoparticles are used mostly as contrast agents although the metallic magnetic nanoparticles were not considered earlier to be applied in biological field because of their chemical instability. Nonetheless, recently the metallic MNP's are being used after coatings with different materials to solve the problem of instability e.g. in case of iron oxide nanoparticles could be coated by oleic acid [12, 13], amine or dextran [14, 15]. The examples of other usage of materials include hyperpolarized carbon imaging for *in vivo* diagnosis, protease activated imaging, *etc.*

Magnetic Resonance imaging (MRI) is one of the important imaging techniques, mostly used now-a-days for the diagnosis and early detection of the diseases. It was first developed by Paul Lauterbur [16] in 1973, and in 1985, it got the FDA (Food and Drug administration) approval for clinical use. In MRI, the radio waves and a magnetic field is used to take high quality images of the body parts e.g. soft tissues, organs and bones. To improve the visibility of the internal body parts, a contrast media is required and iron oxide nanoparticles could be employed as contrast media (as gadolinium and manganese particles)

because of their magnetic susceptibilities in the presence of an external magnetic field and other characteristics like biodegradability and economical advantages. The visibility is increased by enhancing the image contrast between the normal and diseased tissues of the body [17]. Applications in clinics include the contrast enhanced imaging of spleen, liver and lymph nodes in the body. Researchers found that MRI based on MNPs is vastly superior to other non-invasive methods to identify lymph nodes metastasis from solid tumors and histologically positive lymph nodes outside the field of resection [18]. MRI technique could be used in the detection of apoptosis in an early stage and has the advantages as compared to the radionuclide technique and magnetic resonance therapy because of the detection of apoptotic regions with relatively high spatial resolution.

An important property of iron oxide particles is superparamagnetism which is a form of magnetism and a superparamagnetic material has small magnetic points in its structure but with undefined directions so material is not magnetic. On the application of an external magnetic field, these small points change their direction accordingly and then material can become magnetic. This is possible in materials made of small parts may be ranging size below 30 nm. For researchers, superparamagnetic property increases interests in use of iron oxide particles. Superparamagnetic iron oxide nanoparticles (SPION) are highly sensitive to magnetic resonance detection, non-toxic and also have been approved for clinical use as a blood agent for the MRI [19] hence can be used as contrast agent to differentiate the diseased tissue from healthy ones. Two of the best examples of the iron oxide nanoparticles include the cell targeting and the cell tracking. The first cellular imaging studies were performed with non-functionalized iron oxide nanoparticles for labeling leukocytes, lymphocytes, *etc.* [20-22]. Boutry et al. [23] successfully performed the targeting of endothelial inflammatory adhesion molecule E-selectin by MRI with a superparamagnetic contrast agent in the context of *in vitro* and *in vivo* models of the inflammation. Folic acid is also used for this purpose and is grafted on magnetic particles for folate receptors targeting [24]. Cell tracking *in vivo* by MRI can also be done by iron oxide nanoparticles [25, 26]. Cell tracking with a resolution reaching cell size can be obtained by MRI when cell can be loaded sufficiently with magnetic particles [27]. Also to enhance the cellular uptake of magnetic iron oxide nanoparticles, particles can be vectorized with various peptides, fragments of proteins [28] or can be coated with dendrimers [29]. Burtea et al. mentioned cellular magnetic linked immunosorbent assay (C-MALISA), a modified form of enzyme linked immunosorbent assay (ELISA), has been developed for *in vitro* clinical diagnosis can be considered as another application in MRI [14].

Treatment of Cancer

Various methods have already been discovered and many more are in the process of discovery for the treatment of cancer e.g. chemotherapy, hormonal therapy, radiotherapy & surgery [30]. All these methods provide physicians many options and they can choose a suitable method according to the stage and type of disease and more importantly condition of a patient. There is a wide range of drugs being used for cancer treatment and with every passing day numerous new drugs are also being added in the list. A need of proper classification was felt and the proper classification of all anticancer agents is a big task because every drug differs from another in its design, reaction mechanism, structural classification and target tissues, *etc.* An effort has been employed to classify the most commonly used drugs for better understanding and this classification is based on different literature resources [31-33].

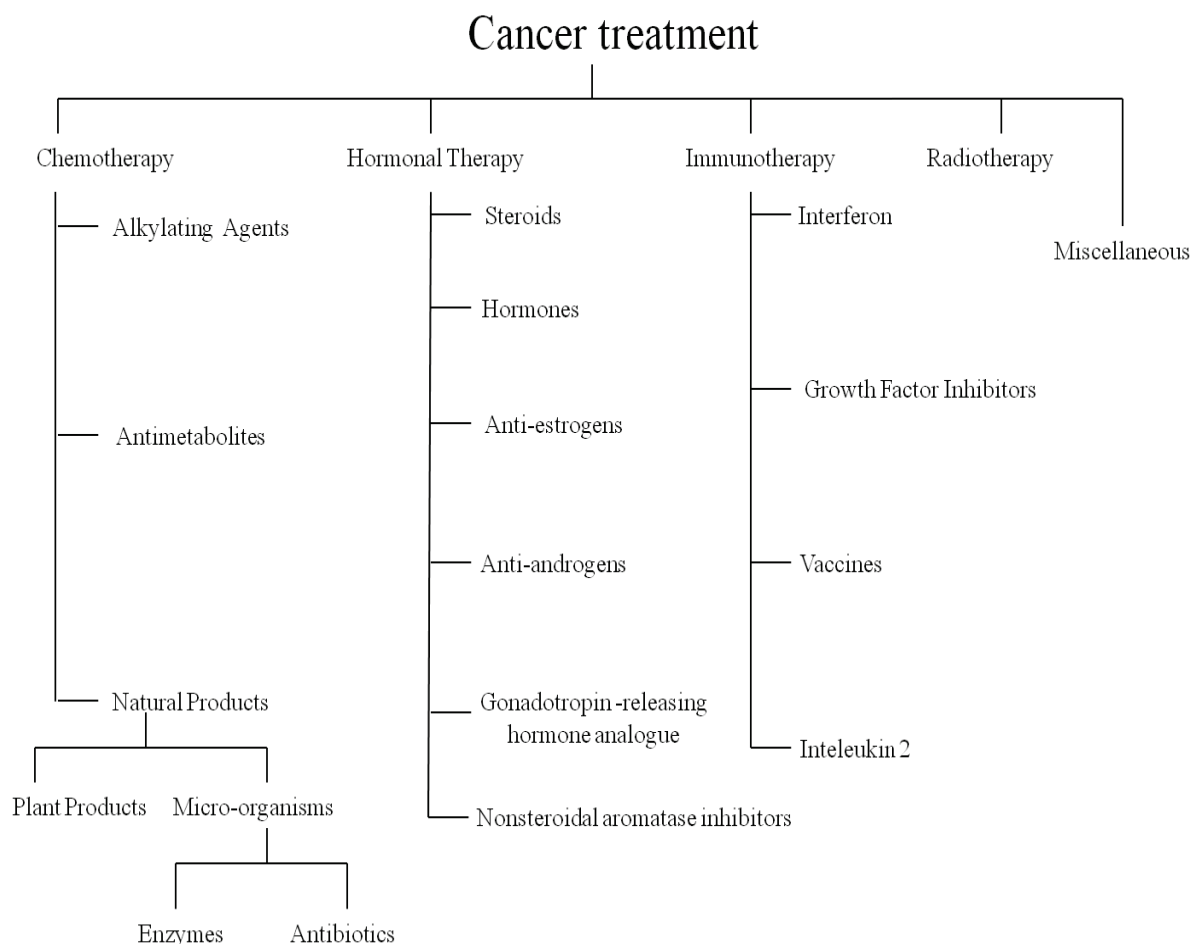


Figure: 1 A Chart for classification of the cancer therapeutic agents

Table.1 Representation of anticancer treatment options with examples

Class	Subclass	Sub-subclasses with examples		
Chemotherapy	Alkylating agents	Nitrogen Mustards e.g. Mechlorethamine, Melphalan, Cyclophosphamide, Ifosfamide, Chlorambucil		
		Nitrosoureas Carmustine, Lomustine		
		Alkylsulfonates Busulphan		
		Ethyleneimines Thiotepa		
		Triazene Dacarbazine, Temozolomide		
		Methyl Hydrazines Procarbazine		
		Platinum Coordination complexes Cisplatin, Carboplatin, Oxaliplatin		
	Anti-metabolites	Folate Antagonists Methotrexate, Trimetrexate		
		Purine antagonists 6-Mercaptopurine (6-mp), 6-thioguanine (6-TG)		
		Pyrimidine antagonists 5-Fluorouracil (5-FU), Cytarabine, Capecitabine, Gemcitabine, Arabinoside, Decitabine.		
	Natural Products	Plants	Vinca Alkaloids Vincristine, Vinorelbine	
			Taxanes: Paclitaxel, Docetaxel	
			Epipodophyllotoxins Etoposide, Teniposide	
			Camptothecins: Topotecan, Irinotecan	
		Micro-organisms	Antibiotics	Anthracyclines Daunorubicin, Doxorubicin hydrochloride, Doxorubicin hydrochloride pegylated, Liposomal epirubicin, hydrochloride, Idarubicin hydrochloride, Valrubicin
				Actinomycins Dactinomycin
				Various Cytotoxic Antibiotics Bleomycin sulfate, Mitomycin, Mitotane, Mitoxantrone hydrochloride
Enzymes e.g. L-Asparaginase				
Hormonal Therapy	Steroids e.g. Prednisolone, Dexamethasone			
	Hormones	Estrogens Diethylstilbestrol sodium diphosphate		
		Progestogens: e.g. Medroxyprogesterone acetate, Megestrol acetate		
	Anti-estrogens Tamoxifen citrate			
	Anti-androgens Bicalutamide, Cyproterone acetate, Flutamide, Nilutamide			
	Gonadotropin-releasing hormone analogs Buserelin acetate, Goserelin acetate, Leuprolide acetate			
	Nonsteroidal aromatase inhibitors Anastrozole, Exemestane, Letrozole, Aminoglutethimide			
Immunotherapy	Interferon Type 1, 2 & 3			
	Interleukin 2 Aldesleukin			
	Vaccines e.g. Melanoma vaccine			
	Growth Factor Inhibitors e.g. Cetuximab, Gefitinib, Erlotinib, Bevacizumab, Rituximab, Imatinib mesylate, Dasatinib			
Miscellaneous Anticancer agents	Retinoic acid derivatives all-trans retinoic acid			
	Others e.g. Porfimer sodium, Amifostine, Arsenic trioxide, Hydroxyurea.			

Therapy

Considering the recent therapies being used for cancer, it might be divided broadly in two parts i.e. various therapies with and without involvement of nanoparticles.

1) Different therapies

Various modalities to treat cancerous tissues include radiation therapy, photodynamic therapy, surgery, chemotherapy and various traditional therapies are used in various regions of the world. Each of these modalities has its own applications and chemotherapy is considered as the most reliable among all. But the anticancer drugs can fail to kill cancer cells for various reasons. One of these reasons can be the transport of drugs to the cancerous cells. The transport of an anticancer drug is normally governed by physiological and physicochemical properties of the target cell and of the drug itself [34]. These properties include pressure, charge, size, configuration, electrochemical properties, hydrophilicity, *etc.* Drugs are usually given systemically and are therefore subject to variations in absorption, metabolism and delivery to target tissues that can be specific to individual patients. Thus for the therapeutic agents delivery to the tumor cells, the following problems can be addressed;

- a. Drug resistance at the tumor levels (non cellular based mechanisms) [35] (For example, a decreased uptake of water-soluble drugs such as folate antagonists, nucleoside analogues and cisplatin, which require transporters to enter cells.)
- b. Drug resistance at cellular level (cellular based mechanisms) [35] (Various changes in cells that affect the cytotoxic capacity of drugs to kill cells, including alterations in cell cycle, increased repair of DNA damage, reduced apoptosis and altered metabolism of drugs.)
- c. Pharmacokinetic properties of the anticancer agent in the body [35].

In last decade the research was inclined to the drug targeting considering a good approach and extensive research is under process in oncology. Targeting of the cancerous tissues within body is a unique approach in the cancer treatment and it was also desirable because mostly anticancer agents demonstrate non specific toxicities significantly limiting their therapeutic efficiencies. There are some factors which can affect the process by modifying the physical and physicochemical properties of the drug and can hinder good targeting. As mentioned above, these factors affect the transport of the particles through the body especially crossing the biological membranes.

With increase in research in oncology, the transplantation phenomenon got fame and is still actively used. The bone marrow transplant and the blood transplantation are the novel applications of this technique for the treatment of cancerous cells and blood cancer.

Gene therapy is another therapeutic technique for cancer where the gene are introduced in the cells to fight against the specific diseased and with the division of the cells, the genes moves to other cells and disease can be mitigated.

Hyperthermia includes the heating of the cancerous tissues to a temperature above the normal body temperature so the cells death caused by heating. It can be local, regional or whole body heating up to the 45 °C thus killing the sufficient amount of the cells. However, it can be used as combine therapy with radiation therapy.

Microparticles were introduced and the anticancer agents/drugs were converted in microparticle formulations for the better efficacy. The concept of the microparticles arisen due to the fact that smaller sized particles allow higher volume to area ratio and thus permitting higher absorption of the drugs in a specific tissue. In the last decade, the microparticle concept is also applied for hyperthermia, radiation therapy, *etc.*

2) Nanoparticles therapy

The struggle for obtaining higher adsorption of the pharmacological agents in the body tissues led to employment of the nanosized agents. The nanoparticles evaluation opened new horizons for all the fields including drug delivery and bringing the term nanomedicines. The nanoparticles are used as diagnostic tools or the carriers for drug or gene delivery. Magnetic nanoparticles with magnetic characteristics were also used for vectorization of the drugs however; the use of magnetic nanoparticles for drug delivery vehicles must address issues such as drug loading capacity, desired release profile, aqueous dispersion stability, biocompatibility with cells and tissue, and retention of magnetic properties after modification with polymers or chemical reaction [36].

Nanomaterials used for medical application

Examples of medical devices utilizing nanotechnology, which are already on the market are surgical tools with enhanced properties, nano-sized contrast agents for molecular imaging, bone replacement materials constructed from nanostructured materials, pacemakers and hearing aids of reduced size and increased power, lab-on-a-chip devices for in vitro diagnostics, wound dressings containing nanocrystalline silver particles, microcantilevers, and

microneedle-based systems for minimally invasive drug administration. Over the next ten to twenty years nanotechnology may fundamentally transform science, technology, and society offering a significant opportunity to enhance human health in novel ways, especially enabling early disease detection and diagnosis, as well as precise and effective therapy tailored to the patient.

At present numerous investigations are being reported on nanotechnology and different materials are being used by different research groups to develop suitable procedures to detect, cure or mitigate the disease. These research groups are working on the applications of this new field on different types of disease like diabetes, nephrology and most importantly on different types of cancer, along with applications in surgery, detection and imaging. Nanotechnology is a suitable tool for delivery of gene, drug and proteins. It is greatly observed that nanoparticles are promising tools for the advancement of drug delivery, medical imaging and as diagnostic sensors. Every research group is working on a specific nanomaterial according to its final requirement that can be improvement in detection, targeting a drug to specific cells, cell repair, *etc.* Surveying literature, a long list of materials including metals, non-metals, metal oxides, semi conductors, polymers, *etc.* along with some combinations of organic and inorganic materials was found that is being used in/as nanomaterials [37].

Nanotechnology tools

In general nanotechnology tools for the biomedical applications include different forms of the nanomaterials designed for a specific purpose. Final applications can play a vital role in designing new shapes and protective layers for the nanomaterials. Table 2 summarizes different forms/tools of nanotechnology being used for cancer treatment.

Table.2 Different nanotechnology tools with applications in oncology

S/No	Name	Applications	References
1	Nanoparticles	Drug delivery, Diagnostic applications	[35,36]
2	Quantum dots	Diagnostics, tumor targeting and live cells study	[38-40]
3	Liposomes	Drug delivery applications	[41,42]
4	Polymeric micelles	Drug delivery, diagnostic applications	[43]

5	Dendrimers	Oral, intratumoral and transdermal drug administration	[44]
6	Nanocantilever	Diagnostic applications	[45,46]
7	Carbon nanotubes	Drug delivery, diagnostic thermal, gene and photodynamic therapy	[47]
8	Stealth nanosystem	Drug delivery	[48]
9	Niosomes	Drug delivery applications	[49]
10	Fullerenes	Drug delivery, diagnostic applications	[50,51]

The different forms of nanomaterial used include nanoparticles, nanotubes, nanopores, dendrimers, *etc.* but nanoparticles are mostly used as the research subject. Further in nanoparticles, there is wide range of materials under consideration because of their different applications e.g. carbon nanotubes, nanocrystals, quantum dots, gold, silver, titanium oxide, zinc oxide, manganese oxide, silicon dioxide, iron oxide, cadmium sulphate, *etc.* Recent studies also show that nanoparticles like Cds can be prepared by microorganisms (fungi, yeast, *etc.*) [52] giving a new direction to nanoparticle research. Another interesting aspect is that nanostructures can be conjugated to biological molecules, including hormones and antibodies which enable their targeting to tissues expressing their cognate receptors [53-55]. For example, fluorescent quantum dots conjugated to various peptides specifically target either the vasculature of normal tissues or alternatively of cancer cells [56]. The rapidly increasing activity of nanotechnology in oncology may be because of factors [57] like a) the discovery of the new forms of matter, buckminsterfullerenes (buckyballs) and nanotubes made of carbon [58,59] b) the increasing ability of material scientists to produce reagents on a small but controlled size scale c) the availability of instruments e.g. atomic force microscope and the scanning tunneling microscope, to complement traditional instrumentation for viewing and characterizing nanoparticles [60] d) the discovery of quantum effects, such as size dependent fluorescent emission in small particles e) Nanoparticulate delivery system provide better penetration of therapeutic and diagnostic substances within the body at a reduced risk in comparison to conventional cancer therapies [61] and f) the stimulation provided by new funding initiatives from worldwide government agencies.

Nanomaterials used in Oncology

Numerous nanomaterials are employed in oncology and a broad classification of the nanomaterials used could be in three main categories depending upon the source; a) organic materials b) inorganic materials c) hybrid materials. This classification is elaborated in Table 3 given below;

Table.3 General classification of nanomaterials with respective applications

Name	Class	Applications	References
Organic polymers	Organic materials	Drug delivery applications	
Silica	Inorganic materials	Diagnostic and drug delivery applications	[62]
Gold	Inorganic materials	Diagnostic and drug delivery applications	[63]
Silver	Inorganic materials	Diagnostic and drug delivery applications	[64]
Gadolinium	Inorganic materials	Diagnostic applications	[65,66]
Cobalt	Inorganic materials	Surgical and diagnostic applications	[67]
Silica	Inorganic materials	Diagnostic and drug delivery applications	[40,68]
Iron oxide	Inorganic materials	Diagnostic applications	[69-71]
Titanium with organic polymers	Hybrid materials	Diagnostic and drug delivery applications	[72,73]
Zirconia with organic polymers	Hybrid materials	Diagnostic and drug delivery applications	[72]
Alumina with organic polymers	Hybrid materials	Diagnostic and drug delivery applications	[72]
Silica with organic polymers	Hybrid materials	Diagnostic and drug delivery applications	[72]
Polyrotaxanes	Hybrid materials	Drug delivery systems	[74]

Although there is a long list of nanomaterials used in oncology with well categorization but mostly they are used in the drug delivery system only. The versatility and the broad applicability of nanotechnology also resulted in the generation of different geometries (e.g. sphere, prism or rod) and structures (e.g. solid, core or shell or dendrimers *etc.*) [57].

Theranostics

“Theranostics” is a new term coined for the drugs or the methods which are used for the diagnosis and treatment at the same time. Simple representation by Warner. S. [70, 75] is
Diagnostics +Therapy = Theranostics

Theranostic nanomedicine is nanotechnology implementation for the preparation of these types of drugs. This term can be defined as “an integrated nanotherapeutic system, which can diagnose, deliver targeted therapy and monitor the response to therapy”. This is a useful idea in designing nanotechnology-based imaging contrast agents and imaging guided therapeutics [76]. It is assumed that this combined technique will result in the acceleration of drug development, improvement of disease management, reducing risks and cost. The diseases which are the main cause of the morbidity and the mortality at present in the world are in the necessity of such type of new trials for the quick diagnosis and treatment because some of these diseases require rapid diagnosis and long treatment duration so theranostics can be proved as a revolutionary technique in this specific aspect. One of these diseases is cancer and due to its significance, the earlier research on theranostics is mostly inclined towards oncology. The important thing in the creation of a theranostics is requirement of a wide knowledge and deep understanding of both mechanisms of detection and therapy. This knowledge requirement includes the understanding of molecular mechanisms, diagnostic strategies, therapeutic efficiency, toxic and side effects of the materials and the nanoparticle preparation techniques for dual purpose of diagnosis and therapy. Hence, a suitable theranostic is expected for all the diseases especially for cancer in future although it will take some time. A general sketch of a nanoparticle for the cancer is given for the structural understanding of the particles.

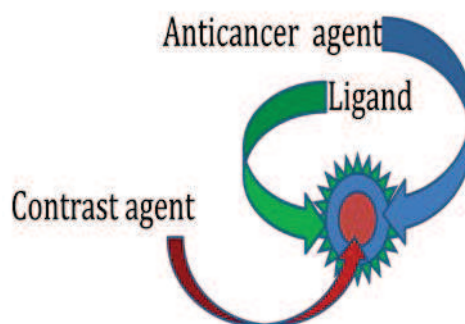


Figure: 2 Structural sketch of nanoparticles to be used as theranostic agent

The principal aim is to prepare a nanoparticulate system that could simultaneously serve purposes of therapy and diagnosis to serve as an effective and efficient theranostic agent. The factors influencing all processes starting from preparation of nanoparticles until the elimination of the metabolites of active molecule and other used materials are important to be considered. Study of these factors requires a keen interest, full concentration and deep investigation of every step before employing it. In a broader sense, these factors can be; the compatibility of the chemicals used, preparation conditions, formulations/modifications according to the route of administration selected, biocompatibility and biodegradability of the materials, toxicity of the materials and their metabolic products, pharmacokinetics and pharmacodynamic parameters evaluation and at the end benefits/disadvantage ratio of all processes.

The nanotechnology involvement made easy the realization of theranostic concept. The minute sized particles containing two different agents have been elaborated. Therapeutic and diagnostic agents has been prepared in nanosized particles and used for theranostic applications. The magnetic particles helped in the realization of this concept, having magnetic properties that can be exploited as diagnostic tools. The choice of the diagnostic tool depends upon the target site. Normally the magnetic particles are involved for imaging of the diseased tissues in the body. Nevertheless, they are involved for other biomedical applications like treatment via hyperthermia (killing of diseased cells using heat), drug delivery, *in vitro* diagnostic techniques (like bioassays), *etc.* Some exemplified works with concept include manganese oxide nanoparticles carrying both drugs and contrast agents [68], silica NPs with magnetic and fluorescent tags [40]. In last decade, a lot of attention focused on magnetoplasmonic nanoparticles preparation by combining both magnetic component and metal NPs or shells [77-83]. Magnetic resonance imaging (MRI) is mostly employed diagnostic technique using magnetic materials as contrast agents for the visualization/

differentiation of diseased cells/tissues from healthy cells/tissues. The researchers are using different contrast agent and especially particles having magnetic properties are being investigated e.g. Gadolinium, Iron oxide, gold, silver and other metals are in the process to find a suitable one with less toxicological effects. Among these, the iron oxide particles are of great interest because of superparamagnetic activity and well understanding of their properties, as aforementioned. Cancer being the main focus of theranostic, nanoparticulate agents incorporating the possibility of cancer diagnostics and therapeutics [84-86] had been developed. A representation of particles prepared as theranostic for cancer including iron oxide is given in following figure 3.

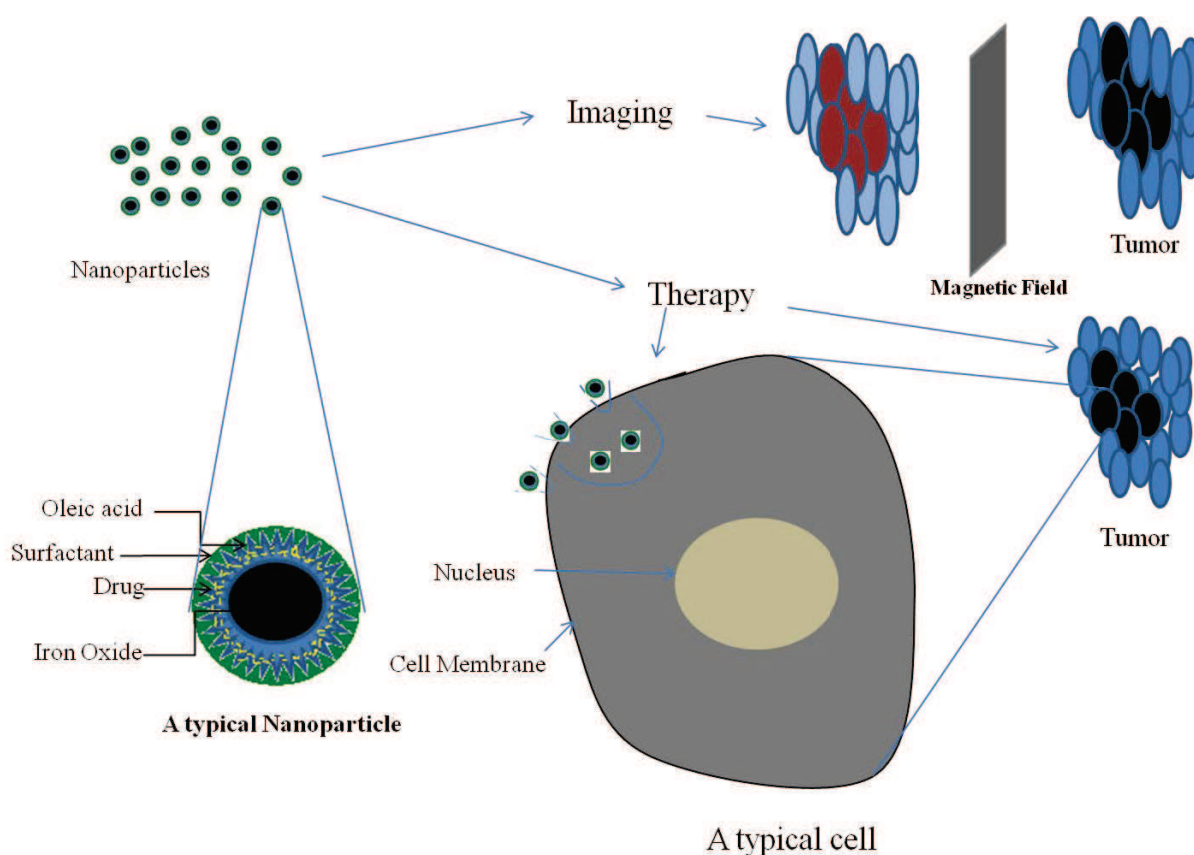


Figure: 3 Representation of iron oxide loaded nanoparticle for therapy and diagnosis.

Toxicities of the nanomaterials

The important parameter in application of the concept of theranostic is the understanding of fate of these particles in the body. In general the pharmacokinetic and pharmacodynamic studies are carried out but special focus should be on the toxicity profile of the materials that particles contained. Attention should be paid on the material being added for diagnostic purposes as therapeutic agent is normally well studied for pharmacological effects.

In nanotechnology application, toxicology profile of nanomaterials used expands to environment also after their removal from bodies or before administration. Thus a broader division of nanomaterials toxicity includes environmental, genetic, cyto and pharmacokinetic toxicity. It is impossible to present a broader aspect of all these types of the toxicities here; a general discussion can be useful.

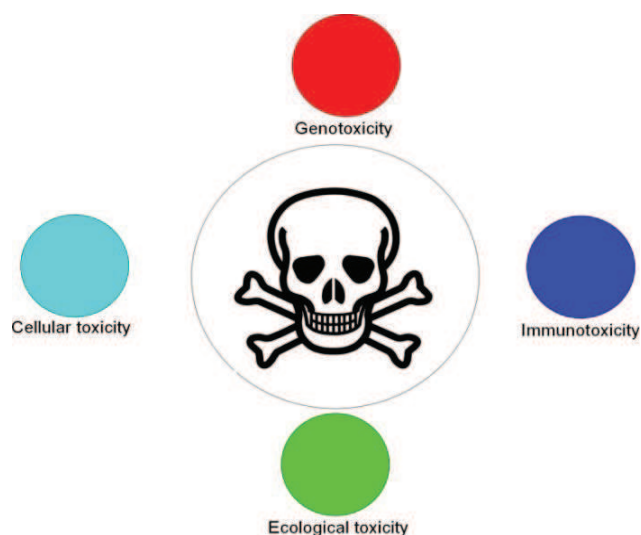


Figure: 4 Nanoparticles toxicity

Main source of the pharmacokinetic toxicity is modification brought to the nanoparticles for enhancement of their bioavailability or delivery to the target site. It could be possible by using the minute size of the nanoparticles that will circulate for long time in the main circulation. However, stealth mechanisms is mainly responsible. The charges on the particles is also an important factor to be considered and could cause the long circulation in the blood. One such study was done by Levchenko et al. [87] on liposomes administered to the rats mentioning the effects and reasons of long circulation.

Conclusion and future perspectives

Application of nanotechnology in biomedical field accelerated the research in various subfields of biomedical field especially in diagnostic mechanisms, drug and gene delivery. Nanomaterials of various origins have been applied in these subfields. Cancer being fatal disease received the nanotechnology application at earliest. As diagnosis is important for any disease, nanomaterials are applied in the diagnosis of cancer both in vivo and in vitro. For the treatment, therapy consisting of nanoparticles has been discussed along with various nanotechnology tools that are being used in oncology. Progress in research developed a new term of theranostics relating to diagnosis and therapy at same time. Various materials are

applied in different ways after the arrival of this specific subfield of biomedical applications. Another such term for dual functionalities has been coined but not related to cancer. It is cosmeto-therapy where nanoparticles can be used for therapy and cosmetics simultaneously. The particles contains the moieties for therapeutic effects and could be used or the cosmetics. One such work has been investigated recently by our group [88]. Focusing on theranostic aspects of the nanotechnology applications in cancer, the diagnostic part in vivo is mostly dedicated to the imaging techniques e.g. MRI and other imaging techniques are involved. The nanomaterials are involved in the enhancement of contrast in these imaging mechanisms. But with the progress and development in this field, it could be hoped that theranostic will bring a lot for the diagnosis and treatment of cancer. The diagnosis and therapy will move towards monitoring of response and hence aheading towards sort of personalized medication. Further it will be extended to treatment of other diseases.

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CHAPTER II.2

THERANOSTIC APPLICATIONS OF

NANOPARTICLES

Résumé

Les nanoparticules sont des objets d'intérêt soumis à de nombreuses études pour des applications biomédicales ces dernières années. Ils sont utilisés aussi bien en oncologie que dans d'autres branches de la nanotechnologie biomédicale. En ce qui concerne le cancer, les nanoparticules, et en particulier les nanoparticules magnétiques, sont étudiées pour le diagnostic, la délivrance de médicament, la vectorisation de gènes, la bio-séparation, l'hyperthermie, la photothérapie, la chimiothérapie, les mécanismes d'imagerie, *etc.* Diverses techniques sont utilisées pour préparer des nanoparticules multifonctionnelles et pour modifier leurs surfaces, nécessaires pour différentes applications. Cette revue porte sur l'approche théranostics de base, les différents matériaux utilisés dans le domaine theranostic, leurs applications et les orientations futures basées sur les développements récents dans ce domaine.

General Summary

Investigation on nanoparticles is among the most researched areas in biomedical field for their various applications. The application of nanotechnologies for anticancer drug delivery has been explored extensively in the hope of improving the efficacy of chemotherapy and reducing its side effects. Various applications for cancer include molecular tumor imaging, early detection (with high-throughput nanosensor devices for detecting the biological signatures of cancers), molecular diagnosis, drug delivery, targeted therapy, gene delivery, bioseparation, hyperthermia, phototherapy and cancer bioinformatics, *etc.* Among them, an important application of nanotechnology is the preparation of dual purpose nanomaterials used for simultaneous diagnosis and therapy. This dual purpose targeted research has given rise to a new term, theranostics: agents used for diagnosis and therapy at the same time. Many techniques have been developed for the preparation of nanoparticles and their relative modification for these large range applications. Variation of the technique may be depends upon the site of cancer to be targeted and active ingredient chosen for therapeutic purposes. Different organic/inorganic moieties are used for this theranostic aspect to be served as whole as contrast agent or as a support for the delivery of other related contrast agents to be used in imaging mechanisms.

In this review, a discussion on the concept of theranostics, different nanoparticles used as theranostics in cancer with an active ingredient (Nucleic acid/drug), a short description of various theranostic applications while in the end an outlook for the future perspectives is given with various recent developments.

Theranostic Applications of Nanoparticles in Cancer

Naveed AHMED, Hatem FESSI and Abdelhamid ELAISSARI*

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

**E-mail: elaissari@lagep.univ-lyon1.fr*
Telephone: 0033-472431841 Fax: 0033-472431682

Published in Drug Discovery Today (2012)

(<http://dx.doi.org/10.1016/j.drudis.2012.03.010>)

Abstract

Nanoparticles are the moieties that have undergone the most investigation in recent years for biomedical applications. They are applied in oncology in the same way as in other branches of biomedical nanotechnology. Regarding cancer, nanoparticles and especially magnetic nanoparticles, are studied for diagnosis, drug delivery, gene delivery, bioseparation, hyperthermia, phototherapy, chemotherapy, imaging mechanisms, *etc.* Different techniques are used to prepare multifunctional nanoparticles and modify nanoparticles surfaces required for different applications. This review focuses on the basic theranostic approach, the different materials used in theranostics, theranostic applications and future directions based on recent developments in these areas.

Key words: Nanoparticles, cancer, drug delivery, imaging, theranostics

Abbreviations used: Nanoparticles (NPs), Iron oxide nanoparticles (IONPs), Carbon nanotubes (CNTs), Magnetic Resonance Imaging (MRI), Doxorubicin (DOX), Methotrexate (MTX), Paclitaxel (PTX),

Introduction

Medical uses of nanotechnology include drug delivery, protein and peptide delivery, nanoparticles targeting, neuro-electronic interfaces, surgery and visualization. Combination of nanotechnology with molecular biology has developed into an emerging research area known as nano-biotechnology [1] while the similar term of biomedical nanotechnology is given to the use of nanotechnology in the medical sector. The development of biomedical nanotechnology research has focused on the detection of molecules associated with diseases like cancer, diabetes mellitus, and neurodegenerative diseases, along with the detection of microorganisms and viruses associated with infections, e.g. pathogenic bacteria, fungi, and HIV viruses. Cancer is one of the major five diseases that caused millions of deaths in the twentieth century, still remains a difficult disease to treat and a significant cause of morbidity/mortality with more than 10 million new cases per year. It is a malignant disease and treatment with anticancer drugs at present is only for palliative purposes or to induce remission, with a small percentage of cures. Various methods for directly introducing anticancer drugs in the body include direct injection into tumor, tumor necrosis therapy, injection into the arterial blood supply of the cancer, local injection into tumor for

radiopotential, localized delivery of anticancer drugs by electroporation (electrochemotherapy) and local delivery by anticancer drug implants. Further specification may include heat-activated drug delivery, targeted drug delivery, tissue selective drug delivery, using vascular targeting agents, using a carrier or selective permeation of the anticancer agent into the tumor, *etc.* Any of these methods can be selected depending on the location of tumor, drug specifications and severity of the treatment, *etc.* With other branches like nano-nephrology, advances in proteomics and genomics, nanotechnology has also emerged as one of the most fruitful fields in cancer treatment and is considered as a medical boon for the diagnosis, treatment and prevention of this disease. The important aspect to be considered in the application of nanomaterials in the medical field, especially in biological organisms, is biocompatibility. Thus to apply nanotechnology in biology and medicine, several conditions must be considered. Firstly, nanomaterials must be designed to interact with proteins and cells without interfering with their biological activities. Secondly, nanomaterials must maintain their physical properties after surface modifications and finally they must be non toxic [2].

The application of nanotechnologies for anticancer drug delivery has been explored extensively in the hope of improving the efficacy of chemotherapy and reducing its side effects [3]. Applications for cancer include molecular tumor imaging, early detection (with high-throughput nanosensor devices for detecting the biological signatures of cancers), molecular diagnosis, targeted therapy and cancer bioinformatics. Above all, an important application of nanotechnology is the preparation of dual purpose nanomaterials used for simultaneous diagnosis and therapy. This dual purpose targeted research has given rise to a new term, theranostics: agents used for diagnosis and therapy at the same time.

In this review, we discuss the concept of theranostics, different nanoparticles used as theranostics in cancer, theranostic applications and the outlook for the future.

Theranostics

“Theranostics” is a new term coined for drugs or methods used for diagnosis and treatment at the same time. A simple definition of theranostics by Warner S. [4] is diagnostics plus therapy. In fact, theranostic nanomedicine is the implementation of nanotechnology for the preparation of these types of drugs. This term defines “an integrated nanotherapeutic system which can diagnose, deliver targeted therapy and monitor the response to therapy”.

This is a useful concept when designing nanotechnology-based imaging contrasting agents and imaging guided therapeutics [5].

It is assumed that a combined technique will result in the acceleration of drug development, improved disease management, with reduced risks and cost. Some of the diseases that are currently the main cause of morbidity/mortality are in great need of such investigation into quick diagnosis and treatment. Cancer is one of these diseases and by chance, initial research into theranostics is oriented towards oncology. The creation of a theranostic agent requires wide-ranging knowledge and deep understanding of both detection and therapy mechanisms. This knowledge includes understanding of molecular mechanisms, diagnostic strategies, therapeutic efficiency, the toxic and side effects of materials and nanoparticle preparation techniques for the dual purpose of diagnosis and therapy. Hence, suitable theranostic approaches are expected for all diseases, especially cancer, in the future, although this will take some time. In the last decade research into theranostics has accelerated enormously and has resulted in different preparation techniques involving different contrast and active ingredients. Examples of all the trials performed are given in the table 1 below.

Table 1 Different theranostic agents used for biomedical applications

Contrast agent	Drug used	Applications	References
Manganese oxide	siRNA	MRI + RNA delivery	[6]
Gold	DOX	Diagnosis, tumor targeting and Photothermal therapy	[7-9]
Iron oxide	siRNA, DOX, Docetaxel	Targeting, MRI and therapy	[10-12]
Silica	Pyropheophorbide (HPPH), DOX	Drug carrier, X-ray/CT imaging, Photodynamic therapy	[7,13-14]
CNTs	DNA Plasmid, DOX, PTX	Diagnosis, DNA and drug Delivery	[15-16]
QDs	DOX, MTX	Imaging, therapy and sensing	[17-18]

Abbreviations: Short interfering ribonucleic acid (siRNA), Carbon nanotubes (CNTs), Quantum dots (QDs), Doxorubicin (DOX), 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-alpha (HPPH), Methotrexate (MTX), Paclitaxel (PTX), Magnetic resonance imaging (MRI), Computed topography (CT).

The aim is to prepare a dual-purpose nanoparticulate system. Hence, it is important to take into account the factors influencing all the processes involved, starting from the preparation of nanoparticles until the elimination of the metabolites of the active molecule and other materials used. Studying these different factors is by no means easy and requires keen interest, great concentration and thorough study of every step before application. In a broader sense, these factors can be the compatibility of the chemicals used, the preparation conditions, the formulations/modifications as a function of the selected route of administration, the biocompatibility and biodegradability of the materials, the toxicity of the materials used and their metabolic products, the assessment of pharmacokinetics and pharmacodynamic parameters and finally the trade-off between the benefits and disadvantages of all the processes.

The diagnostic aspect of theranostics mostly turns around imaging mechanisms using different contrast agents. Among different imaging mechanisms, MRI is the technique, studied most and much research has been devoted to magnetic particles used as contrast agents. Particles of gadolinium, iron oxide, gold, silver and other metals are being investigated in view to finding a suitable one with the fewest toxicological effects. The purpose of using these particles in MRI is to highlight diseased tissues/cells in contrast to healthy tissues/cells. As mentioned above, cancer is now one of the most dangerous diseases so theranostic research has mostly concentrated on cancer. Much research has already been done in this area and more is in progress. Nanoparticulate agents incorporating the possibility of cancer diagnostics and therapeutics [19-21] have been developed. To improve understanding of basic concept of cancer diagnosis and therapy, a schematic illustration is given in the Figure 1.

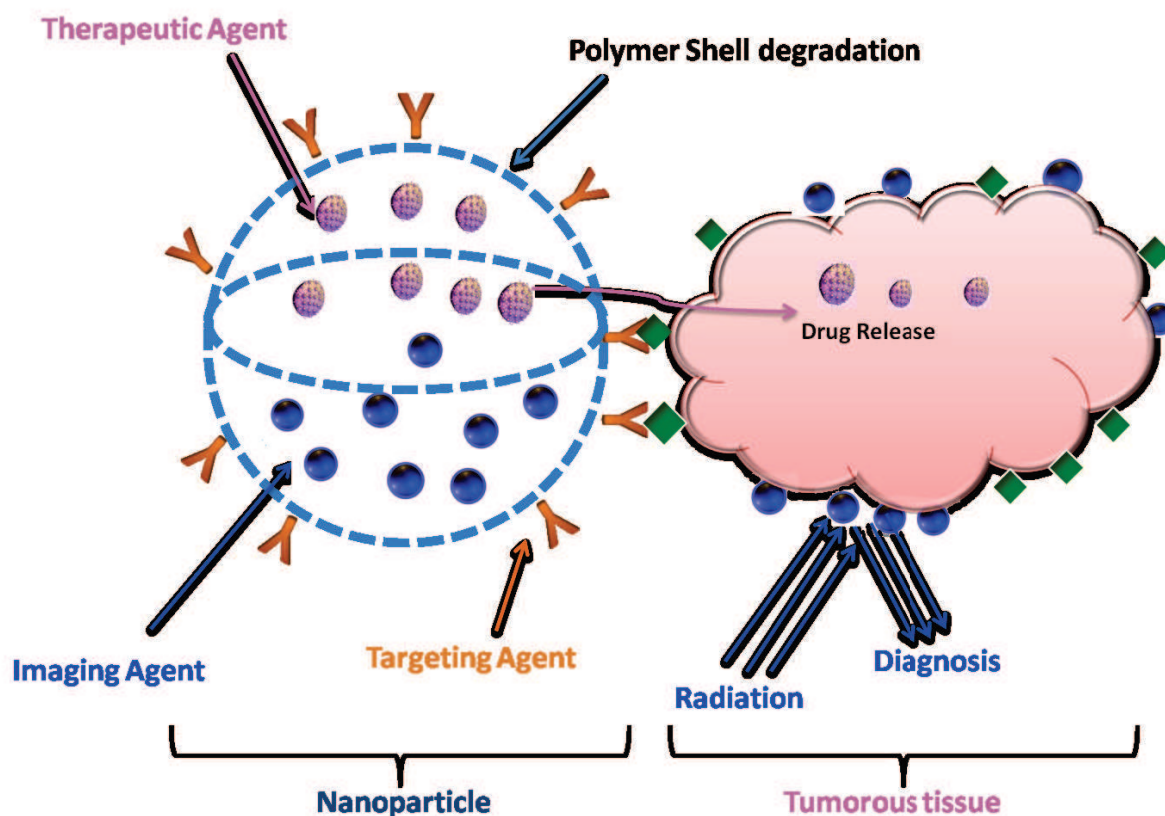


Figure: 1 Schematic illustration of cancer diagnosis and therapy with nanoparticles

Theranostic agents can be exemplified by manganese oxide nanoparticles carrying both drugs and contrast agents [22], and silica NPs with magnetic and fluorescent tags [23]. In the last decade, much attention has been given to magnetoplasmonic nanoparticle preparation by combining a magnetic component and metal NPs or shells [24-29].

Iron oxide nanoparticles in cancer theranostics

Iron oxide nanoparticles (IONPs) are of great interest as contrast agents because of their superparamagnetic activity, biocompatibility and very low cost, allowing research benches to use them easily. IONPs are used in magnetite or hematite form. Further surface modifications with different inorganic molecules, ligands, and polymeric and non polymeric stabilizers provide the opportunity to use IONP-based agents for different applications. Therapy with diagnosis is such an application of IONPs that combines different active agents. Kohler et al. [30] prepared MTX modified superparamagnetic iron oxide nanoparticles (SPIONs) for MRI and drug delivery purposes. MTX is a folate antagonist and is used as a chemotherapeutic agent for cancer. Trastuzumab, a monoclonal antibody interfering with cell membrane receptors, was coupled with modified IONPs by Huh [31] and Lee et al. [32]. The

same group reported the preparation of all-in-one target cell specific magnetic nanoparticles for imaging and siRNA delivery [10]. Veisheh et al. [33] developed magnetic nanovectors to exploit the cell transcytosis intracellular trafficking pathways, offering a safer and more efficient route for siRNA delivery. Magnetic properties were obtained for IONPs for cancer gene therapy. DOX, an anthracycline, was coupled with modified IONPs for lung carcinoma [34]. Recently, Piao et al. [35] investigated hollow IONPs loaded with doxorubicin by physical adsorption. IONPs were also coupled with PTX, a taxane, and doxorubicin in the same preparation to procure enhanced therapy and diagnosis [36]. Yang et al. [37] prepared cRGD-functionalized, DOX-conjugated, and ^{64}Cu -labeled SPIONS for targeted anticancer drug delivery and PET/MR imaging of tumors with integrin $\alpha_v\beta_3$ expression. An anticancer drug was conjugated onto PEGylated SPIO (superparamagnetic iron oxide) nanocarriers while tumor targeting ligands were conjugated onto the distal ends of the PEG arms. Stable and tumor-targeting multifunctional wormlike polymer vesicles loaded with (SPIONS) and DOX were developed for targeted cancer therapy and ultrasensitive MR imaging [37]. These multifunctional wormlike polymeric vesicles were prepared by hetero-bifunctional triblock copolymers R (methoxy or FA)-PEG₁₁₄-PLA_x-PEG₄₆-acrylate *via* a double emulsion technique. Both active and contrast agent loading contents were analyzed. However, Kievit et al. [38] investigated the loading of DOX on SPIONS using the pH labile hydrazone bond to overcome multidrug resistance *in vitro* for cancer. To increase the efficacy of chemotherapeutics, PEG coated SPIONS were conjugated to DOX already bound to polyethylene amine through hydrazone linkage, and different *in vitro* assays were performed for evaluation. Paclitaxel was also conjugated alone with iron oxide and gold nanoparticles by Hwu et al. (39). Cheng et al. (40) loaded cisplatin, an alkylating agent, on porous hollow IONPs. Ling et al. [12] reported dual docetaxel/SPION loaded nanoparticles for ultrasensitive MRI and prostate cancer therapy. However, multimodel nanoparticles consisting of IONPs are new trends providing multi-diagnostic opportunities. Xie et al. [41] prepared PET/NIRF/MRI triple function IONPS for a better diagnostic approach. Nanoconjugates were prepared by modifying the surface of IONPs using dopamine for easy encapsulation into Human serum albumin (HSA) matrices used as drug carriers. By contrast, Xu et al. [42] reported a nanocomposite of polymer encapsulated upconversion nanoparticles/IONPs. These nanocomposites were prepared for multimodel (upconversion luminescence (UCL)/down-conversion fluorescence (FL)/magnetic resonance (MR) imaging and magnetic drug delivery for *in vitro* and *in vivo* use.

Carbon nanotubes in cancer theranostics

Carbon nanotubes (CNTs) are single or multilayered cylindrical structures and can be considered as allotropes of carbon. They have unique mechanical and electronic properties valuable for material chemistry and nanotechnology. CNTs are used for imaging thermal ablation and for gene and drug delivery. The importance of CNTs has increased due to their efficient uptake by cells, although the exact mechanism is still unclear. They are used as a template for loading different active agents for delivery purposes. A study on this topic was presented by Pal et al. [43] in which they coated core shell nanotubes with zirconium oxide, hence proving the possibility of loading other active materials. Their strong optical absorbance makes CNTs an interesting tool for NIR photothermal ablation therapy. Theranostic applications are investigated by stacking active ingredients onto CNTs, which is means of drug loading. Liu et al. [44] investigated *in vivo* and *in vitro* detection and drug delivery by loading DOX on CNTs. Pantarotto et al. [15] used CNTs for gene delivery while Boucetta et al. [45] demonstrated DOX loading on copolymer coated CNTs. Yang et al. [37] prepared magnetic functionalized CNTs to deliver gemcitabine, an antibiotic for the treatment of cancer lymph node metastasis. Sitharaman et al. [46] coupled paramagnetic gadolinium nanoparticles with CNTs for high performance MRI. Loading and delivering DNA plasmid was demonstrated by Pantarotto et al. [15] and Singh et al. [47] while Kam et al. [48] investigated CNT functionalization using enzymatically cleavable disulfide bonds for siRNA delivery. Liu et al. (49) used CNTs as transporters for the siRNA delivery to human T cells and primary cells. In another study, Liu et al. (16) reported paclitaxel delivery by coupling surface modified CNTs for *in vivo* cancer treatment. Fullerenes are closer to carbon nanotubes from the structural standpoint while Krishna et al. [50] reported their use for photoacoustic imaging and photothermal ablation therapy. The important point in his report was rapid tumor reduction by photoacoustic tomography.

Quantum dots in cancer theranostics

Quantum dots (QDs) are inorganic semiconductor fluorophores based on the elements belonging to transition groups of the periodic table. QDs are used for biological applications, especially for imaging and drug delivery, though they have been subjected to less research due to their toxicological profile. The quantity of drug delivered is increased due to flexible linking with active ingredients and other properties like optical spectral changes as a function of the size of the nanoparticle core. However, toxicity can be minimized by preparing QDs of

silicon and carbon [51]. QDs are mostly used for therapeutic purposes and less for imaging, especially *in vitro* imaging. Gao et al. [52] prepared quantum dot based conjugates and investigated accumulation inside tumors in mice. In this case quantum dots were modified to form conjugates with other ingredients. Bagalkot et al. [53] reported a novel conjugate based on QDs-aptamer and DOX for targeted cancer imaging, therapy and sensing. The aim was to prepare a system to deliver DOX to targeted prostate cancer cells. Sensing of DOX delivery and imaging was done using fluorescence imaging with QD. Savla et al. [17] reported a pH-responsive quantumdot-mucin1 aptamer-doxorubicin (QD-MUC1-DOX) conjugate for chemotherapy of ovarian cancer along with imaging. In this study QD was conjugated with a specific DNA aptamer and DOX was attached to QD via a pH-sensitive hydrazone bond. *In vivo* imaging studies have presented higher toxicity of QD-MUC1-DOX conjugate in multidrug resistant cancer cells and preferential accumulation in ovarian tumors. Yuan et al. [18] investigated methotrexate loading via simple adsorption by studying DNA-drug interactions using a photoinduced electron-transfer mechanism based on luminescent QDs. Derfus et al. [54] prepared conjugates and studied them for siRNA delivery. Their main goal was a composite preparation for diagnostic imaging and treatment of metastatic cancer at the same time. Using a PEGylated quantum dot (QD) core as a scaffold, they conjugated siRNA and tumor-homing peptides (F3) to the functional groups on the particle's surface. In addition, they estimated the ratio between the number of F3 peptides and the number of siRNAs per particle.

Gold in cancer theranostics

Melancon et al. [55] prepared multifunctional gold based nanoshells with both optical and magnetic properties, conjugated with the targeting agent, superparamagnetic gold coated iron oxide nanoshells. This preparation was investigated for head and neck cancer. Tunable gold nanoprobe have been prepared for theranostic application in prostate cancer [56]. The later suggested a method of cell theranostics with rainbow plasmonic (gold) nanobubbles. Plasmonic nanobubbles are not nanoparticles but nanoparticle-related mechanisms. This mechanism improves the selectivity of bubble generation in specific target cancer cells, providing cell level selectivity for diagnosis and therapy. Similar work was also done by Yang et al. [11] but they targeted built-in hotspots in gold nanoshells for diagnosis and delivery with slow drug release. Fales et al. [57] mentioned a potential nanoplatform for theranostics by coating gold nanostars with silica. This nanocomposite consisted of a

methylene blue sensitizing drug for singlet oxygen generation for photodynamic therapy. Such types of nanocomposites were also reported. Nanocomposites containing silica-coated gold-silver nanocages and yb-2, 4-dimethoxyhematoporphyrin were reported for multifunctional IR-luminescence detection, photosensitization, and photothermal lysis. Huang et al. [7] studied folic acid-conjugated silica-modified gold nanorods for X-ray/CT imaging-guided dual-mode radiation and photo-thermal therapy. Gold nanorods were modified with silica instead of the usual CTAB and folic acid was anchored to the gold nanorod surface by silane coupling agents. The results were obtained through X-ray/CT imaging with highly selective targeting, enhanced radiation therapy (RT) and photo-thermal therapy (PTT). Gold is also used for targeted drug delivery. For example, Gibson et al. [58] prepared Paclitaxel functionalized gold nanoparticles. A photodynamic therapeutic agent Pc4 with gold nanoparticles vectors was studied by Cheng et al. [59] for in vivo photodynamic therapy. Prabaharan et al. [9] loaded DOX on gold nanoparticle cores for tumor targeting and drug delivery. Moreover, Ji et al. [60] reported bifunctional gold nanoshells with a superparamagnetic iron oxide core suitable for MRI and PTT. Drug loading on such types of core shells can also be used for this purpose.

Silica in cancer theranostics

Silica has been used for implants for a long time and is considered as toxicologically safe. In nanomedicine, it is normally used as a coating material to provide or avoid different nanoparticle characteristics. Silica particles are frequently used in mesoporous form, making them a suitable choice of researchers. Vivero-Escoto et al. [61] prepared gold capped mesoporous silica nanospheres loaded with anticancer Paclitaxel while Giri et al. [62] proposed a mesoporous system capped with magnetic nanoparticles. Lee et al. [10] reported a silica-based nanoparticle preparation for MRI, fluorescence imaging and drug delivery. This method increased magnetic and optical imaging capabilities and DOX was loaded successfully. Park et al. [14] loaded DOX on luminescent nanoparticles of silica for in vivo applications. By contrast, Roy et al. [13] devised silica-based particles that entrapped a water insoluble anticancer drug for photodynamic therapy. On the same lines, Kim et al. [63] co-encapsulated a water insoluble drug with two photon absorbing fluorescent dye aggregates for photodynamic therapy. Huang et al. [7] reported novel multifunctional nanoprobe for imaging and therapy. Folic acid-conjugated silica-modified gold nanorods were prepared for

X-ray/CT imaging-guided dual-mode radiation and photo-thermal therapy, as mentioned in the section on gold.

Theranostic applications

The theranostic approach in cancer is mostly oriented towards diagnosis and drug delivery. Nevertheless, as development progresses, the concept opens up other perspectives and it has been found to have a wide range of uses and applications. It is impossible to review all the approaches here but some examples are provided while a brief overview is given in the Figure 2.

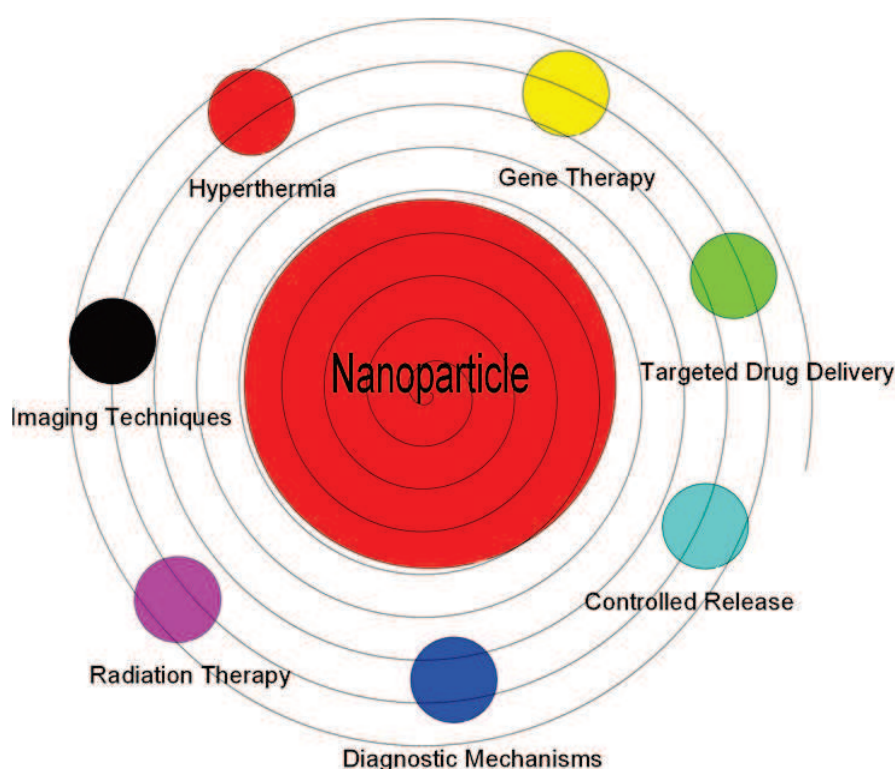


Figure: 2 Different axes of nanoparticles applications as theransotic agents

Multifunctional nanoparticles for simultaneous cancer diagnosis and therapy prepared by Yang et al. [64] illustrate theranostic applications. In this study, poly (aspartic acid) was used as carrier for drug delivery being biodegradable. IONPs synthesized by thermal decomposition method were loaded on poly (aspartic acid) nanoparticles via an emulsion method while DOX was incorporated into Multifunctional nanoparticles via solvent diffusion method. IONPs were used for the enhancement of T2 contrast while DOX was loaded for cancer therapeutic applications. They showed that DOX was released succesfully from nanoparticles and MRI study was done using IONPS as contrast agent for diagnosis purposes.

Similar results were produced by Xu et al. [42] using DOX and IONPS with a block copolymer. They also proved the in vitro and in vivo imaging and drug delivery and imaging guided therapeutic applications of prepared nanocomposites. Recently another interesting work is done by Yang et al. [65] for preparation of theranostic agents and observation of theranostic applications in vitro and in vivo. They prepared nanocomposite of graphene-oxide with iron oxide for imaging and intratumoral drug delivery. Nanocomposite was prepared and functionalized with a biocompatible polymer Polyethylene glycol (PEG). This functionalization was done to reduce the toxic effects of graphene. With imaging guidance they were able to design a photothermal study for treatment of tumor and obtained results using ultra efficient tumor ablation. Different doses were tested and no side effect in vivo or in vitro toxicity was observed, hence providing another probe for in vivo multimodal tumor imaging and imaging guided photo thermal therapy.

Conclusion and future strategies

The concept of theranostics agents is new and based on the application of different diagnostic and therapeutic mechanisms combined in a single moiety. Nanomaterials are the first materials to be used as theranostic agents. Existing preparation methods for theranostics have been developed on the basis of nanoparticles preparation performed by different polymerization and nanoencapsulation techniques. The multiple emulsion method is an easy non-polymerization technique for preparing nanoparticles with hydrophobic and hydrophilic agents. One noteworthy method is the double emulsion technique that provides the possibility of emulsifying hydrophilic and hydrophobic agents with the dual choice of w/o/w and o/w/o. [66] Detail study of the factors influencing the process will allow developing a general methodology for the encapsulation of different anticancer and contrast agents together, irrespective of their hydrophilic/hydrophobic nature. Surface modifications and ligand attachment using silica, dextran, CTAB, *etc.* will improve the pharmacokinetic properties of the final preparation.

Furthermore, polymerization techniques have been used to develop Janus-like particles for different applications. The name 'Janus' comes from the Roman god with two faces. Likewise, these particles have two faces that can be hydrophilic and hydrophobic, hence allowing the loading of different active materials. This loading can be used for theranostic purposes as it is possible to load a hydrophilic contrasting agent with a hydrophobic active ingredient and vice versa. The selection of different polymers and other materials has to be

done as a function of their biocompatibility and biodegradability profile. Sotiriou et al. [67] prepared silica coated Ag/Fe₂O₃ nanoparticles with two faces using aerosol technology for drug delivery and imaging purposes. The advantage of silica coating is the reduction of toxic effects of silver and increased stability, with easy dispersion in biological solutions. Same group is working on other organo-metallic particles for various biomedical applications. [67]

As a summary, it can be said that trials are underway for an ultimate remedy of cancer. This dual purpose approach is an important consideration to arrive a permanent solution of cancer. The discussion was aimed towards theranostics and among all the methods mentioned the diagnostic applications were the result of metallic part in the nanocomposites irrespective of choice which is used (e.g. IONPs or gold) while therapeutic applications are because of an anticancer agent. The various investigations and trials were made on the basis of characteristics of inorganic/metallic portions. For example IONPs were used for MRI and different preparations were proposed with iron oxide nanoparticles as combination with number of anticancer drugs. Then studies done for the modification of these preparations or nanocomposites on the basis of the use of anticancer drug or simply site and mode of action of the drug selected. These studies were further divided in vivo and in vitro analysis. Nonetheless, the basic purpose of IONPs use was MRI. CNTs are being used as carrier of different materials including some contrast agent and sometimes for imaging purposes. QDs are also in use for therapeutic purposes more than for imaging techniques. For diagnostic purposes, they have been used in fluorescence imaging. Gold particles are used for the diagnosis taking help from X-ray/CT scan techniques, *etc.* To mask the toxic effects of gold particles and to gain the required characteristics, various modifications were brought e.g. silica coating on gold particles that is also used as theranostic material being worked as carrier of different dyes for imaging mechanisms and active agents for therapeutic purposes. However, among all aforementioned materials, IONPs are mostly used because of properties like superparamagnetism, biodegradability, biocompatibility, remote control as well as an economical approach for every research bench.

From above discussion it can be concluded that nanoparticles provide interesting perspectives for application in oncology. Recent developments in applications ranging from diagnosis to treatment provide a mirror image of future horizons. The development of multifunctional nanoparticles has increased positive expectations for cancer treatment, leading to early diagnosis and enabling in-time disease management. Multifunctional nanoparticles in diagnosis (both in vivo and in vitro) provide multiple imaging platforms for rapid disease

verification and exact localization of the diseased tissues. In addition, they permit the rapid delivery of therapeutic agents to the target. The concept of theranostic agent development based on the 'all-in-one approach' is showing great potential in the emerging field of personalized medicine, because such agents allow detecting and monitoring an individual patient's cancer at an early-stage, and deliver anticancer agents over an extended period for enhanced therapeutic efficacy. At present, this all-in-one approach is limited to imaging techniques but will extend to more precise and less time consuming diagnostic methods. Multiple emulsion and Janus-like particle preparation techniques are now undergoing rapid development to this end. In short the research for an ultimate remedy for cancer is on way and it can be hoped that day is not far away when this disease will be timely diagnosed and properly cured.

Acknowledgement

Thanks to the Science and Technology cooperation programme between Pakistan & France and between SFERE (Société Française d'Exportation des Ressources Educatives) and Higher Education commission (HEC) of Pakistan for supporting Ph.D. studies of Mr. Naveed Ahmed. Authors also thank Dr. M. Essa for his carefully reading and corrections.

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CHAPTER II.3

ENCAPSULATION OF HYDROPHOBIC DRUGS: STATE OF THE ART

Résumé

Au cours des dernières décennies, l'optimisation des systèmes de vectorisation de médicaments a fait l'objet de nombreux travaux. Parmi la grande diversité de systèmes étudiés, les nanoparticules ont fait l'objet d'une attention particulière concernant la vectorisation de molécules hydrophobes. Aussi bien les méthodes de préparation des nanoparticules que la supériorité de ces systèmes sur les microparticules sont désormais bien caractérisées. Les nanoparticules sont des systèmes ayant une taille comprise entre 1 et 500 nm et peuvent être classées en deux catégories : les nanocapsules et les nanosphères. Les polymères sont largement utilisés dans la préparation des systèmes particuliers et occupent donc une place importante dans ce domaine. Ceux-ci peuvent être utilisés comme polymères préformés ou directement synthétisés à partir de monomères lors de la préparation de nanoparticules, permettant ainsi de diversifier la nature des matériaux disponibles pour des applications biomédicales. Puisque l'objectif de ce travail est la préparation de nanoparticules pour la vectorisation d'actifs pharmaceutiques pour des applications *in vivo*, les questions de sécurité concernant l'administration de ces systèmes ont guidé le choix des polymères. Ainsi, les polymères préformés ont été préférés pour leur biocompatibilité. Récemment, des particules dites intelligentes ont été conçues à partir de polymères préformés sensibles à différents facteurs extérieurs comme la température ou le pH du milieu. Les propriétés physico-chimiques de la molécule active à vectoriser conditionnent le choix de la méthode de préparation et les polymères susceptibles d'être utilisés. Une fois les systèmes nanoparticulaires préparés, ceux-ci sont caractérisés en termes de taille, charge de surface et morphologie. En effet, ces propriétés revêtent un rôle fondamental pour le relargage contrôlé et la vectorisation des molécules actives.

Les méthodes de préparations des nanoparticules font l'objet de ce chapitre. Néanmoins étant donné la grande diversité des techniques disponibles, cette étude se limite à la préparation de nanoparticules à partir de polymères préformés destinées à la vectorisation d'actifs hydrophobes. La première partie de ce chapitre est consacrée à la biocompatibilité des polymères dans le domaine pharmaceutique. La deuxième partie concerne les méthodes de préparation des systèmes nanoparticulaires. Les méthodes de préparations conventionnelles aussi bien que les méthodes émergentes ont fait l'objet d'une attention particulière. Ainsi, les méthodes de préparation par émulsification, nanoprecipitation, polymersome, adsorption couche par couche de polymères et par fluides supercritiques sont discutées en détail. Enfin,

dans la dernière partie, les propriétés des nanoparticules, leurs modifications ainsi que les conséquences de celles-ci sur la libération des principes actifs sont décrites.

General Summary

In recent decades, extensive work has been done on the improvement of drug delivery systems. In this enormous investigation, the nanoparticles received the attention and methods to prepare nanoparticles were studied in detail because of resolving solubility problems and advantages over microparticles. Bringing the active ingredients under hydrophilic and hydrophobic divisions, different pharmaceutical techniques have been devised for the preparation of nanoparticles of both types of active ingredients separately. Normally a particle with 10-500 nm size is considered nanoparticle and further classification of nanoparticles in nanocapsules and nanospheres has been evolved. Polymers are playing an important role in the preparation of nanoparticles and being used widely. They could be used as preformed polymers. Also methods exist that synthesize polymers from monomers directly and used for the nanoparticles preparation, hence increasing the choice of polymers for biomedical applications. As aim is the nanoparticles preparation for *in vitro* and *in vivo* applications with focus for drug delivery purposes therefore the preformed polymers are preferred keeping their safety and biocompatibility in mind. Furthermore, recently intelligent particles have been prepared using stimuli responsive polymers. Various methods are elaborated for the preparation of nanoparticles (nanospheres and nanocapsules) from these preformed polymers. The choice of a pharmaceutical method can be done importantly with nature of the drug and the polymer to be used. Then the characterization of the prepared particles can be done with already existing specific parameters like size zeta potential and morphological studies. These all properties play a significant role in the drug delivery or targeting. The modification in these properties can bring change at the level of targeting or release.

In this chapter, methods for the preparation of nanoparticles have been discussed limiting to the use of preformed polymers. Another restriction of this work is the preparation of the nanoparticles containing hydrophobic drugs only. Hence first of all, the safety and biocompatibility of the polymers has been discussed. Then various pharmaceutical techniques used for the preparation of nanoparticles containing hydrophobic drugs have been studied. The basic phenomena and progress in various emulsification methods, nanoprecipitation, polymersome, layer by layer and supercritical fluid technology have been examined in detail. Finally the properties of the nanoparticles and their modifications have been dealt with release characteristics and futures perspectives have also been discussed.

POLYMERIC DRUG DELIVERY SYSTEMS FOR ENCAPSULATING HYDROPHOBIC DRUGS

Naveed Ahmed, C.E.Mora-Huertas, Chiraz Jaafar-Maalej, Hatem Fessi and Abdelhamid
Elaissari.

(Accepted as a book chapter in 'Drug delivery strategies for poorly water -soluble drugs' will
be published by John Wiley & Sons,Ltd)

Abstract

This review is based on drug delivery systems involving hydrophobic drug encapsulation. It explains different methods of preparation for polymeric nanoparticles containing hydrophobic drugs with biocompatibility profiles of polymers. The mechanism, merits, demerits and examples of hydrophobic drugs, already used by nanoprecipitation, various emulsification methods, polymersome, layer-by-layer and supercritical fluid technologies are explained. Characterization of the nanoparticles prepared by these methods is given in a comparative way. It permits to select a suitable method for nanoparticles preparation of a specific drug on basis of polymer choice, advantages, disadvantages and characterization of prepared nanoparticles. Already published data is the basis of this review.

Introduction

The application of nanotechnology in biomedical field is praiseworthy. In last two decades, it has been resulted in enormous work leading to hundreds of articles with new ideas, trends and methods for the development of a suitable drug delivery system. Among these, nanoparticles for the drug delivery and targeting attracted researcher's attention, the most. This can be considered as result of advantages over micro-particles and liposomes in terms of small size, which provides high surface to volume ratio and resolve the solubility problem of hydrophobic active molecules, resulting in better efficacy, precise delivery and safety for organisms.

Different researchers defined nanoparticles depending upon their size and normally these are particles having size range of 1-500 nm. It should be noted that the nanoparticles is a collective term used for nanospheres and nanocapsules in biomedical nanotechnology. The nanoparticles based on polymers are well investigated without the differentiation of the natural and synthetic polymers but the important point to be taken in account is the biodegradability and biocompatibility of the polymers. Polymeric nanoparticles can be prepared by the preformed polymers or by direct polymerization of the monomers resulting in nanoparticles by polymerization techniques. The development in polymeric nanoparticle field has enabled us to divide the preparation methods of nanoparticles in two main divisions that are via preformed polymers and by polymerization techniques. However, in this chapter focus will be the use of preformed polymers for nanoparticles preparation. Knowledge of safety and biocompatibility of polymers being used for nanoparticles preparation will be helpful in

choice of suitable one, keeping in view the preparation method as well as *in vivo* and *in vitro* applications of the final particles.

Preformed polymers are used for encapsulation of both hydrophilic and hydrophobic drugs. The researchers have devised specific techniques for both types but here the discussion will focus on hydrophobic drugs. The different methods being used for the hydrophobic molecules include nanoprecipitation, emulsification, polymer coating, polymersomes, supercritical fluid and layer-by-layer technologies. Generally, is a requirement of an aqueous and an organic phase for the preparation of nanoparticles, further selection of a suitable method should be on basis of the drug nature, the selected polymer and solvent to be used. The study of different characteristics of nanoparticles prepared by a method can be helpful to compile a suitable data sheet and can give an idea about success in preparation of conceived nanoparticles. Nevertheless, as mentioned above, it is a dynamic research topic so resulted in the preparation of more intelligent particles and specifically leading to concept of taking multiple advantages at same time that can be seen by the recent developments in preparation of theranostics/theragnostics targeted for therapy and diagnosis at same time.

In this chapter, first of all the safety and biocompatibility of the polymers used for nanoparticles preparation then the techniques being used for preparation and at the end the properties of the nanoparticles will be discussed.

Safety and biocompatibility of polymers

Polymeric nanoparticles are widely used in medical and pharmaceutical applications. They exhibit several desirable properties; safety, biocompatibility and biodegradability are the most important ones that nanoparticles must meet for *in vivo* applications [1].

Delivered nanoparticles are always in direct and sustained contact with biological tissues, and some of them degrade *in situ*. Thus, a fundamental understanding of the *in vivo* degradation criteria and mechanisms as well as an appreciation of cellular and tissue responses which determine the biocompatibility of polymeric nanoparticles are of great interest and vital components in the design and development of biocompatible vectors [2]. For this aim, both polymer materials and degradation derivatives must be devoid of toxicity.

As shown in table 1, among the synthetic polymers, biodegradable aliphatic polyesters such as poly (ϵ -caprolactone) (PCL), poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and their copolymers received considerable attention [3]. Owing to the high degree of crystallinity and hydrophobicity, PCL degrades rather slowly and is less biocompatible with

soft tissue, which restricts its further clinical applications. Therefore, modification is proposed; Poly (ethylene glycol) (PEG), appreciated for its hydrophilicity, non-toxicity, absence of antigenicity and immunogenicity, have been selected to be attached to PCL. The preformed PCL–PEG copolymers improved the hydrophilicity, biodegradability and mechanical properties of PCL leading to wider applications [3]. Due to biodegradability, good biocompatibility, excellent shaping and modelling properties, both PLA and poly lactic-glycolic acid (PLGA) had been widely applied in biomedical field [4]. Furthermore, poly glycolic acid (PGA) and poly methyl methacrylate (PMMA), typically included in biodegradable nanoparticles formulation, were frequently used for improving bioavailability and reducing the patient risks of toxicity [5]. On the other hand, chitosan has already drawn increasing attention as a polymeric drug carrier because of desirable properties such as biocompatibility, biodegradability, and biological activities [6-7]. In addition to this, it has been reported that, chitosan metabolic derivatives are biodegradable, biocompatible and poorly immunogenic.

The interactions between polymers and cells are known primarily to depend on physical and chemical properties of the biomaterial surfaces, such as microstructure, surface wettability and the hydrophobic to hydrophilic ratio. The influence of surface hydrophilicity or hydrophobicity on biocompatibility of polymers has been reported in many studies [8-9]. Recent data highlights the impact of surface characteristics on nanomaterials biocompatibility and point to the inadequacy of the current size-dependent mechanistic paradigms [10]. Bulk and surface modifications have been used to improve the compatibility of materials with the surrounding tissues as well as the interactions between two different materials [11].

Generally, the evaluation of a formulation's biocompatibility progresses through *in-vitro* and *in-vivo* phases. In many cases, the types of experiments involved are quite similar. Nonetheless, the specifics of the evaluation depend on nature of the problems that can be anticipated given the involved compounds [12].

In vitro studies provide a rough assessment of relevant cell type survival in presence of an investigated polymer and derivatives. Numerous tests such as MTT assay, measures of DNA synthesis and cell proliferation, and dye-based cell membrane integrity tests can be applied. For further investigation, the mechanism of toxicity can be addressed by looking at cell death or tissue injury markers [12].

In vivo studies, an essential tool for biocompatibility understanding, can considerably vary depending on the context. A common assay for injected or implanted nanoparticles is

hematoxylin-eosin stained paraffin-embedded sections. These are generally excellent for looking at gross tissue morphology and tissue reaction including inflammation [12]. There is a range of specialized stains, as well as immune-histo-chemical methods, for detecting specific biological changes. Epon-embedded sections or electron microscopy may allow detection of more subtle signs of tissue injury [12].

Table 1. Examples of polymers used in the nanoparticles preparation via different methods.

Polymer	Reference
Nanoprecipitation	
PACA	13
Methacrylic acid copolymers: Eudragit [®] S90, S100, RS, L100-55	14-17
PCL Mw 10 kDa, 14 kDa, 40 kDa, 42,5 kDa, 64 kDa, 65 kDa, 80 kDa, 100 kDa	14, 18-33
PLA Mw 9 kDa, 42 kDa, 75 kDa, 88 kDa, 100 kDa, 110 kDa, 200 kDa.	20-22, 24-25, 34-44
PLGA 50:50 Mw 40 kDa, 50-75 kDa; 75:25 Mw 75-120 kDa	20-21, 24-25, 45-47
PCL-PEG 40-5 kDa, 45-5 kDa	20-21, 24, 48
PLA-PEG 45-5 kDa, 90% PLA Mw 45 kDa - 10% PEG Mw 20 kDa, 90% PLA Mw 49 kDa - 10% PEG Mw 5 kDa	20-21, 24, 43-44
PLGA-PEG 45-5 kDa, 45-20 kDa,	20-21, 24
PCLLA	49
Poly[H ₂ NPEGCA-co-HDCA]	13
Poly(N-acryloylamide)	16
Poly(vinylene)	16
PVM/MA	50
Gliadin	51-52
Cellulose acetate	53
Emulsification-diffusion	
Methacrylic acid copolymer	54-57
PCL Mw 10 kDa, 14 kDa, 40 kDa, 42,5 kDa, 60 kDa, 80 kDa	54-55, 58-66
PLA	54-55, 67-71

PLGA 50:50, 65:35, 70:30, 75:25, 85:15 Mw 14.5 kDa, 45 kDa, 85 kDa, 137 kDa, 213 kDa	54, 56,69, 72-79
PHBHV Mw 23 kDa, 300 kDa	32-33
Modified starch	80
PLGA-mPEG	81
Emulsification-coacervation	
Sodium alginate Mw 75 - 120 kDa	82-83
Swine skin gelatin type II	84-85
Poly (1,4 butadiene) (PB)-block-polystyrene (PS)-block-poly(ethylene oxide) (PEO) triblock terpolymer Mn 76 - 86 kDa	86
Emulsification-evaporation	
PLA	84,87-88
PCL Mw 10 kDa	89
PLGA 75:25 Mw 10 and 92 kDa	90
PLGA 50:50 Mw 7, 14, 24, 48, 63 and 85 kDa; 65:35 Mw 97 and 114 kDa; 85:15 Mw 87 kDa)	78,88
Dextran derivatives	91
Double emulsification	
PLGA Mw 34 kDa	92
Polymersome preparation	
PCL-PEG	93-97
PLA-PEG	93-94,96-99
PB-PEG	93,97-98,100
Poly(FA/SC)-co-PEG	101
PEG-co-poly(FA/SC)-co-PEG	101
PTMC-PEG	96
PMPC-PDPA	102
PEO-PDPA	102
PB-b-PGA	103
CMPC	104
Supercritical fluid technology	
PLA Mw 10, 100 kDa	105-106
Polymer coating	
Chitosan oligomers or medium molecular weight chitosan	18,107
PCL Mw 40 kDa - poly-L-lysine	18
PLA Mw 28 kDa/PLGA - chitosan Mw Mn >50 kDa	108
Sodium alginate Mw 80 - 120 kDa - chitosan Mn 41 and 72 kDa	109

Layer-by-layer	
Chitosan Mw 250 kDa - sodium alginate Mw 70 kDa	110
PDDA Mw 200 kDa - sodium alginate Mw 70 kDa	110
Gelatin Mw 500 kDa - sodium alginate Mw 70 kDa	110
PAH Mw 15 kDa - PSS Mw 70 kDa	111-112
PDDA - PSS	112
Chitosan Mw 400 g/mol - Lambda-carrageenan ^{*1}	113
<p>* PACA: Poly(alkylcyanoacrylate) derivate; PCL: Poly(ϵ-caprolactone); PLA: Poly(lactide); PLGA: Poly(lactide-co-glycolide); PCL-PEG: copolymer of poly(ϵ-caprolactone) and poly(ethylene glycol); PLA-PEG: copolymer of poly(lactide) and poly(ethylene glycol); PGLA-PEG: copolymer of poly(lactide-co-glycolide) and poly(ethylene glycol); PCLLA: copolymer of ϵ-caprolactone and L-lactide; poly(H₂NPEGCA-co-HDCA): Poly[aminopoly(ethylene glycol)cyanoacrylate-co-hexadecyl cyanoacrylate]; PHBHV: poly(3-hydroxybutyrate-co-hydroxyvalerate); PB-PEG: copolymer of poly(butadiene) and poly(ethylene glycol); PEG-co-poly(FA/SC)-co-PEG: copolymer of poly(ethylene glycol) and poly(fumaric acid-co-sebacoyl chloride); PTMC-PEG: copolymer of poly(trimethylene carbonate) and poly(ethylene glycol); PMPC-PDPA: block copolymer (2-(methacryloyloxy)ethyl phosphorylcholine)-poly(2-(diisopropylamino)ethyl methacrylate); PEO-PDPA: poly(ethylene oxide)-poly(2-diisopropylamino)ethyl methacrylate; PB-b-PGA: poly(butadiene)-b-poly(g-L-glutamic acid); CMPC: cholesterol-end-capped poly(2-methacryloyloxyethyl phosphorylcholine); PDDA: poly(dimethyldiallylamide ammonium chloride); PAH: poly(allylamine hydrochloride); PSS: sodium poly(styrene sulphonate).</p>	

For further progress, it is important to address appropriately nanoparticles safety profile including possible immunogenicity and inflammation reactions [114]. Given the increasing novelty of materials proposed for second-generation polymer therapeutics development (with increasing complexity of conjugate composition), and growing debate as to the safety of nanomedicines, the need for evolution of an appropriate regulatory framework is at the forefront of the scientific discussion. The adequacy of the current tests and models used to define safety are also constantly being reviewed [115].

In spite of numerous advantages, cytotoxic effects has been reported for polyester polymers, induced by particles nanometric size range which is conducive to internalization by cells (macrophages), and degradation inside the cell [116]. Furthermore, high tissue accumulation of non-biodegradable nanoparticles caused toxicity problems. The toxicity and safety of non-biodegradable nanoparticles are subject to future research and more studies on the toxicological effects of nanoparticles in humans need to be done [117].

Encapsulation techniques of the hydrophobic drugs

1. Nanoprecipitation method

The nanoprecipitation method can also be called as solvent displacement or interfacial deposition method. But Reis et al. [118] described minute difference between solvent displacement and interfacial deposition explaining as first forms nanospheres or nanocapsules while the later restricts only to nanocapsules. Nevertheless, both are based on spontaneous emulsification of organic phase into aqueous phase. According to Fessi et al. [119], for synthesis of nanoparticles, solvent and non solvent phases are essential. This division of solvent or non solvent phases is based on dissolution of polymer and can be termed as organic and aqueous phase respectively. It's because mostly solvent is organic medium for the dissolution of polymer or active substance and the non solvent phase consists of water.

The solvent phase consists of a single or a mixture of solvents (e.g., ethanol, acetone or dioxane) and in same way non solvent phase can be a single or a mixture of non solvents. Hence keeping in view the miscibility, solubility and stability, two aqueous phases and two organic phases can be used. The solvent phase can contain the active substance to be encapsulated, stabilizing agent and polymer that can be natural, synthetic or semi-synthetic while the non solvent phase can contain one or more stabilizing agents.

The resulting particles will be used for the drug delivery purposes. Therefore selection of polymer should be on the biodegradability and biocompatibility. The mostly used polymers include poly ϵ -caprolactone (PCL), poly (lactic acid) (PLA), poly (lactic-co-glycolic acid) (PLGA), Eudragit and poly (alkylcyanoacrylate) (PACA), *etc.* According to Khoee and Yaghoobian [120] synthetic polymers show greater reproducibility and higher purity over natural polymers and can be modified according to the requirements e.g., to avoid recognition of nanocapsules by mononuclear phagocyte system, some polymers can be copolymerized with PEG [43]. This discussion does not imply that biodegradable nanoparticles only prepared using preformed polymers; it's possible with monomers or macromonomers passing through polycondensation reactions [121-122]. For nanocapsules preparation via nanoprecipitation method having size range of 150-200 nm, a composition is suggested in table 2. However in practical research, only a few of all these are in use and among them different capric/caprylic triglyceride types having ability to solubilise most of active substances are frequently used as oily phase. For w/o surfactants, sorbitan esters and phospholipids are considered. As a polymer solvent, acetone is mostly preferred while solvents like ethanol are used for dissolution of the active molecule or oil. The non solvent can contain only water with a

surfactant or a buffer solution. In preparation, organic phase is added slowly under stirring to the aqueous phase and a colloidal suspension containing the nanocapsules is obtained. This simple method is presented in figure 1.

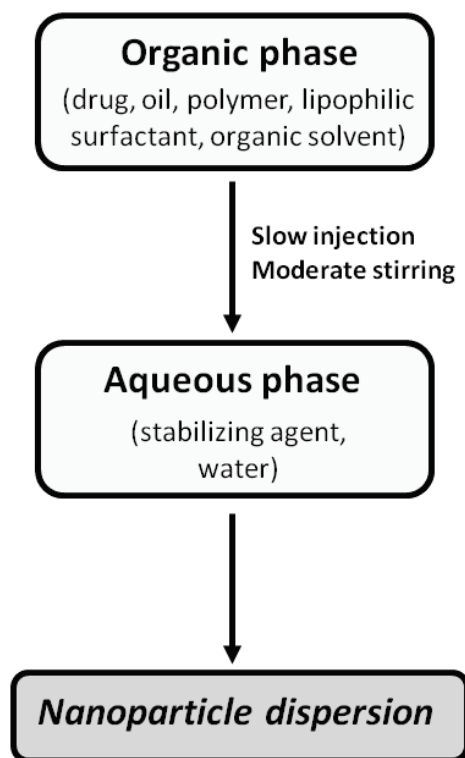


Figure: 1 Schematic representation of nanoprecipitation method

Other techniques of nanoparticle preparation such as dialysis method have been proposed considering the fundamental principles of nanoparticle formation using the nanoprecipitation method [123-125].

The different parameters affecting the process are stirring speed, method and rate of organic phase addition to the aqueous phase and organic to aqueous phase ratio. However, nature and concentration of substance to be encapsulated are additional/external to the process parameters that affect the characteristics of resulting particles [126-129]. For detailed understanding of mechanism involved for nanoparticles preparation via solvent displacement technique, some researchers explained two types of effects; Maragani effect [130-132] and classical precipitation or ouzo effect [133-134] (for better understanding, it can be called as classical precipitation when nanospheres are prepared and ouzo effect referring to nanocapsule preparation). But mostly studies are based on understanding of the physicochemical principles and emphasizes specially on type of solvent, molecular weight of

polymer, concentration, viscosity and mixing speed of polymer solution with non-solvent phase and interfacial tension of the non-solvent phase [135].

Cyclosporin A encapsulation with entrapment efficiency of 98 % is an excellent illustration of interfacial deposition method application. [136] Spironolactone nanocapsules with entrapment efficiency of more than 90 % have been successfully prepared by Blouza et al. [137].

2. Emulsification methods

Some widely used techniques for nanoparticle preparation are the emulsification-based methods, which require the formation of a nanoemulsion previously to the particle formation. They include the techniques of emulsification-diffusion, emulsification-evaporation, emulsification-coacervation and double emulsification.

A) Emulsification-diffusion method

Emulsification-diffusion method is one of the most commonly used methods for polymeric nanoparticles preparation. In broader sense, this technique is a combination of emulsification process and diffusion process. This technique was utilized by Leroux et al. [54] for nanosphere preparation and then by Quintanar-Guerrero et al. [67] for obtaining nanocapsules. Generally, both organic and aqueous phases are necessary but Quintanar et al. [55,138] suggested the requirement of three phases, considering dilution by water as a third phase, hence, termed as organic, aqueous and dilution phases. The organic solvent should be partially miscible with water and capable of dissolving polymer, active substance, oil or any other compound (excipient) in the preparation. The aqueous phase normally contains stabilizing agent and for dilution water is used. Hence, preparation of o/w emulsion followed by dilution, induce diffusion of organic solvent resulting in nanoparticles formation. Benzyl alcohol was used by Leroux et al. [54], propylene carbonate by Quintanar-Guerrero et al. [55] and ethyl acetate was considered the best by Moinard-Chécot et al. [65]. A general recipe for the nanocapsules preparation by emulsification-diffusion method resulting size in between 150 – 200 nm is shown in Table 2.

Solvents such as benzyl alcohol, propylene carbonate and ethyl acetate are used along with dichloromethane (DCM). However, poly vinyl alcohol (PVA) is a common choice as stabilizing agent although poloxamer and ionic surfactants have been used. Mostly used

polymers are based on different capric/caprylic triglyceride types including PLA, PCL, eudragit but Poly (hydroxybutyrate-co-hydroxyvalerate) (PHBHV) are also considered.

For the nanoparticles preparation by this technique, the water saturated organic phase (dissolving all the required compounds) is firstly emulsified in organic solvent saturated aqueous phase containing stabilizing agent. This emulsification process is accompanied by high energy stirring. Then more water (dilution phase) is poured within this emulsion to induce diffusion of solvent in the external phase that results in nanoparticles formation. Finally according to the boiling point of solvent, it is eliminated by distillation, filtration, or by evaporation in some cases.

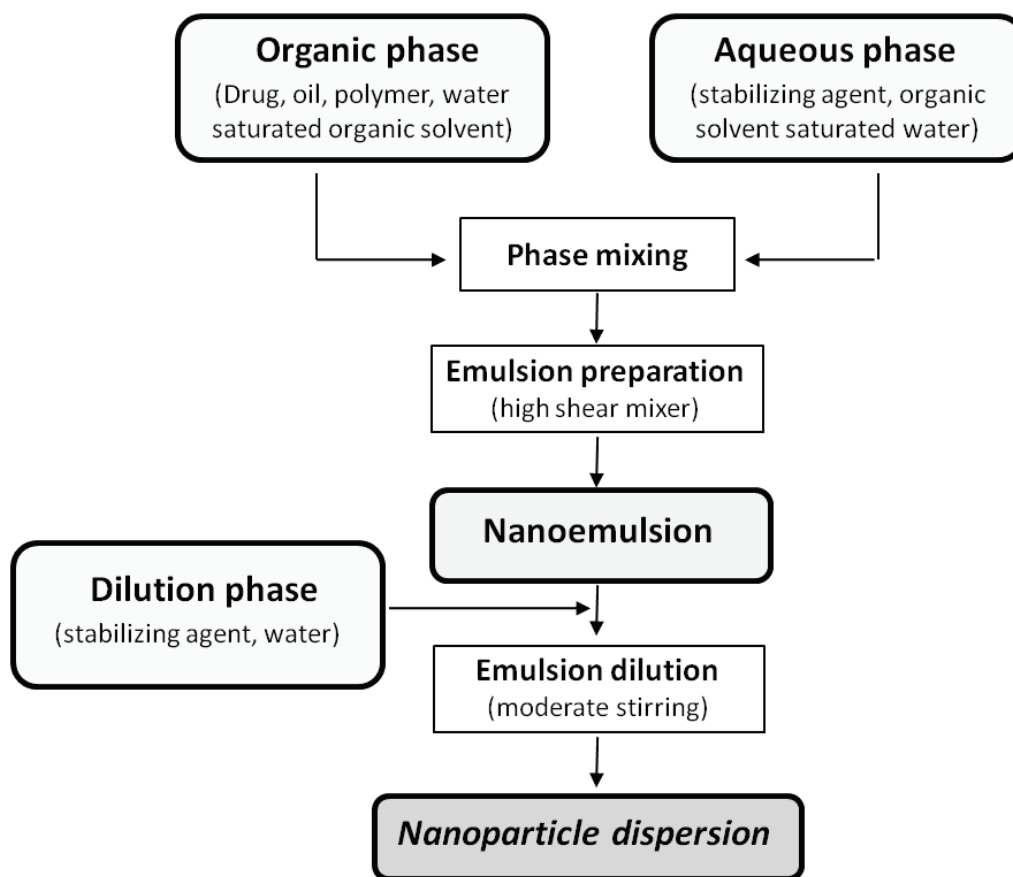


Figure: 2 Schematic representation of emulsion diffusion method

According to Guinebretière [139] and Moinard-chécot et al. [65], in emulsion formation process, shear rate, oil to polymer ratio, polymer concentration in the organic phase, and the chemical composition of the solvent phase are the key factors affecting the final particle size. This technique is used for drug loaded nanoparticles preparation and some efficiently

encapsulated drugs applying this method include; coumarin [140], cyclosporine A [141], indocyanine [142] *etc.* Plasmid DNA was also encapsulated by this technique [143].

B) Emulsification-coacervation method

This method is renowned for the naturally occurring polymers *i-e.*, gelatine and sodium alginate although some synthetic polymers can also be used like carboxy methyl cellulose (CMC) and polystyrene derivatives [54]. The emulsion coacervation method is based on emulsion formation and then coacervation that results in polymer precipitation as a thin layer on the template forming the nanocapsules. Then stability is achieved by addition of a cross-linking agent or by altering temperature or pH which result in physical intermolecular or covalent cross-linking. The active agent is dissolved in a solvent and then a poor solvent/non solvent is added to induce coacervation based on the phase separation [144]. In o/w emulsification step, the organic phase contains oil, active substance and if necessary some other organic material (e.g., active substance solvent) while the external aqueous phase consists of polymer and stabilizing agent in water. The coacervation process can be done by electrolyte addition, [83,109] temperature modification [86] or by addition of a dehydration agent [84] and is finalized by cross-linking step to have a stable nanocapsules aqueous dispersion. Nonetheless, work of Lutter et al. [86] is normally used as principle method for emulsion coacervation technique. A general concept of this method is explained here via figure 3.

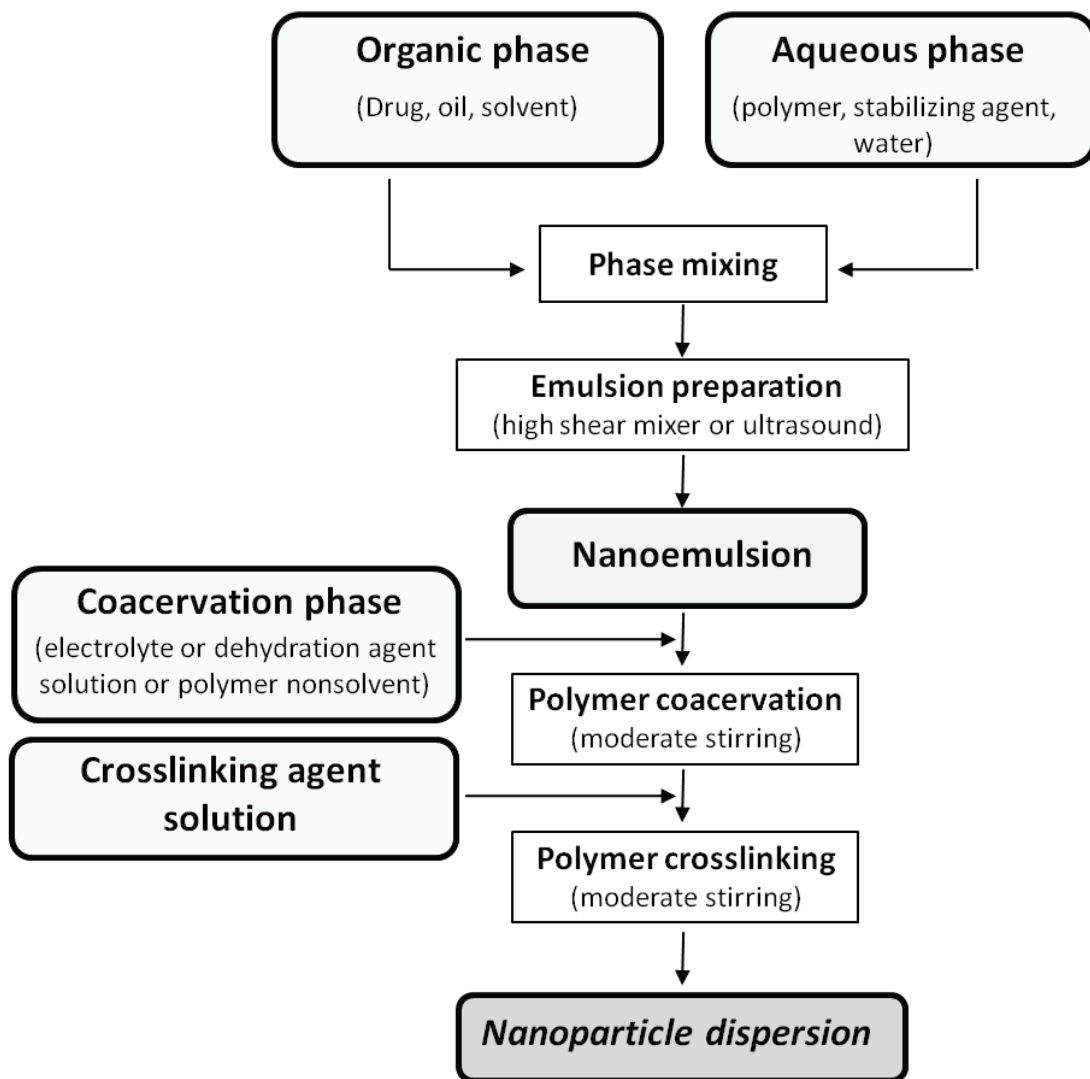


Figure: 3 Schematic representation of the emulsion coacervation method

The coacervation step is the most important in this technique and is investigated in detail to establish the pH effects, electrolyte effects and change in polymer type such as cellulose acetate trimellitate [145], ethylcellulose [146-147] and polyvinyl chloride [148]. This type of research leads to devise emulsion coacervation technique to have nanocapsules size in nanometers. The use of a template and a technique to form a concentrated, colloid-rich dispersed phase to cover the template can be useful to form nanoparticles. The polymers are precipitated on the core material and these core nanoparticles are then dispersed in another solvent mostly aqueous one. According to Gander et al. [149], the polymer functional groups are solvated by water molecules. Coacervating agents are added to avoid solvation, inducing a thin solvated shell and allowing the attraction among functional groups to form a network with increasing polymer concentration.

As discussed above, temperature modifications, electrolytes, or dehydrating agents are used for the completion of coacervation process so in case of temperature modification to induce polymer precipitation, Flory-Huggins theory on interaction parameter χ is important. According to this theory if the interaction parameter value is lesser than a specific value *i-e.*, critical value χ_c , the polymer will dissolve in solvent and this χ_c value depends on the degree of polymerization of polymer. Similarly for electrolytes efficiency in desolvation process, Hofmeister or lyotropic series is followed responsible for arranging the ions to their capacity to immobilize water molecules in solvation. Yin et al. [150] performed a practical study on polymer coacervation behaviour with respect to lyotropic series. The electrolytes used in polymer desolvation can be designed as salting out. In case of dehydrating agent, the polymer concentration increase is due to solvent-solvation competition process that leads to desolvation of polymer chains resulting in phase separation. However, other factors such as pH change or addition of other incompatible materials can also be used for reducing polymer solvation.

Capsaicin, a poorly soluble active agent, obtained from red hot pepper is successfully nanoencapsulated by Xing et al using coacervation method [151].

C) Emulsion-evaporation

The emulsion-evaporation is another widely used technique for nanoparticles preparation particularly by Pisani et al. [152]. It is also known as emulsion evaporation method or solvent evaporation method. It is based on the nanoemulsion preparation followed by evaporation of the organic solvent leading to point of insolubility and precipitation of the polymer encaging active material. Aqueous phase outside behaves as dispersion medium therefore, involvement of two phases; aqueous and organic. For evaporation, simple evaporation by stirring or evaporation under reduced pressure may be used depending on the nature of solvent. The factors affecting the size of particles can be stirring time and stirring speed for emulsification, the nature and concentration of the stabilizing agent. A simple schematic representation is here in figure 4.

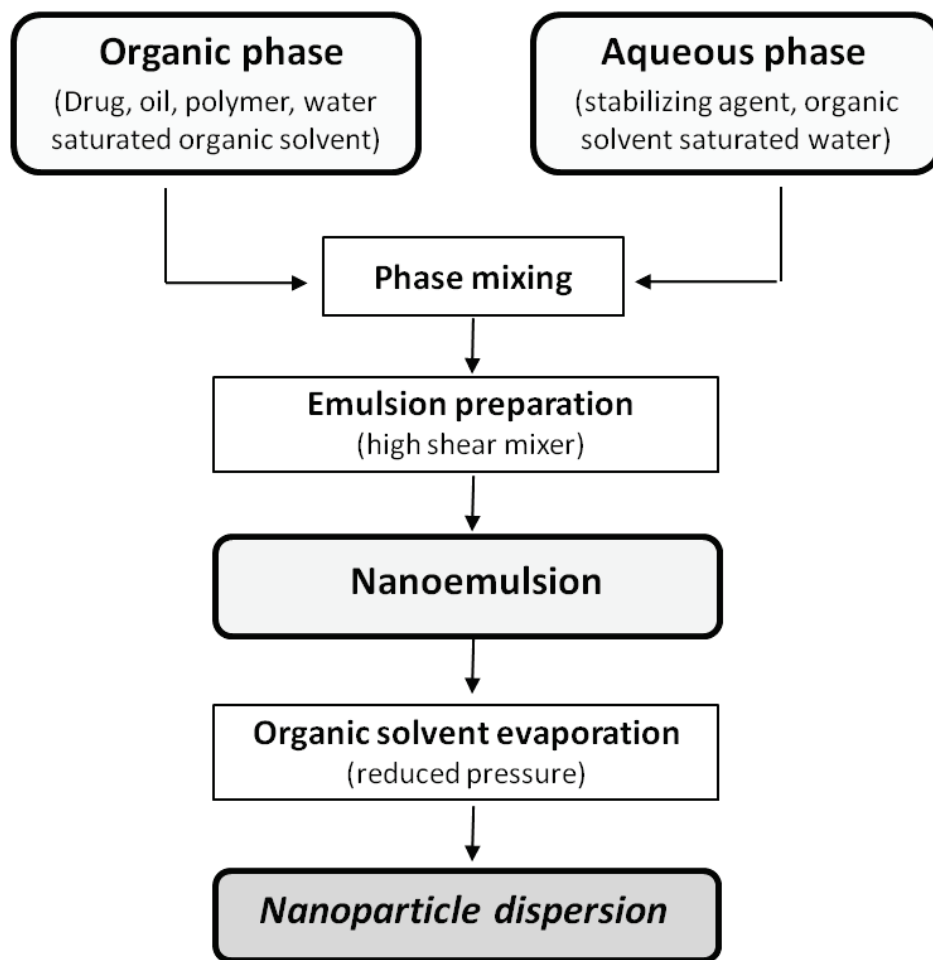


Figure: 4 Schematic representation of the emulsion-evaporation method

The main drawback of this technique is the susceptibility of the formation of different interfaces inside organic and aqueous phases which can result in restriction of solvent diffusion in presence of polymer, hence, creating a difficulty in nanocapsules formation during rapid solvent removal via evaporation. [152] However, Moinard-chécot et al. [65] described that resistance in evaporation can be explained as mechanical stress inside the emulsion due to gas bubbles formation. The nanoparticles of docetaxel were prepared by Quaglia et al. [153] using emulsion evaporation method.

D) Multiple emulsions method

Multiple emulsions are emulsification of already prepared emulsions. However, in this multiple emulsion technique, double emulsion technique is praiseworthy allowing encapsulation of both types of materials; hydrophilic and lipophilic. According to researchers, [154-155] double emulsions is considered as the complex heterodisperse systems and called

as “emulsions of emulsions”. On the basis of two phases, aqueous and organic, the double emulsion can be classified in two categories *i-e.*, oil-water-oil emulsion (o/w/o) and water/oil/water (w/o/w) emulsion. For encapsulation of hydrophobic drugs both are used depending upon nature of drug and choice of starting materials. Among these double emulsion methods, the mostly employed is double emulsion evaporation method, in which, a primary emulsion is prepared that again emulsified by another phase to prepare the secondary emulsion and after this the evaporation is done. For example, in case of (w/o/w) emulsion, primary emulsion is prepared by emulsifying aqueous phase in organic phase, this primary emulsion is again emulsified in aqueous phase to form w/o/w double emulsion and finally the organic phase is evaporated. Hence contents of the organic phase (mostly polymer) act as the film between two aqueous phases in particles and secondly that film can also act as a barrier for release of drug from particles [120].

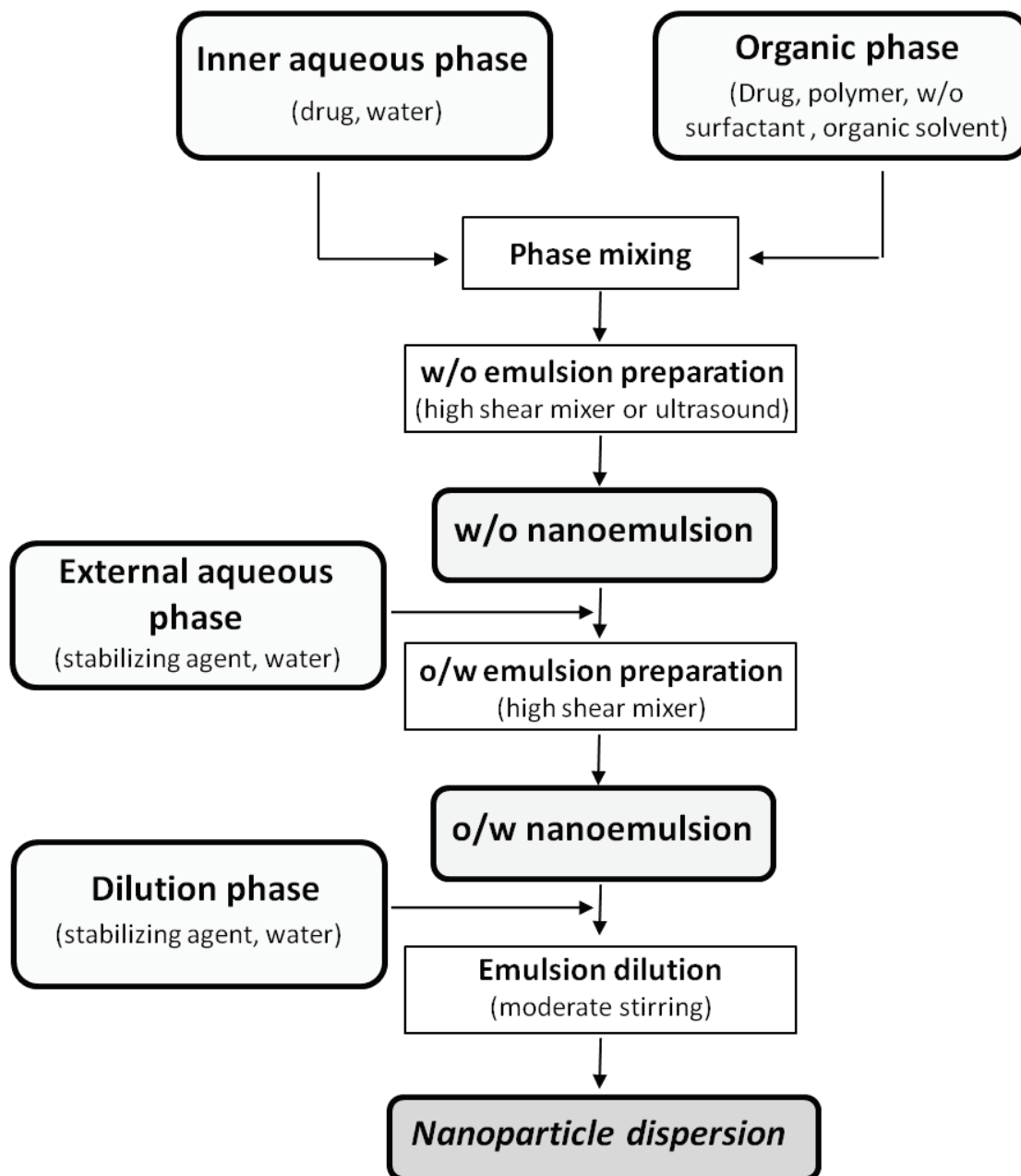


Figure: 5 Schematic representation of the double emulsion method

Like other encapsulation methods, the polymer choice accounts for its biodegradability and biocompatibility profile. These emulsions are normally prepared with two stabilizing agents, a hydrophobic and hydrophilic one but in some cases, a single stabilizing agent is sufficient, normally in second emulsification phase. For evaporation, simple evaporation by stirring or evaporation under reduced pressure is used depending on nature of the solvent. Different experimental variables affecting physicochemical characteristics of the final particles are; ratio between phase volumes, stirring speed; stirring time, nature and concentration of both polymer and stabilizing agents, preparation temperature, method of

solvent evaporation, *etc.* This method was earlier used for the preparation of microparticles [156-157] but later on, applied to nanoparticles preparation using high energy stirring techniques [158-160]. The advantage of this technique is ability to encapsulate both hydrophilic and hydrophobic drugs thus other constituents like polymer, solvent and stabilizing agent, *etc.* can be selected accordingly depending upon the nature of the drug.

Different polymers used in this technique include PLA, PCL and PLGA and as stabilizing agents, polysorbates and poly vinyl alcohol (PVA) are studied. In case of solvents, ethyl acetate, dichloromethane, and chloroform are the choices. However, ethyl acetate is preferred because of less toxicological effects and smaller particle size as compared to that of DCM and chloroform. The table 2 shows the different compositions based for nanocapsules preparation via double emulsion method.

Table 2 Different composition used for nanoparticles preparation via different methods

	Nano precipitation ^{*1}	Emulsification-diffusion ^{*1}	Emulsification-coacervation ^{*2}	Emulsification-evaporation ^{*3}	Double emulsification ^{*4}	Polymerosome preparation ^{*5}	Supercritical fluid ^{*6}	Polymer-coating ^{*7}	Layer-by-layer ^{*8}
Active substance (g)	0,1 - 0,25	0,1 - 0,5	≈ 0,07	≈ 0,00024	≈ 0,01	≈ 0,45	1 - 1,5	≈ 0,1	≈ 0,095
Polymer (g)	0,5 - 1,25	1,0 - 2,0	≈ 0,7	≈ 0,4	0,5 - 2	≈ 10	1 - 1,5	≈ 2	≈ 0,005
Oil (g)	2,5 - 5,0	2,5 - 5	N.A.	N.A.	N.A.	N.A.	N.A.	≈ 5	N.A.
Lipophilic surfactant (g)	0,5 - 1,25	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	≈ 1	N.A.
Salt (g)	N.A.	N.A.	≈ 10	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Crosslinking agent (g)	N.A.	N.A.	≈ 0,7	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Oxidizing agent (g)	N.A.	N.A.	≈ 4	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Stabilizing agent (g)	1 - 2,5	8 - 20	N.A.	0,4 - 1,6	≈ 0,6	≈ 2,5	N.A.	≈ 1	N.A.
Water (q.s.; ml)	100	100	≈ 100	≈ 100	≈ 100	≈ 100	N.A.	≈ 100	N.A.

*Estimated composition for 100 ml of nanoparticle dispersion after the total evaporation of the organic solvents and before any additional purification step. N.A.: non-applicable. ^{*1}Based on (161,162). ^{*2}Based on (84) ^{*3} Based on (152) ^{*4}Based on (163) ^{*5}Based on (95). ^{*6}Based on (164). ^{*7} Based on (18). ^{*8} Based on (110).

3- Polymersome preparation

Another method for nanoparticles preparation is the polymersome preparation for drug delivery and diagnostic purposes. Polymersome is a kind of artificial vesicle, enclosing a solution, made of amphiphilic block copolymers and forming a vesicle membrane that recalls the structure of lipids in cell membranes [94]. The amphiphilic block copolymers have been widely studied for drug delivery systems because of self-assembling ability on aqueous solutions and the polymersome are developed on same idea [165]. Polymersome are important as artificial cells because of having large compartment rendering stability properties, tunable membrane and encapsulation of biofunctional compounds [94].

The copolymers used are poly (2-methylaxone) derivatives, PEG (polyethylene glycol) or PEO (polyethylene oxide) derivatives and cholesterol derivatives along with some new polypeptides [99,104,166-167]. Nevertheless, other polymers applied in polymersome formulation include diblock copolymers, such as, poly(ethyl ethylene)-*b*-poly(ethylene glycol) (PEE-PEG), poly(lactic acid)-*b*-poly(ethylene glycol) (PLA-PEG), poly(ϵ -caprolactone)-*b*-poly(ethylene glycol) (PCL-PEG), poly(*N*-isopropylacrylamide)-*b*-poly(ethylene glycol) (PNIPAAM-PEG), poly(styrene-*b*-acrylic acid) (PS-PAA), poly(2-vinylpyridine)-*b*-poly(ethylene glycol) (PV2P-PEG), and polybutadiene-*b*-poly(glutamic acid) (PBD-PGIA), and also triblock copolymers including poly(ethylene glycol)-*b*-poly(propylene sulfide)-*b*-poly(ethylene glycol) (PEG-PPS-PEG). Graft copolymers and branched polymers have also been described for polymersome formulation [168].

The techniques used for polymersome preparation are similar to that of liposomes in which different structures of the block copolymers are used. For the methods applied Kita et al. [169] described two main divisions' *i-e.*, solvent accompanied and solvent free method. The solvent free method is without any organic solvent, hence for the preparation amphiphile in dry state contacts the aqueous medium and is hydrated subsequently. In solvent accompanied method, block copolymer is dissolved in a specific organic solvent and mixed with water then removal of solvent is done by evaporation or any other technique. As it is difficult to remove the solvent completely, there is possibility that solvent residue can cause problems in biological and pharmaceutical applications. A number of factors, including both the structure of amphiphile (chemical constitution and the relative lengths of the individual blocks) and the properties of the solution (such as concentration, pH, temperature and the solvent) can control the size and shape of nanocapsules in this process. [169] Figure 6 shows a

scheme to be followed for polymersome preparation method including both solvent free and solvent displacement techniques.

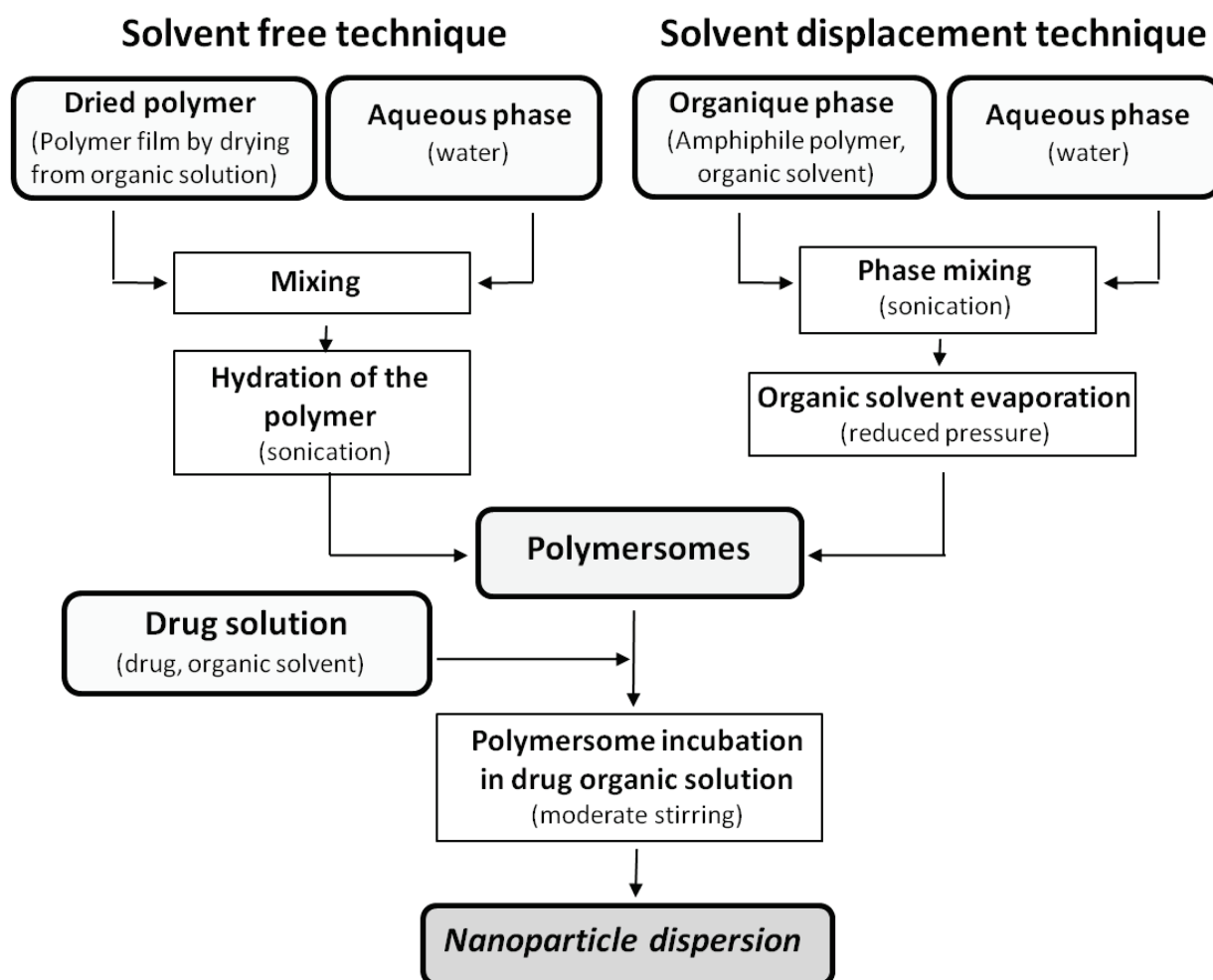


Figure: 6 Schematic representation of the polymersome preparation

In general, the active substance encapsulation in polymer vesicles is done by techniques based on incubation and choice of the polymer is based on nature of the active molecule. However, both hydrophilic and lipophilic molecules can be encapsulated. According to Ahmed et al. [98] polymersome can be used in controlled release of different drugs against tumor offering enhanced permeability, retention effect and high drug loading efficiency as compared to liposomal formulation. The encapsulated active molecules are mostly anticancer agents *e.g.*, paclitaxel [170-171]. The combination of doxorubicin (a hydrophilic active molecule) and paclitaxel into polymersome exhibited greater effect in reducing tumor growth due to increased synergistic effect and less cardiac cytotoxicity [155]. Other examples include

the development of biotin functionalized leuko-polymerosomes [172] to monitor or treat inflammation, cancer and cardiovascular disease, and of Tat peptide-conjugated polymerosomes [173] for cellular trafficking. Early developments in polymerosomes application were focused on parenteral and oral administration with controlled release of active molecules. However, recently the drug targeting applications by ligand incorporation within the membranes, biosensing applications and other routes of administration are being explored [174-175].

4- Supercritical Fluid technology

Nanosized particles can be prepared by use of supercritical fluid technique. Any liquid or gas can be used as supercritical fluid (SCF); although the most commonly used are carbon dioxide and water. SCF can be defined as any substance above its critical temperature and pressure (i.e., $T > T_c$ and $P > P_c$) where no distinct gas and liquid phases exist and the physical properties of SCF exist between those of gas and liquid. The phase diagram of SCF is given for better understanding of the phenomenon.

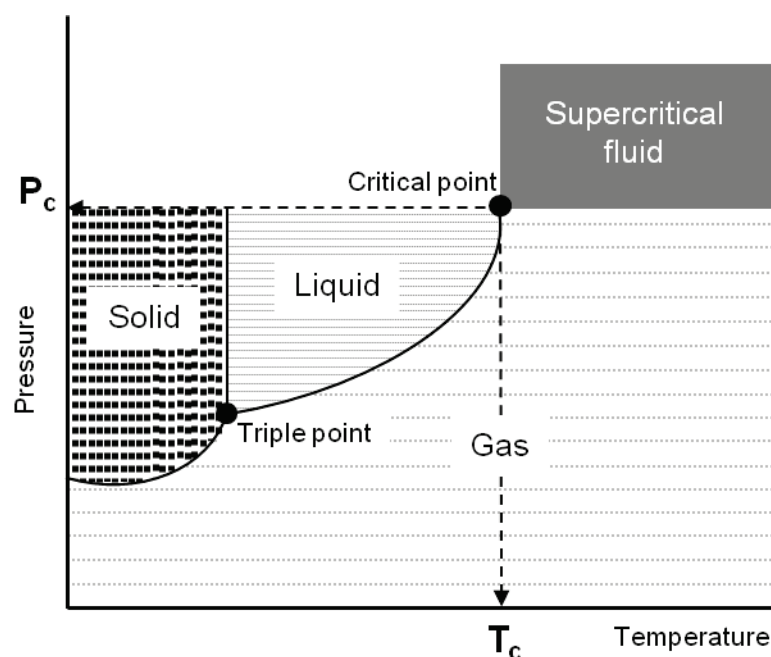


Figure: 7 Phase diagram on supercritical point via pressure temperature

SCFs are being used as organic solvent substitutes in laboratory as well as on industrial scale being harmless to environment. Like other fields, SCF based technology is a new potential technology for nanoparticle preparation and has been adopted by many researchers

for nanoparticles involved in drug delivery purposes. These methods are preferred because of environment friendly solvents and rapid unistep process. Nanoparticles can be obtained without traces of organic solvents, hence; with high purity. SCF technology is a good choice in comparison to existing nanoparticles preparation methods providing an opportunity to control the particle size and morphology in a more efficient manner [175-177].

The preparation of nanoparticles using supercritical technology can further be divided on basis of the method used and this has been done by various authors [178-180] in different ways but the important one is by Mishima [175], who has divided all the processes in four general methods depending on whether SCF was used as a solvent, a solute and an antisolvent.

- 1) Rapid expansion of supercritical solution (RESS),
- 2) Particles from gas-saturated solution (PGSS) process
- 3) Gas antisolvent (GAS) process and
- 4) Supercritical antisolvent (SAS) process and its various modifications

Explanation of all the processes will not be given; a general technique will be discussed. As a summary mentioned in literature [175,178-181]; a mixture of polymer, drug and other excipients (if present in recipe) put in a vessel and SCF is passed through this vessel to absorb/dissolve drug and pressure treatment (expansion of the solution) is done. Then nanoparticles are obtained in a vessel as precipitate after passing the solution through a capillary tube. A general scheme of this process is explained in following figure 8.

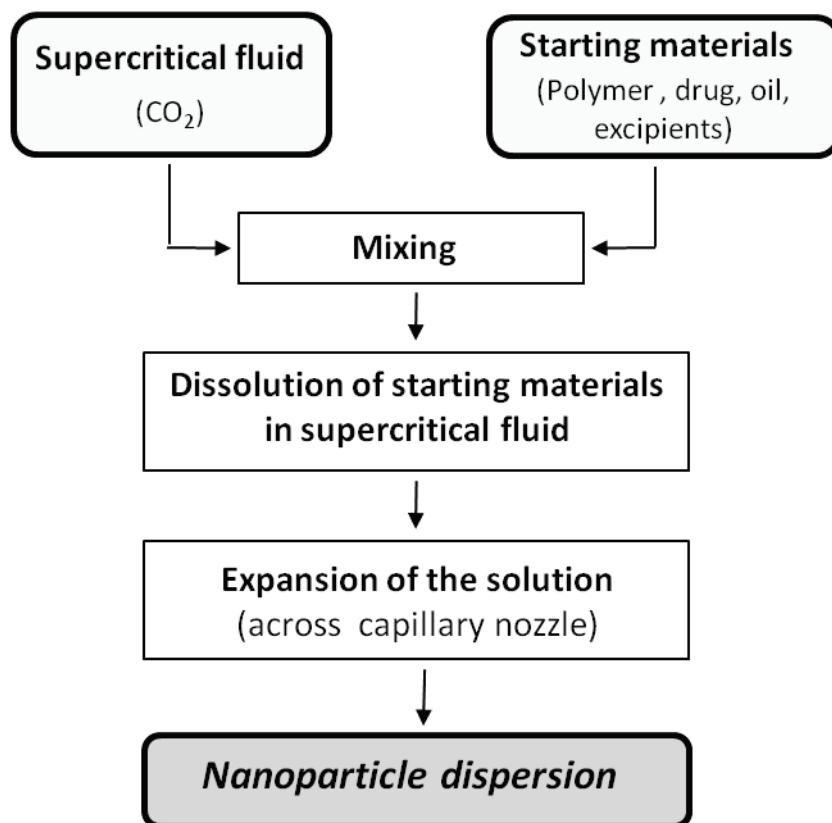


Figure: 8 Schematic representation of the supercritical fluid technology technique

The characteristics of nanoparticles prepared by supercritical fluid technology can be influenced by solute properties (drug, polymer and other excipients if present), parameters used in the technique (temperature and pressure applied, solute and solvent flow rate, nozzle geometry) and the most important the nature and type of SCF used. Less popularity of SCF technology is appreciated to special equipment requirements difficult for every laboratory, involvement of processes like extraction, partitioning and expansion of solvents that may cause porosity resulting in quick release of drugs and low encapsulation efficiency [175] and the last but not least ability of SCF to dissolve only small number of hydrophobic drugs and polymers [182].

5- Polymer-coating method

Polymer coating is a relatively newer technique. Actually coating is deposition of a new layer on the particles and in case of nanoparticles for biological applications; this is used to acquire stability, good solubility or specific functionality for good targeting. In surface engineering of these nanoparticles both inorganic and polymeric materials [183-184] are used. Polymers used for coating purposes can easily be classified as natural and synthetic ones,

although some polymeric surfactants are also in use for coating but here the focus is polymer used for coating technique. The natural polymers used are gelatine, chitosan and dextrane, *etc.* [185-187] while synthetic polymers include poly (lactic-*co*-glycolic acid) (PLGA), poly (vinyl alcohol) (PVA), poly (vinylpyrrolidone) (PVP), poly (ethylene-*co*-vinyl acetate), and poly (ethylene glycol) (PEG) [188-189].

The mechanism of deposition of this new layer on the particle surface can be considered as a result of van der waals forces, hydrogen binding, or other forces for adsorption of polymer on particle surface depending on nature of surface and polymer. For methods to prepare nanoparticles by polymer coating technique there are different propositions. According to Calvo et al. [18] this can be done by putting non coated nanoparticles in a stirred polymeric dispersion for a specific time and hence thin layer of polymer is obtained. It can be done by little modification in the traditional nanoparticle preparation methods (e.g., nanoprecipitation) adding a polymer to external aqueous medium leading to charged particles precipitation and diffusion of solvent [18,108]. However, Prego et al. [107] mentioned the preparation of nanoemulsion template and addition of polymer in continuous phase for its precipitation on droplets and then solvent diffusion and polymer coacervation as shown in Figure 9.

It is impossible to present a general criteria and starting materials for preparing nanoparticles using this technique although some references about nanocapsules preparation by different polymer coating methods are given in the table 3 below.

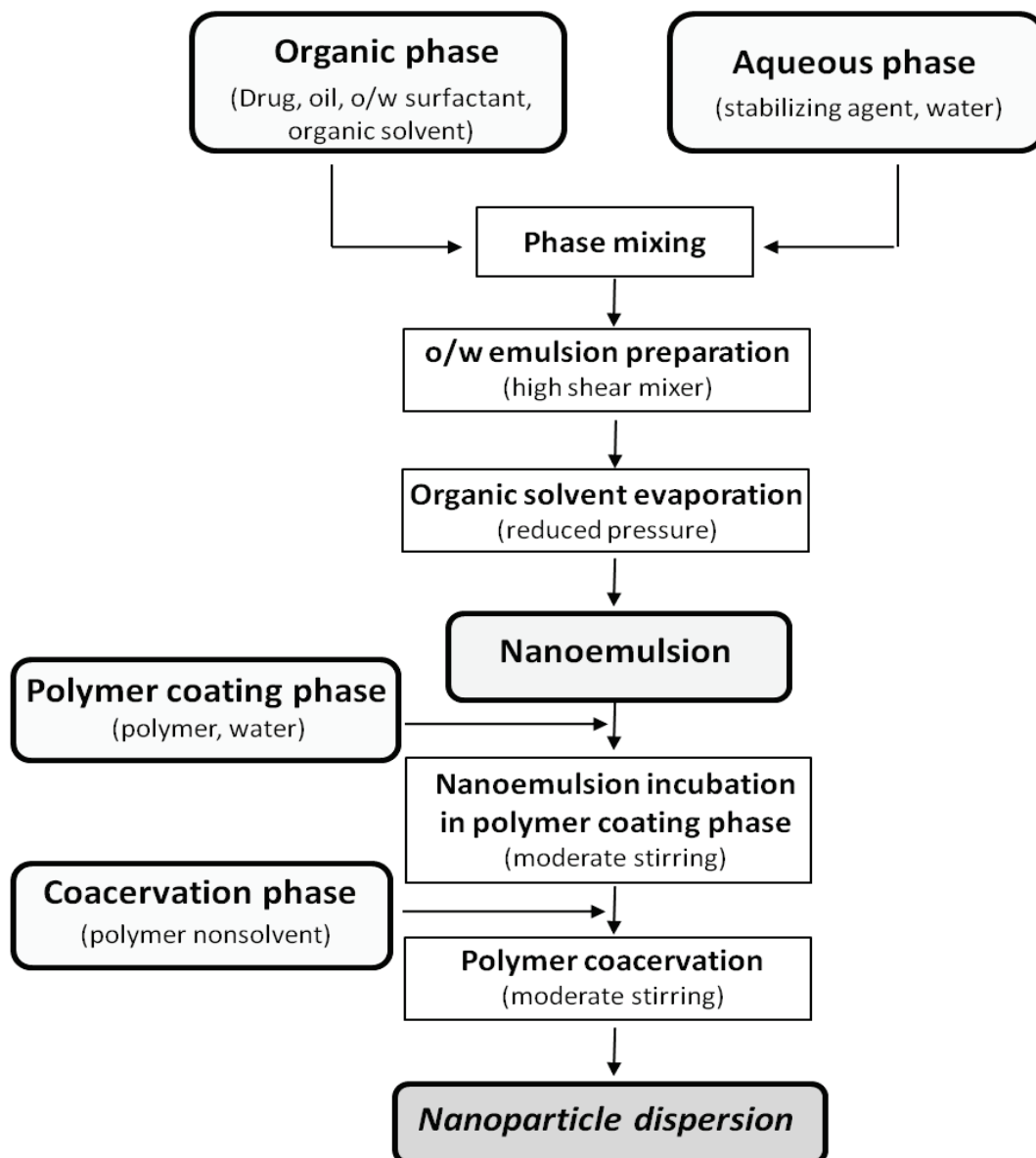


Figure: 9 Schematic representation of the polymer coating method

6 - Layer-by-layer method

The idea of nanoscale objects assembling by electrostatic attraction in layer-by-layer fashion was first coined by Iler [190] however, work of follow researchers [191-196] especially work done by Sukhorukov et al. [197] for biotechnological applications gave a big fame to this technique. Almost every type of macromolecular species including inorganic molecular clusters, [198] nanoparticles, [199] nanotubes and nanowires [200-201] nanoplates, [202] organic dyes, [203] organic nanocrystals, [204] dendrimers, [205] porphyrin, [206] polysaccharides, [207-208] polypeptides, [209] nucleic acids and DNA [210] proteins [211-212] and viruses [213] are successfully used as assembly components [214]. Remarkable versatility has further led to a number of novel designs and applications, such as

superhydrophobic surfaces [215-216] chemical sensors and semi-permeable membranes [214,217-219] drug and biomolecules delivery systems [214,220-221] memory devices [222] optically active and responsive films [223-225] cell and protein adhesion resistant coatings [214,226] fuel cells and photovoltaic materials [227] biomimetic and bio-responsive coatings [228] semiconductors [229-230] catalysts [231-232] magnetic devices [233-234] and many others [214,235].

The mechanism of this newer technique for the nanocapsules preparation is described as irreversible electrostatic attraction resulting in polyelectrolyte adsorption which needs molecules with a sufficient number of charged groups in order to allow a good adsorption on opposite surface. The alternation of the negative and positive surface charges results in formation of continuous assembly between opposite charged materials providing freedom in number of layers and their sequence. However, method is similar to polymer coating except it requires multiple coatings on the template. According to Radtchenko et al. [236] adsorption of polymer layer on template is done by its incubation in polymer solution or by decreasing polymer solubility using an immiscible solvent.

According to Chen et al. [110] like inorganic particles, the solid form of active agent to encapsulate can act as template for layer-by-layer deposition of the polymer. Adsorption can occur by subsequent core dissolution and then hollow nanocapsules can be loaded with required substance [237-242]. A general mechanism is explained in following figure 10.

Radtchenko et al. [236] mentioned penetration of molecules in the polyelectrolyte multilayers; small solutes can easily penetrate while for large macromolecules it is difficult task. Large macromolecules will be present only inside capsules resulting in difference of physicochemical properties between the bulk and capsule interior; a polarity gradient will be established across the capsule wall, a useful step for the precipitation of hydrophobic molecules. On the same lines, Antipov et al. [238] and Ai and Gao [240] explained pH effect on permeability properties of hollow polyelectrolyte nanocapsules and the temperature effect on reversible behaviour of the nanocapsules. Preetz et al. [113] have mentioned the nanocapsules having an oily core covered with polyelectrolytes were designed for the incorporation of lipophilic drugs and prepared by high pressure homogenization. The different polyelectrolyte layers were deposited in a way as for complex coacervation process and then nanocapsules dispersion was treated by homogenization and centrifugation at the end. The advantage over other mostly used methods mentioned by Preetz et al. [113] is deletion of the separation step.

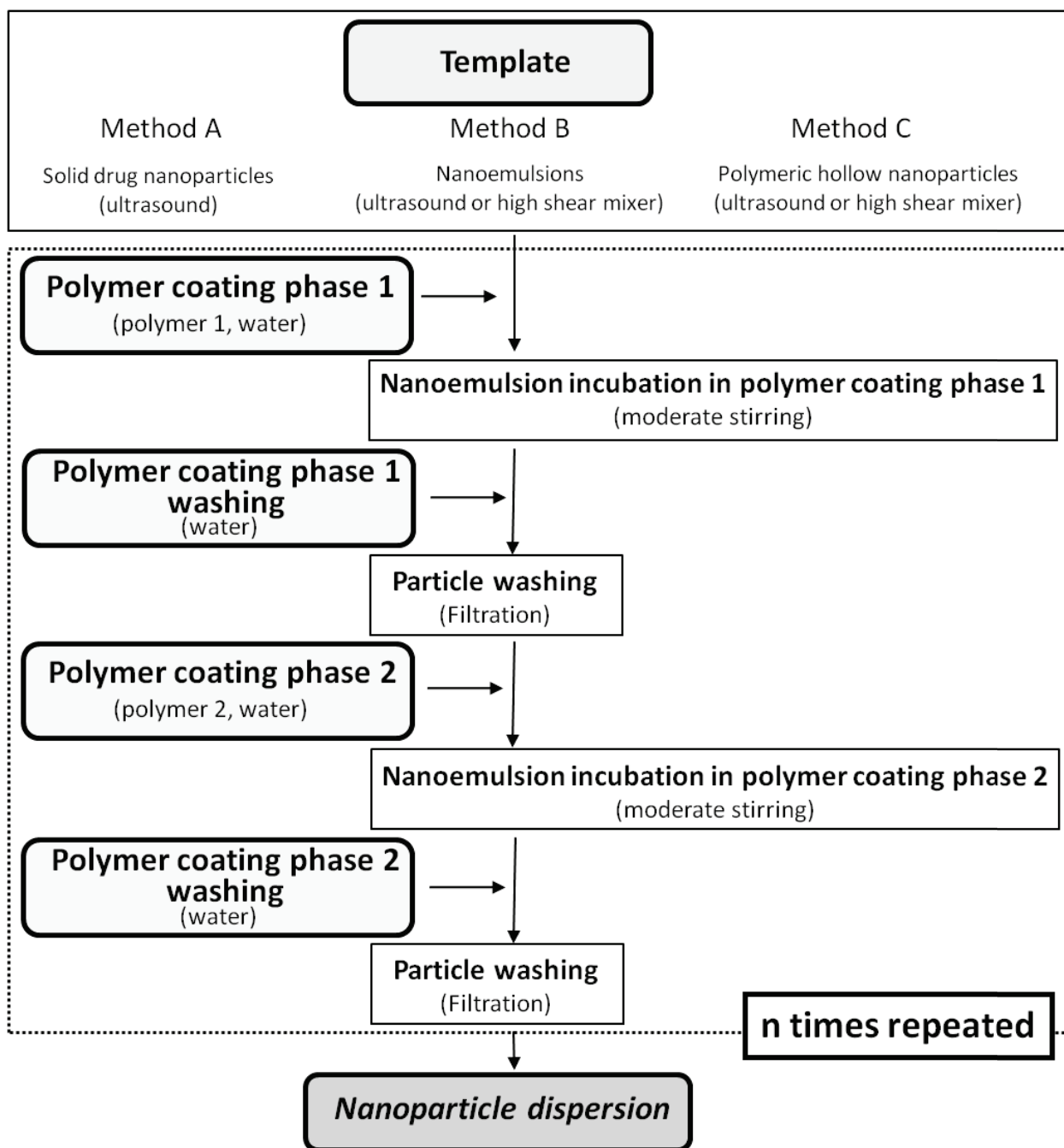


Figure: 10 Schematic representation of the polymer coating method

Small sized particles are obtained by ultrasonic sonication of the suspension to decrease the size of individual drug particles which can further be stabilized by coating using ultra-sonication and hence thin polyelectrolytes are assembled on their surfaces [112]. The other difficulties include formation of contraion aggregates, separation of free polyelectrolytes from the particles before next layer deposit and polyelectrolyte bridging during centrifugation.

According to Sukhorukov et al. [197] a close particle-particle encounter can result in unnecessary interaction with films leading to aggregate formation and film destruction.

As mentioned above, the essential for this method is the choice of polymers having sufficient number of charged groups, utilizing polyanions e.g., poly (styrene sulfonate) (PSS), sodium alginate, poly (acrylic acid), dextran sulfate, carboxymethyl cellulose, hyaluronic acid, gelatin A, chondroitin heparin and polycations e.g., polylysine, chitosan, gelatin B, poly (allylamine) (PAA) poly (ethyleneimine) (PEI), amini-dextran and protamine sulphate [112,243-244]. The examples of hydrophobic drugs encapsulated include paclitaxel, tamoxifen, and curcumin [112,245-247].

Behavior of nanoparticles as drug delivery systems

This section covers the discussion of some nanoparticles properties including size, zeta potential, drug encapsulation efficiency, release of the active ingredients and performance related to above mentioned preparation methods.

1. Mean size

The particle size is an important characteristic of any particulate system especially one used in pharmaceutical applications. The importance increases in view of *in vitro* and *in vivo* applications. Table 3 shows some examples of size of particles prepared via the different methods discussed. According to Hans et al. [248] the molecular weight and concentration of polymer has effects on size of the nanoparticles but targeted size depends on purpose of delivery system e.g. if the goal of administration is rapid dissolution or arterial uptake then approximately 100 nm particles are suitable but if purpose is targeting mononuclear phagocytic system (MPS) or prolonged dissolution then 800 nm particles have the preference as the drug release kinetics depends on mean size and size distribution [249]. In different nanoparticles preparation techniques, several factors can be modified to have the required nanoparticle size as well. The most important of these factors are nature and concentration of polymers and surfactants, solvent choice, stirring rate (when emulsification is involved), compatibility and ratio of external/internal phase but there are contradictory views for nature and concentration of the drug to be encapsulated. Different methods being used for size measurement include dynamic light scattering (DLS), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) but their detail is out of the context.

2. Zeta-potential

Zeta potential gives idea about charge of particle; higher the zeta potential value higher will be the charge on the particle surface. Zeta potential is important to characterize nanoparticles because the stability, adhesion and rheological properties of colloids depend on their zeta potential value [250] and indirectly on the repulsive and attractive forces that a colloidal particle experience. It gives an idea about the potential at the diffused double layer [251] but unable to explain completely the phenomena of coagulation. However, it is considered important by researchers to characterize nanoparticles and more descriptive behaviour about coagulation is related to critical coagulation concentration (c.c.c.) along with consideration of slow coagulation regions [252].

Table 3. Examples of mean size and zeta potential of nanoparticles prepared by different methods

Active ingredient	Nanoparticle type	Mean diameter range (nm)	Zeta potential (mV)	Reference
Nanoprecipitation				
All-trans-retinoic acid	Nanospheres	70 - 460	nr.	253
Atovaquone	Nanocapsule	200 - 270	nr.	25,37
Benzathine penicillin G	Nanocapsule	130 - 230	nr.	45
Cyclosporin A	Nanospheres	110 - 215	nr.	254
Dexamethasone	Nanocapsule	270 - 320	nr.	34
Diclofenac	Nanocapsule	120 - 420	nr.	14,35
	Nanospheres	85 - 195	nr.	14
Flubiprofen	Nanospheres	150 - 290	-25 to -30	255
5-Fluorouracil	Nanospheres	75 - 255	nr.	256
Gemcitabine derivatives	Nanocapsule	180 - 300	nr.	13
	Nanospheres	150 - 190	+25 to +35	
Griseofulvine	Nanocapsule	380 - 400	nr.	27
	Nanospheres	250 - 325	nr.	
Ibuprofen	Nanospheres	105 - 145	nr.	57
Indomethacin	Nanocapsule	120 - 450	-35 to -50	18,22-23,28,34,36
	Nanosphere	170 - 180	nr.	22
Indomethacin ethyl ester	Nanocapsule	230 - 295	-45	28,30
Isradipine	Nanosphere	200 - 210	-20 to -35	257
Melatonin	Nanocapsule	185 - 295	-30	15
Oridonin	Nanosphere	105 - 195	~ -15	258
Paclitaxel	Nanosphere	115 - 160	-20 to -35	259

Primaquine	Nanosphere	150 - 165	-35 to +1.5	260
Primidone	Nanocapsule	222	nr.	19
Progesterone	Nanocapsule	225 - 275	nr.	34
Rifabutine	Nanocapsule	200 - 500	nr.	37
Spironolactone	Nanocapsule	310 - 410	nr.	29
Tacrolimus	Nanocapsule	350 - 400	nr.	17
Tamoxifen	Nanosphere	180 - 800	-15 to 800	261
Taxol	Nanocapsule	240 - 280	nr.	34
^{99m} Tc-HMPAO complex	Nanocapsule	240 - 360	-60	44
Tretinoin	Nanocapsule	220 - 230	-5 to -7	31
Usnic acid	Nanocapsule	140 - 290	-30	262
Vitamin E	Nanocapsule	260	nr.	26
Vitamine K	Nanocapsule	240 - 310	nr.	34
Xanthone	Nanocapsule	265 - 300	-40	46
Emulsification-diffusion				
17-b-estradiol benzoate	Nanosphere	75 - 350	nr.	72
Chlorambucil	Nanocapsule	330 - 335	nr.	131
Clofibrate	Nanocapsule	365 - 375	nr.	
Cyclosporine	Nanosphere	60 - 270	nr.	77
Enalaprilat	Nanosphere	180 - 615	-30 to - 60	56
Estradiol	Nanocapsule	335 - 345	nr.	131
	Nanosphere	95 - 650	+70 to +95	75,78-79,
Eugenol	Nanocapsule	320 - 325	-6	66
Hinokitol	Nanocapsule	225 - 600	nr.	64
Ibuprofen	Nanosphere	310 - 430	nr.	57
Indomethacin	Nanocapsule	310 - 520	nr.	59,131, 139
4-Nitroanisole	Nanocapsule	550 - 600	nr.	68
Progesterone	Nanocapsule	505 - 515	nr.	131
Vitamin E	Nanocapsule	355 - 365	nr.	
Tacrolimus	Nanosphere	215 - 220	-20 to -30	81
p-THPP	Nanosphere	90 - 160	-4 to -8	69
Triclosan	Nanosphere	175 - 450	nr.	71
Emulsification-coacervation				
HAuCl ₄	Nanocapsule	185 - 219	nr.	86
Hydrocortisone	Nanosphere	150 - 230	-4 to -12	92
Triamcinolone acetonide	Nanocapsule	300	nr.	84
Turmeric oil	Nanocapsule	195 - 470	-17	83
Double emulsification				
Triptorelin	Nanosphere	350 - 600	nr.	163
Polymersome preparation				
NC-1900	Nanocapsule	90 - 110	-21	95
Paclitaxel	Nanocapsule	80 - 120	nr.	98

Supercritical fluid technology				
Cefurexime axetil	Nanosphere	< 1000	nr.	164
Dexamethasone acetate	Nanosphere	500 - 1000	nr.	175
Polymer coating				
Indomethacin	Nanocapsule	245 - 255	+30	18
Salmon calcitonin	Nanocapsule	255- 270	+35	107
Layer-by-layer				
Artemisinin	Nanocapsule	800	-30	110
Tamoxifen	Nanocapsule	100 - 120	-20 to 50	112
*nr.: Non-reported data; ^{99m} Tc-HMPAO complex: Technetium-99m D,L-hexamethylpropyleneamine oxime; HAuCl ₄ : Hydrogen tetrachloroaurate; NC-1990: Vasopressin fragment analog; p-THPP: Mesotetra(p-hydroxyphenyl)porphyrin.				

In nanocapsule preparation, the zeta potential depends on nature of the polymer and the stabilizing agent and at medium pH values. Hence, unable to explain any general trend about zeta potential of nanoparticles prepared by above mentioned methods because of unknown pH and salinity of the solution. Some examples of particles zeta potential are included in Table 3. Generally, in water and other polar solvents, the adsorption of anionic surfactants induces negative zeta potential, and adsorption of cationic surfactants induces positive values while in hydrocarbons and other non-polar solvents, the case is reversed and anionic surfactants induce positive zeta potential, and cationic surfactants induce negative zeta potential [263]. According to Mora-Huertas et al. [162] zeta potential values are dependent on the adsorption of stabilizing agent on the nanocapsule surfaces and hence it can be expressed as non-ionic stabilizing agents that give negative zeta potential values with polyester polymers/methacrylate derivatives and positive values with cationic polymers. They further explained that negative zeta potential values are obtained by using negative charged stabilizing agent and polymers and positive values are obtained by using positive charged stabilizing agents and polymers.

3. Encapsulation efficiency

Encapsulation efficiency is one of the important criteria for the characterization of the drug delivery systems. It evidences the success of a selected technique for encapsulation of active ingredient. There are various factors associated both to the preparation and the starting materials used for preparing nanoparticles that can affect the drug entrapment efficiency. As can be seen, drug encapsulation data is variable even by using the same method. Then

further it depends on the operational conditions involved as the volume of organic solvent [19], the volume of aqueous phase [29] or the active substance concentration [46] and of the type of nanoparticle prepared. The encapsulation efficiency of nanocapsules and nanospheres obtained by above mentioned different preparation method is given in table 4.

Table 4. General tendencies on drug encapsulation by different methods for the nanoparticle preparation

Nanoparticle preparation method	Encapsulation efficiency (%)	
	Nanocapsules	Nanospheres
Nanoprecipitation	50 - 100	20 -100
Emulsification-diffusion	30 - 100	20 - 85
Emulsification-coacervation	~ 45	~ 40
Double emulsification	nd.	4 - 80
Polymersome preparation	~ 20	-
Supercritical fluid technology	-	nd.
Polymer coating	40 - 90	nd.
Layer-by-layer	85 - 100	nd.

*nd. Non-disponibile information; -: It is not possible the preparation of this kind of nanoparticles by this method.

However, taking particular examples of nanoparticles prepared via nanoprecipitation technique and the emulsification-diffusion method, it seems that nanocapsules render the most efficient encapsulation rates, as mentioned in the table 5.

Table 5. Drug encapsulation by using nanoprecipitation and emulsification-diffusion methods for the preparation of nanocapsules and nanospheres.

Active molecule	Encapsulation efficiency (%)	Reference	Active molecule	Encapsulation efficiency (%)	Reference
Nanocapsules			Nanospheres		
Nanoprecipitation					
Indomethacin	100	34	Indomethacin	100	22
Indomethacin	100	28	Primaquine	85 - 94	260
Atovaquone	100	25	Cyclosporin A	82 - 98	264
Atovaquone	100	37	Cyclosporin A	90 - 98	254
Rifabutine	93	37	Pentamidine	40 - 76	26
Tretinoin	100	31	Isradipine	74 - 97	257
Primidone	75 - 67	19	Nimodipine	20 - 90	49

Spirolactone	76 - 96	29	Rolipram	2 - 20	89
Griseofulvine	78 - 99	27	Tyrphostin AG-1295	70	127
Melatonin	50	15	Paclitaxel	15 - 100	259
Diclofenac	97 - 100	14	Acyclovir	1 - 3.5	266
Diclofenac	100	35	Diclofenac	100	14
Xanthone	85 - 89	46	Ibuprofen	40 - 50	57
All-trans retinoic acid	68 - 97	253	Xanthone	26 - 40	46
			Griseofulvin	78 - 98	27
			Docetaxel	10 - 23	267
			Flurbiprofen	74 - 97	255
			Haloperidol	0,25 - 4	88
			Coenzyme Q10	49 - 72	268
			Silymarin	20 - 62	269
			Carvedilol	41 - 56	270
			Phenobarbital	94	90
Emulsification-diffusion					
Indomethacin	100	131	17- β -estradiol benzoate	67	72
Vitamin E	92		Enalaprilat	24 - 46	56
Progesterone	99		Ibuprofen	62 - 86	57
Estradiol	52		Estradiol	46 - 73	75
Chlorambucil	32		Cyclosporine	16 - 23	77
Hinokitol	92 - 94	64	Estradiol	35 - 68	78
			Estradiol	48 - 95	79
			Tacrolimus	50 - 60	81

Regardless of the technique and operational conditions used, molecule properties are relevant for the successful encapsulation of active substances. They include drug solubility in aqueous and organic media, the drug stability and the drug-polymer interactions [178]. As mentioned above, nanoparticle preparation methods often require organic and aqueous phases. Usually hydrophobic drugs and the polymeric matrix are dissolved in organic solvents doing it essential to determine the drug solubility in different organic solvents in order to select the best option for getting optimal drug quantities to be processed. On the other hand, considering the particularities of the method and the mechanism of nanoparticle formation, it could be advisable to guarantee the active molecule in its non-dissociate state in order to lead to the highest encapsulation efficiency via the manipulation of aqueous phase properties (e.g., by the pH adjustment). Likewise, nanoparticles are dispersed in aqueous media which depending on its composition (e.g., containing surfactant agents) or its pH could favour the drug leakage

from the nanoparticles during the storage. As particular case, it is interesting to highlight the effect of pH on the encapsulation efficiency of triptorelin by using the double emulsion evaporation method [163]. Here, the highest encapsulation efficiency is achieved when isoelectric point arrives for internal and external aqueous phases.

Drug stability at the operating conditions also governs its encapsulation. Then, emulsification conditions in some cases use high shear forces rendering high local temperatures which could induce degradation of temperature sensitive drugs. On other hand, high drug-polymer interactions could be considered when polymer is chosen mainly because of chemical affinity could govern the drug trapping. It is expected that good interaction between the active molecule and the polymeric matrix will allow a drug partition favouring its encapsulation.

4. Drug release Properties

The active substance release from nanoparticles is an important parameter to consider in their development as drug carriers being the basic point for their *in vivo* performance.

In vitro release of the active substance depends on concentration and physicochemical properties of drug, biodegradability, nature and concentration of polymer, nature of solvent and its compatibility with other starting materials, size of the nanocapsules, the conditions of preparation and also the conditions of *in vitro* testing e.g., temperature, pH, presence of surface-active agents, *etc.*

Unfortunately, drug release data reported by the different research works on nanoparticle preparation is limited and it is not possible to have a comparative study using the same active molecule. Moreover, same active molecule is encapsulated by different methods; the experimental conditions used by research team for investigating *in vitro* drug release are different. Considering the formers drawbacks which limit the discussion, although as an attempt to do disposable an overall view on the drug release from nanoparticles prepared using the different methods, Figure 11 compiles the behaviors of typical examples. It is visible that immediately or extended drug release profiles can be obtained. Nanoparticles prepared via the supercritical fluid technology lead to the drug availability in the dissolution medium during the first 15 min of start of the test. On the contrary, polymersomes and nanoparticles prepared by using the double emulsification seem to release the active very slowly (50% of the molecule is released after one day of beginning the dissolution study).

Active substances encapsulated by using the other nanoparticle preparation methods exhibit release behaviors included between this range.

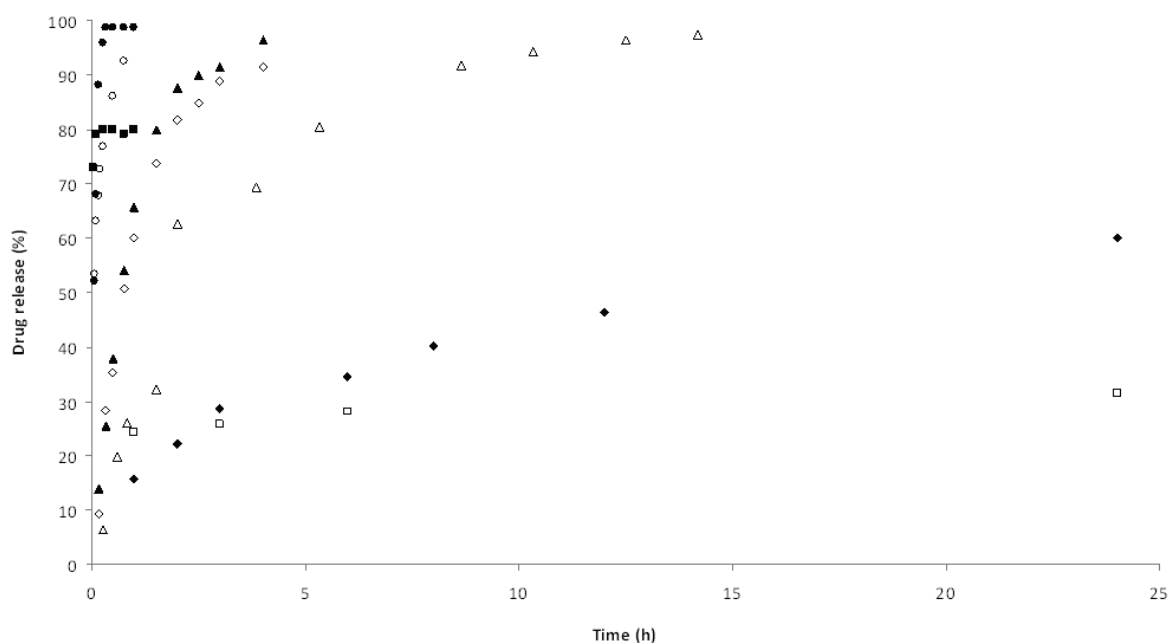


Figure: 11 Drug release behaviors of nanoparticles prepared by different methods. NP: Indomethacin nanocapsules by nanoprecipitation [18]; ED: Indomethacin nanocapsules by emulsification-diffusion [139]; EC: Triamcinolone acetonide nanocapsules by emulsification-coacervation [84]; DE: Triptorelin nanospheres by double emulsification [163]; Pms: NC-1900 nanocapsules by polymersome preparation [95]; SCF: Cefuroxime axetil nanospheres by supercritical fluid technology [164]; PC: Indomethacin nanocapsules by polymer coating [18]; LbL: Artemisin nanocapsules by layer-by-layer [110].

5. General performance of the nanoparticles

The nanoparticles have been developed for better delivery of drugs and targeting specific cells in body to overcome the problems related to the conventional systems. The most important problem was the insolubility of drugs in water but detailed research on nanoencapsulation is for other purposes like sustained drug delivery, targeting of specific organs, overcome the multidrug resistance, achieve higher bioavailability, minimize the side effects, protection of the drugs from specific body defence mechanisms and most importantly required therapeutic activity. For example in cancer research use of nanoparticles can overcome the drug resistance at tumour and cellular level, to enhance pharmacokinetic properties of anti-cancer agents and the most important to reduce the toxic effects to normal cells [271]. However, these targets are not easy to achieve because of many influencing

factors like preparation difficulties, *in vitro* and *in vivo* analysis problems and interaction of body with contents of the nanocapsules including drug, polymer, and other ingredients, *etc.* although the success is there and with passing time, the techniques are improving with discovery of new approaches. e.g., Soma et al. [272] report encapsulation of doxorubicin, a water soluble molecule, with cyclosporine A to reduce the side effects of both drugs with increasing their therapeutic efficacy giving a new idea of co-encapsulation.

Keeping in view all the above mechanisms and requirements, researchers are working hard on *in vitro* and *in vivo* modelling, for more than a decade to find a good solution. The delay in success or less success to achieve these targets can be attributed to the limitations of the nanoparticles. A review of the success and results of researcher's trails is shown in table 6 below.

Table 6. *In vitro* and *in vivo* performance of polymeric nanoparticles prepared by several methods

Active	Preparation method	Test	Conclusion	Reference
Atovaquone	Nanoprecipitation	Antiparasitic activity.	Increasing of the therapeutic effect.	37
Indomethacin	Nanoprecipitation	Pharmacokinetic study and potential irritant effect on the rectal mucosa in rabbits.	Improvement of the extravascular distribution. A limited protective effect on the rectal mucosa was evidenced.	36
Indomethacin	Nanoprecipitation	Active ocular distribution and acute ocular tolerance studies in rabbits.	Good ocular tolerancy and ocular bioavailability.	18
Indomethacin ethyl ester	Nanoprecipitation	Antiedematogenic activity study in rats.	Nanoencapsulation was not able to target the prodrug to the site of action and the antiedematogenic effect observed was due to metabolite effect.	30
Melatonin	Nanoprecipitation	Acute antioxidant effect of intraperitoneal administration in mice.	Improvement of the drug antioxidant effect.	15
Muramyltripptide cholesterol (MTP-Chol)	Nanoprecipitation	Immunomodulating capacity towards a mouse macrophage cell line <i>in vitro</i> .	MTP-chol included within biodegradable polymeric nanoparticles activates mouse macrophages.	38

Oridonin	Nanoprecipitation	Pharmacokinetics and tissue distribution studies.	Drug could obtain prolonged circulation time and accumulate in liver, spleen and lung.	258
4-(N)-Stearoylgemcitabine	Nanoprecipitation	Cytotoxic activity on human cancer cell lines.	Active incorporation in nanocapsules did not change the IC ₅₀ compared to the free active.	13
Tacrolimus	Nanoprecipitation	Pharmacokinetic study in rats and pigs.	Nanocapsules yielded significantly higher drug levels than an active emulsion, resulting in a more enhanced bioavailability.	17
Usnic acid	Nanoprecipitation	Antitumor activity in Sarcoma 180-bearing mice and subchronic toxicity in healthy animals.	Nanoencapsulation was able to maintain and improve the usnic acid antitumor activity and considerably reduce the hepatotoxicity of this drug.	47
Salmon calcitonin	Polymer coating	Hypocalcemic effect in rats.	Pulsatile pharmacological profile and enhancement of the hypocalcemic effect when compared to the peptide solution.	107
Tamoxifen	Nanoprecipitation	Cell uptake and distribution studies using breast cancer cells.	Drug vectorization could serve as a useful form of targeted drug delivery system towards breast cancer.	273
Docetaxel	Nanoprecipitation	Interaction with biomembrane models.	Drug was not able to diffuse into the external medium through the polymer matrix, then longer times are needed to obtain complete drug release.	267
Estradiol	Emulsification-diffusion	Drug release behavior in rats.	Nanoparticles can be effective in improving the drug oral bioavailability.	78
Hinikitol	Emulsification-diffusion	Permeation study in hairless mice.	The active nanoencapsulated was more skin-permeable than active in propyleneglycol.	64
Tacrolimus	Emulsification-diffusion	Drug targeting to the lymphatic system.	Nanoparticles accumulate to the target lymph site during continuous systemic circulation. They easily pass through the walls of lymphatic capillaries acting as "passive targeting" drug delivery system.	81
Triclosan	Emulsification-diffusion	Study in dogs with induced periodontal defects.	Nanoparticles penetrate through the junctional epithelium, and then they could help decrease gingival inflammation.	71

Paclitaxel	Polymersome	Antitumor activity in human breast carcinoma xenograft model and <i>in situ</i> apoptosis in isolated tumor tissue.	Polymersomes persist in the circulation and accumulate drug in solid tumors following a pattern of controlled release.	98
Paclitaxel	Layer-by-layer	Cytotoxic activity.	Targeted particles increase cytotoxicity of tumor cells.	112

Conclusion

The primary goal of nanoencapsulation is to solve the insolubility problem of different active ingredients especially hydrophobic drugs which can be accomplished by nanoparticles preparation providing increased surface area and especially increased surface to volume ratio. The other factors responsible for the choice of nanocapsules include improvement in pharmacokinetic and pharmacodynamic profiles of the drugs (the most important is controlled release properties of the drugs) and decrease in the side effects especially on the neighbouring normal cells of the target. The main focus of the earlier research in nanotechnology application in biomedical field includes the work on antineoplastics, anti-inflammatory drugs, antigens, hormones, vitamins, antivirals, antibacterials, antifungals, antipneumocystics and diuretics. The nanocapsules preparation methods are almost based on the use of preformed polymers. The properties of the polymer (e.g., type, molecular weight, solubility, biodegradability, biocompatibility, *etc.*) and the properties of the active molecules (e.g., nature, molecular weight, charge, solubility, *etc.*) play an important role in the selection of the appropriate technique of nanocapsules using preformed polymers. The different researchers proved that the nanoencapsulation of hydrophobic drugs can be achieved via nanoprecipitation, emulsification methods, supercritical fluid technology, polymersome, polymer coating and layer-by-layer methods according to the specific requirements and with modifications of the technique. The required properties can be enhanced encapsulation, controlled release, improved bioavailability, targeting specific site (tissues or cells), reduced toxicological effects and suitability for a specific route of administration. However, there are factors to be considered that can affect significantly each method resulting in effects on the physicochemical properties of the nanocapsules, the most important is size.

The progress in research opened new horizons enriched with new ideas to obtain many requirements/advantages at the same time from the nanocapsules. The preparation of hybrid compounds having a metal and the active ingredients lead to several advantages especially use

of the nanoparticles as theranostic i.e having both diagnostic and therapeutic properties. The metals used normally include gadolinium, gold, silver, and iron, *etc.* For sake of example, use of such type of theranostic agents for cancer includes diagnostic purposes mostly imaging of cancerous cells e.g., MRI (Magnetic resonance imaging). In this case iron oxide nanoparticles are encapsulated with active ingredients to give the therapeutic and diagnostic advantages. The further research leads to the creation of intelligent/smart particles e.g., thermo-sensitive particles which release the active ingredients at a specific temperature.

After above discussion it can be simply concluded that the preparation of nanoparticles and their use in drug delivery systems is not less than a milestone in biomedical field and also it results in setting new trends for the researchers.

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PART III
EXPERIMENTAL PART

General Summary

As described earlier, cancer being fatal disease is among various diseases with high rate of incidents and still spreading at high rate. This might be due to living style e.g. smoking or any other factor. The ultimate solution of the illness is necessary on urgent basis. Numerous research groups are working for finding a solution but till now none among suggested ones is trustable. This work is a struggle for finding a trustworthy solution. It consists of the advanced technique including application of nanotechnology that is beneficial in various ways. It does not deal with pharmacological aspects of a drug (nothing to do with mechanism of action of drug) but with the delivery of drug to the site affected. In other words, it emphasizes on the pharmaceutical technique to prepare the nanoparticles for the delivery of the required moiety to the specific site. It's the advancement in nanotechnology that allows us to use this technique for having multiple advantages. This multiple advantage includes rapid diagnosis and delivery of the drug for therapeutic purposes. To diagnose cancer various tests and modern techniques are involved and imaging modalities are one of the important weapons in this regard. Different imaging techniques used various types of ingredients, called as contrast agents, to visualize/differentiate the affected part of the body from the normal body tissues. In this case MRI was selected as diagnostic mechanism to help our therapeutic moiety in creation of theranostic agent. The iron oxide is used as contrast agent in MRI and selection of which has been discussed already.

Many pharmaceutical techniques are in process of development and are used for the preparation of nanoparticles including nanoprecipitation, emulsion diffusion, emulsion evaporation, layer by layer and supercritical fluid technology. This method is modification of emulsion evaporation in which multiple emulsions and especially double emulsion technique is used. It can be further specified as water/oil/water double emulsion coupled with evaporation. The choice of method especially double emulsion was done due to its permission for the use of both hydrophilic and hydrophobic drugs. The evaporation using a rotavapor is coupled for the removal of oily phase. Reason for the evaporation is the prevention from possible toxicity of oily phase. Evaporation allowed us to choose any type of organic solvent as oily phase. To prepare these polymeric nanoparticles a biodegradable polymer PCL was chosen due to its biocompatibility properties. Choice of the oily phase was based on the solubility of PCL. DCM was brought into account as oily phase due to its low boiling point so easy to remove via evaporation under pressure using rotavapor. For emulsion preparation, an important ingredient is the stabilizing agent, the ingredient responsible for stabilization of two

phases. To investigate this, a preliminary study was performed using various stabilizing agent in second emulsification step like triton-405, tween 80, poloxamer, PVP (Polyvinylpyrrolidone), PEG (poly ethylene glycol) and PVA (poly vinyl alcohol), *etc.* Nonetheless, the best results were obtained using PVA in concentration of 0.5%. In first emulsification step, no stabilizing agent/surfactant was introduced as second emulsification step was instantaneous. Furthermore the validation of the method was done by encapsulation of a model hydrophilic drug i-e a fluorescent agent, a derivative of Stilbene. Along with encapsulation of Stilbene various parameters affecting the hydrodynamic size of final particles were studied. This study revealed some important parameters to be considered including stirring speed and stirring time for both emulsification steps. The concentration of polymer and stabilizing agent also play role in determining size of the final particles.

MRI is one of the mostly used techniques being non invasive for distinguishing cancerous cells/tissues in the body than the normal tissues. It needs a specific ingredient, called contrast agent for the visualization or differentiation of these affected cells from normal cells. Various inorganic materials are used as contrast agents for MRI like gadolinium, manganese oxide, gold and iron oxide. Iron oxide was selected as contrast agent for MRI because of the facile preparation mechanisms and economy of the process along with its paramagnetic and superparamagnetic properties. A classical chemical method was used for the preparation of iron oxide nanoparticles called co precipitation method in which ferrous and ferric chloride were dissolved in water and allowed to react in the basic media provided by NH_4OH . After preparation of iron oxide nanoparticles, surface modification was done for the dispersion of nascent particles in respective solvent. Iron oxide nanoparticles dispersible in organic and aqueous media were prepared. This preparation was done envisaging the double emulsion method that allows the encapsulation of both types of material (hydrophilic and hydrophobic) and hence on the selection of anticancer agent to be encapsulated. With preparation of both types of iron oxide nanoparticles and using double emulsion method a choice can be made on the basis of the solubility of the active ingredient. In beginning of the work, the target was to prepare the nanoparticles finally to be used for the dual functions. This preparation will allows us to encapsulate both active ingredient and contrast agent together in same nanoparticles to be used as theranostic agent. The iron oxide or contrast agent encapsulated will be used as diagnostic tool in MRI while the chemotherapeutic agent will serve the purpose of therapy. For this reason, the prepared iron oxide nanoparticles were encapsulated using newer modified emulsion evaporation method and whole characterization

was done. The characterization includes the determination of size and zeta potential values as a mark for the stability via DLS technique, TEM for morphological study and size estimation too, magnetization of the particles to determine their paramagnetic or superparamagnetic behaviour and thermogravimetric analysis for estimation of IONPs encapsulated. Encouraging results were obtained for the prepared particles with high percentage of encapsulation of iron oxide and morphology of the particles.

III.1. PREPARATION OF NANOPARTICLES

Résumé

Ayant des avantages sur les systèmes classiques de vectorisation de médicaments, les dispersions colloïdales améliorent la solubilité des molécules actives, la biodisponibilité et la perméabilité des médicaments encapsulés. Les nanoparticules sont des systèmes de vectorisation de médicaments restent une stratégie prometteuse pour l'industrie biopharmaceutique. Actuellement, la préparation de nanoparticules biodégradables contenant des molécules actives attire l'attention d'un grand nombre de chercheurs. Différentes méthodes sont utilisées pour la préparation de ces nanoparticules encapsulant des molécules d'intérêt. Le but de cette étude est de réaliser pour la première fois des particules submicroniques par la méthode de double émulsion et évaporation de solvant ($e_1/h/e_2$) pour encapsuler des substances hydrophiles. Des nanoparticules contenant un agent fluorescent, une dérivée du Stilbene comme modèle de principe actif, chimiquement stable et hydrophile ont été préparées. Dans cet objectif, PCL (polycaprolactone) a été choisi comme polymère et le DCM (dichlorométhane) comme phase organique et un agent stabilisant. Plusieurs types de stabilisants [Triton-405, tween 80, poloxamère, PVP (polyvinyle pyrrolidone), PEG (polyéthylène glycol) et l'alcool polyvinylique] ont été examinés et les résultats ont montré que l'alcool polyvinylique (à 0,5%) favorise la formation de double émulsion stable avec une taille des particules submicronique.

Pour la préparation de l'émulsion, un appareil d'homogénéisation, Ultra-turrax a été utilisé et l'évaporation a été réalisée sous pression réduite. Plusieurs paramètres affectant la taille des particules telles que le temps d'agitation et la vitesse d'agitation (pour la 1^{ère} et la 2^{ème} émulsification), la concentration de polymère et la teneur en agent stabilisant. Les émulsions résultantes ont été caractérisées en termes de potentiel zêta, la taille finale des particules et la distribution de taille. Le taux d'encapsulation de la molécule active après stockage des émulsions préparées pour une durée d'un mois a été mesuré en utilisant un spectrophotomètre UV et la quantité encapsulée a été trouvée de l'ordre de 85%. L'analyse par microscopie électronique à transmission (MET) a montré que les particules préparées ont une forme sphérique. La taille est submicronique comprise entre 150 à 400 nm et que la distribution de taille est relativement large.

En conclusion, la méthode modifiée de double émulsion-évaporation de solvant a été examinée via une étude systématique. Il ressort de cette étude qu'il est possible d'obtenir des particules submicroniques en utilisant le PCL comme polymère, le DCM comme phase organique et l'alcool polyvinylique comme agent stabilisant de dispersion.

CHAPTER III.1. (A)

PRELIMINARY STUDY OF ENCAPSULATION PROCESS USING DOUBLE EMULSION TECHNIQUE

MOHAMED AYOUB^{a, b}, NAVEED AHMED^a, NADER KALAJI^a, CATHERINE
CHARCOSSET^a, AYOUB MAGDY^c, HATEM FESSI^a and ABDELHAMID ELAISSARI^{a*}

^{a)} Université de Lyon, F- 69622, Lyon, France; Université Lyon 1, Villeurbanne, CNRS,
UMR-5007, Laboratoire d'Automatique et de Génie des Procédés

^{b)} Department of Pharmacognosy, Pharmaceutical Sciences Division, National Research
Centre, Dokki, Giza 12622, Egypt

^{c)} Polymers and Pigments Department, National Research Centre, Dokki, Giza 12622, Egypt

(Published in Journal of Biomedical Nanotechnology (JBN)

Vol 7, 1-8, 2011)

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 for the first time by 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 stabilizers [triton-405, tween 80, poloxamer, PVP (polyvinylpyrrolidone), PEG (poly ethylene glycol) & PVA (poly vinyl alcohol)] were investigated and results indicate that PVA (0.5% concentration) leads to the 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 1st & 2nd emulsion), stirring speed (for 1st & 2nd emulsion), polymer and stabilizer concentrations, *etc.* After duration of one month, the encapsulation efficiency of obtained particles 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 drug, encapsulation, biodegradable polymer, nanoparticles.

1. Introduction

The administration of a pharmaceutical compound to show its therapeutic effects in targeted tissues comes under the drug delivery definition. To deliver pharmaceutical preparations to a specific tissue with a possibility to show 100 % therapeutic efficacy is the main target in pharmaceutical field at present. Many researchers work in this regard to devise new methods and technologies to achieve 100 % concentration of the specific active molecule/drug in order to provide the best possible benefits to the recipient with lesser secondary effects. Biomedical nanotechnology is the field which is being considered by the researchers to apply in this context and it has shown many advantages, which include increased solubility, enhanced dissolution rate and improved bioavailability. The objective is to achieve the required pharmacokinetic and pharmacodynamic properties by involving nanotechnology in pharmaceutical field and thus obtaining adequate stability, controlled release, improved absorption; [1] good targeting and timely excretion from the body but this can also leads to toxicological responses [2] which will not be discussed here because it is beyond the goals of this study. Hence, it can be expected that nanotechnology will be helpful to i) increase the drug activity and ii) to decrease the side effects. In addition, biodegradability is another important characteristic to be required for the nanoparticles because non biodegradable materials are not ideal for therapeutic use and they can only be helpful for mechanistic study [3]. Hence the development of biodegradable micro and nanomaterials as effective drug delivery device for different therapeutic and diagnostic purposes has taken much attention in the last few decades [4-6]. In this research area different polymers found their place because of effective drug delivery to the target site resulting in increased therapeutic efficacy and minimized undesired effects [7]. Different biocompatible and biodegradable polymers are preferably used for the preparation of micro and nanoparticles in medicines e.g. poly (ϵ -caprolactone) (PCL) [8], poly (D, L-lactide-co-glycolides) (PLGA) [9, 10] and amino methacrylate copolymers (Eudragits RL and RS). This may be because of two reasons i) minimum toxic effects and ii) no need of surgical removals as in case of implants. Also they degrade in biological fluids and their bi-products can be removed easily by the normal body functions [11].

Different methods have been explored to encapsulate the various types of drugs and the choice of the technique is perhaps determined by nature of the drug [12]. Among the most important techniques to encapsulate the drug include; single emulsion process [13], double emulsion, super critical fluids, organic phase separation, spray drying techniques [9,14],

miniemulsion polymerization [15], nanoprecipitation [16-18] and solvent diffusion [19]. The basic purpose of all these techniques was to enhance bioavailability of poorly soluble drugs [20] although there are factors other than solubility that can cause lower bioavailability. An interesting technique being used in drug delivery is colloidal drug carrier system because their small size allows the permeation through biological barriers [21]. In different colloidal drug delivery systems, nanoparticles (NP) preparation is the most promising approach for increasing the bioavailability of a certain drug [22] and the double emulsion is one of the best methods to prepare these nanoparticles. Many authors [23, 24] have investigated and showed the double emulsion technique (multi-emulsion technique) as the most appropriate method to encapsulate hydrophilic drugs and proteins within microparticles. However, in all cases the size of the particles obtained was above 1 μm . This technique has later been applied to nanoparticles [25-27] by using sonication or ultra-turrax. Garti et al [28] defined double emulsions as “complex, heterodisperse systems called “emulsions of emulsion”. There are two major types of double emulsions; water in oil in water emulsion (W/O/W) and oil in water in oil emulsion (O/W/O). Normally emulsion is a phase dispersed in another phase but here in case of double emulsion one can consider that dispersed phase itself is an emulsion. In other words, double emulsion consists of three phases and mostly the inner and the outer layers are of the same phase. Double emulsions are prepared in two step emulsification process using two surfactants e.g. in case of W/O/W emulsion a hydrophobic surfactant to stabilize the interface of the W/O internal emulsion and a hydrophilic to stabilize the external interface of the oil globules [29]. Researchers have already encapsulated different drugs and proteins by this technique e.g. Bovine serum albumin (BSA) [30], Plasmid DNA [31], and Ciprofloxacin [32], *etc.* Moreover, different parameters in micro/nanoparticles preparation, that can affect the results (e.g. particles size, zeta potential, *etc.*), have been studied [1, 22, 23, 30, 33, 34]. Nonetheless there are several challenges which remain to understand and to control in order to enhance the encapsulation efficiency. Considering this, our research group has initiated efforts to better explain the process of encapsulation of drug. Current work is continuation of our efforts, and the aim is to encapsulate the hydrophilic model drug and to study the different parameters affecting the size of the particles obtained by double emulsion technique such as stirring speed (1st and 2nd emulsions), stirring time (1st and 2nd emulsions), polymer concentration and stabilizer concentration. The size of particles obtained was measured by light scattering and TEM and the encapsulation efficiency of the water-soluble stilbene derivative as a model drug inside the obtained particles was estimated after one

month using U.V. spectrophotometer. As discussed below, it is found that different parameters can be adjusted to significantly optimize the encapsulation of drug for system based on double emulsion techniques. Such studies are vital to the development of the future nanoparticulate drug delivery systems and increase our understanding about the bioavailability and permeability of the drugs encapsulated inside the particles.

2. Materials and methods

2.1. Materials

Water soluble Stilbene (CBS-X) was kindly provided by Digast (Lille, France). 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.

2.2. Methods

2.2.1. 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 20 mg of active molecule dissolved in 1 ml distilled water and then 0.5 ml of this solution was poured into organic phase containing 1 g of PCL dissolved in 4 ml of DCM along with stirring using ultra-turrax (T-25 basic IKA® -WERKE) for 15 minutes at a speed of 16,000 rpm. This 1st emulsion (W₁/O) was then transferred to the aqueous phase (a mixture of 25 ml distilled water and 25 ml 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 (W₁/O/W₂) was subjected to evaporation under vacuum using rotavap (Buchi rotavapor R-144) until the whole organic solvent has been removed. To ensure the complete removal of organic solvent, the volume of emulsion after evaporation was measured. Figure 1 shows a diagrammatic representation of the double emulsion evaporation process.

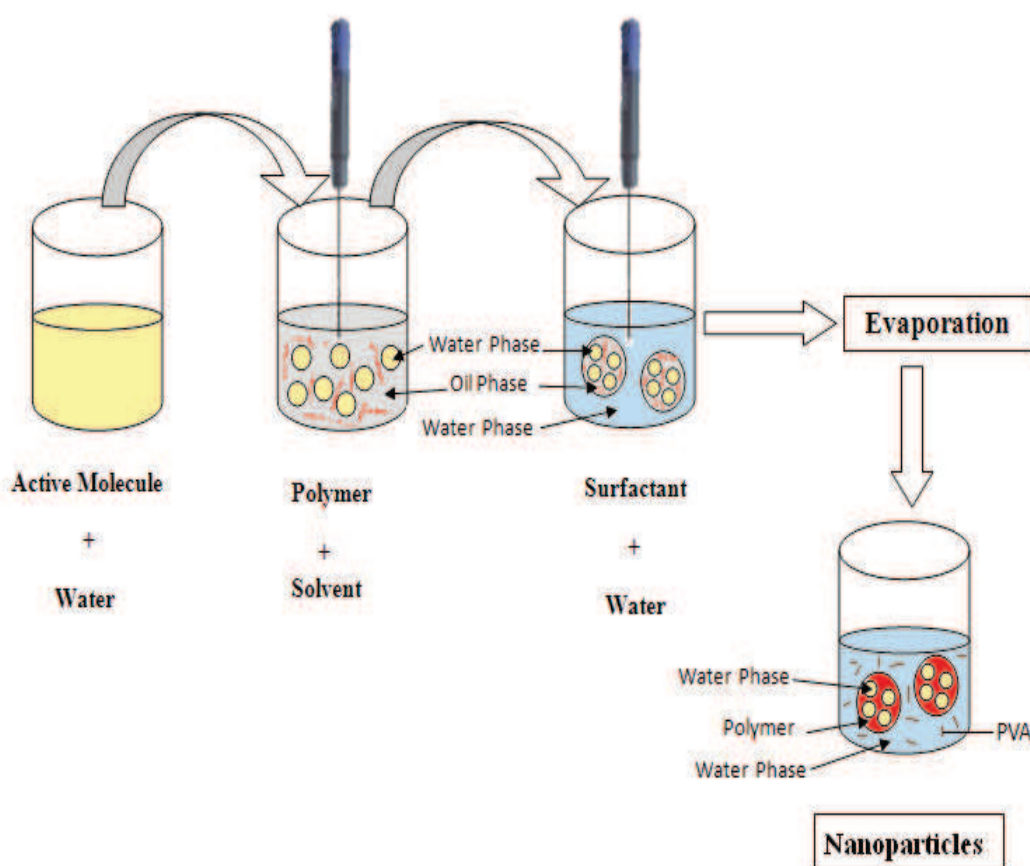


Figure: 1 Illustration of the double emulsion-evaporation method.

2.3. Size measurement

The hydrodynamic size of the prepared particles was measured in 1 mM solution of NaCl (the dispersion was diluted with 1 mM NaCl solution); using Zetasizer nanoseries “zs” (Malvern, England) at 25 °C where each measurement was performed at least 5 times and then the average hydrodynamic diameter was taken.

2.4. Encapsulation efficiency measurement

The efficiency of encapsulation of the active molecule in the final emulsion particles was analyzed after one month of preparation, using U.V. spectrophotometer (CARY 50 probe U.V. Visible spectrophotometer from Australia). 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) using Optima Max-XP ultracentrifuge, Beckman Coulter made in USA. 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 350 nm.

2.5. 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, Claude Bernard University Lyon1, France) 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.

3. Results and discussion

The aim of this work was to achieve the nanosize particles and then to investigate the different parameters which can affect the size of the particles obtained 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 stabilizing agents 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 were separated when PVA (0.1%) was used.

Table 1. Study of various stabilizers 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

Table 1 shows the results of examination of different stabilizers studied for emulsion preparation using this specific method. The table indicates that PVA at a concentration of 0.5 % is suitable to prepare emulsion by this method.

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 (1st and 2nd 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, thrice and six times. For the twice and the three times 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 because ultra-turrax was unable to prepare nanoparticles in larger volume.

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. Besides this, a study of parameter's effect was done to have the idea about importance of changing the conditions/parameters in this double emulsion based method.

It was noticed that in some samples (i.e. batches), in addition to the representative peak of the mean particles size, a second small peak showing the particles size in micro range was present. 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 fibers only.

3.1. Effect of parameters on the formulation

3.1.1. Effect of time of stirring

In order to enhance the dispersion of any liquid 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, high performance stirring apparatus ultra-turrax T-25 was used for stirring purpose and the effect of stirring time was investigated. This study of hydrodynamic particles size versus stirring time for 1st emulsion preparation as reported in Figure 2 shows that by changing the time of stirring for 1st emulsion, keeping other parameters (including the parameters for 2nd emulsion preparation)

constant, the particles size have not been affected except a small reduction in particles size for 15 minutes that gives the particles size less than 300 nm proving 15 minutes as the most suitable time.

The stirring time of 2nd emulsion can be a significant factor because if the second emulsification is not sufficiently dispersed via the stirring process, large and highly polydisperse particles results and the aggregation of particles can occur resulting in larger particles size. Different experiments were performed by changing the time of stirring for 2nd emulsion keeping other parameters (including the parameters for 1st emulsion preparation) constant, in order to point out suitable time duration for stirring. The results reported in Figure 2 for the 2nd emulsion preparation indicate that 10 to 20 minutes are suitable for stirring of 2nd emulsification and prove that the selection of 15 minutes was appropriate for performing basic experiment. The results showed that time duration less than 10 minutes is not appropriate for this process because it may not be sufficient for the good fragmentation/dispersion of the particles and on the other hand a long time duration for stirring (e.g. more than 20 minutes) is not suitable that might be because of aggregation. An interesting fact can be concluded from this diagram that the 15 minutes is a suitable time for preparation of both (1st & 2nd) emulsions. Both curves are superimposed at 15 minutes justifying the selection of 15 minutes for basic experiment.

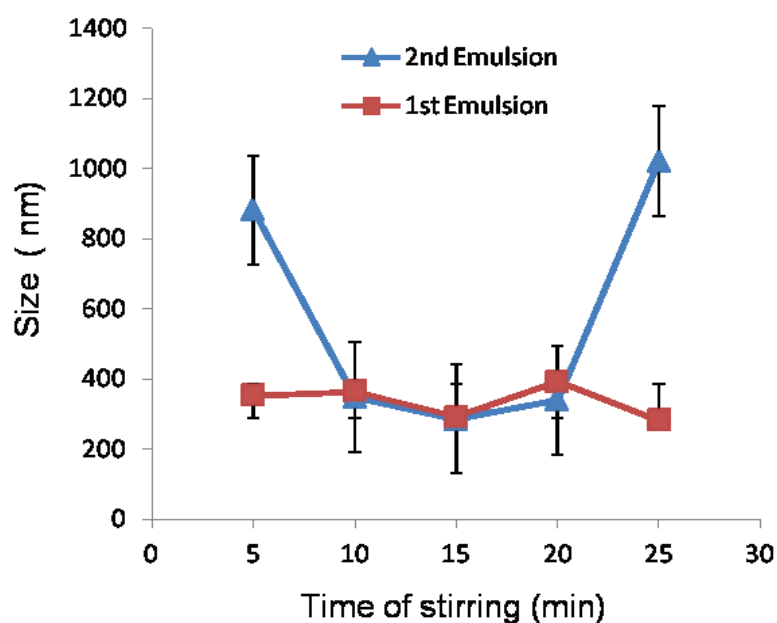


Figure: 2 Effect of stirring time on the size of drug loaded particles prepared at 0.5 % PVA and 25 % polymer concentrations and at a speed of 16,000 rpm and 21,500 rpm for 1st and 2nd emulsion respectively.

3.1.2. Effect of stirring speed

The stirring speed for the emulsion preparation is a dominating factor to determine the particles size because it provides the adequate energy to disperse the oil phase in water phase. According to Jeffery et al [35] and Yang et al [36], by increasing the stirring speed of 1st emulsion, the particles size is reduced but they also mentioned that very high speed can destroy 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 this case since the particles size is in nano range. 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 of 13,000 rpm, the particles become slightly larger (356 nm) but by increasing the speed to 16,000 rpm the particles become smaller and within the range of 297 nm which is in accordance with the literature survey.

Increasing the speed beyond 16,000 rpm has the same effect but not so prominent and approximately remain constant even by increasing 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 due to the unavailability of oily phase (O) to encapsulate the water phase (W₁). This is what was done in basic experiment that proves 16,000 rpm as a good selection.

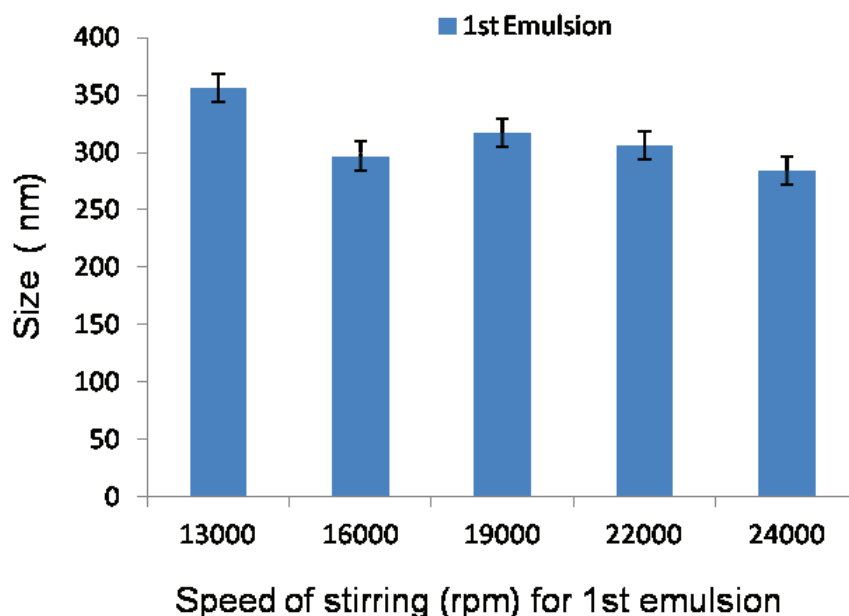


Figure: 3 Effect of stirring speed (1st emulsion) on size of drug loaded particles prepared at 0.5 % PVA and 25 % polymer concentrations and at a speed of 21,500 rpm for 2nd emulsion for 15 minutes.

It is clear from Figure 4 that 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 particles size is increased and by increasing the speed, the particles size started to decrease and become within range of nanosize. It means that a suitable stirring speed in preparation of both emulsions can produce good results rather than by using either a very high or a very low speed.

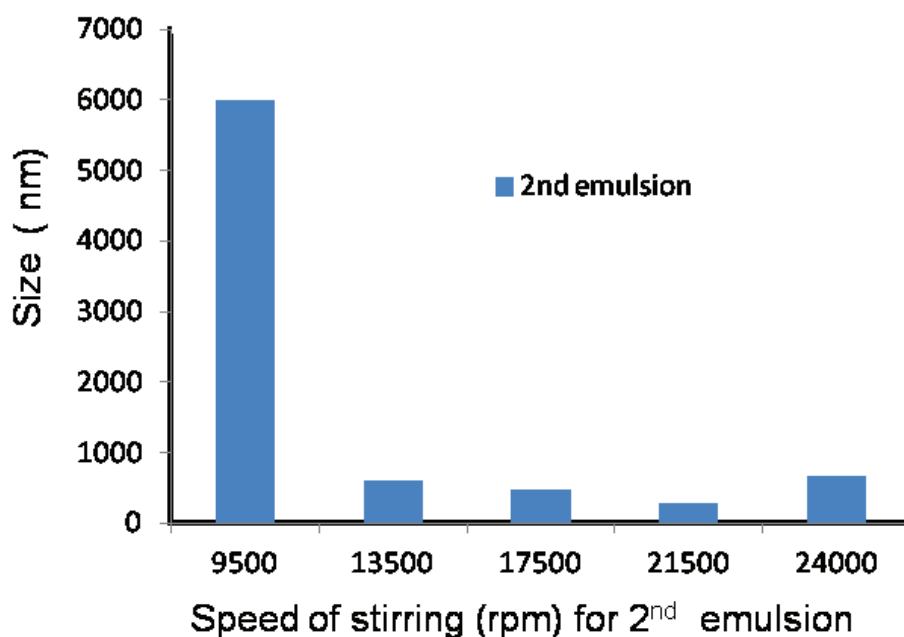


Figure: 4 Effect of stirring speed (2nd emulsion) on size of drug loaded particles prepared at 0.5 % PVA and 25 % polymer concentrations, at a speed of 16,000 rpm for 1st emulsion for 15 minutes.

3.1.3. Effect of polymer concentration

The polymer concentration can be a critical factor influencing the characteristics of the particles and it may also affect 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 shown in a form of curve in Figure 5, implies that at lower concentration of polymer, there is no significant effect on the size of resulting particles. But increasing the concentration of polycaprolactone beyond the 3g (25 %), 4 and 5 g (for 33 % and 41 %), the particles size increased from 260-430 nm.

This showed that the addition of polymer has no significant effect until a specific percentage (25 %) and after that by increasing its concentration; it increases the particles size and these results are in accordance with those reported by Lamprecht et al [30]. If one assumes that the stirring process controls the particles number, then by increasing the

concentration of the polymer can result in large sized particles formation and also highly polydisperse system.

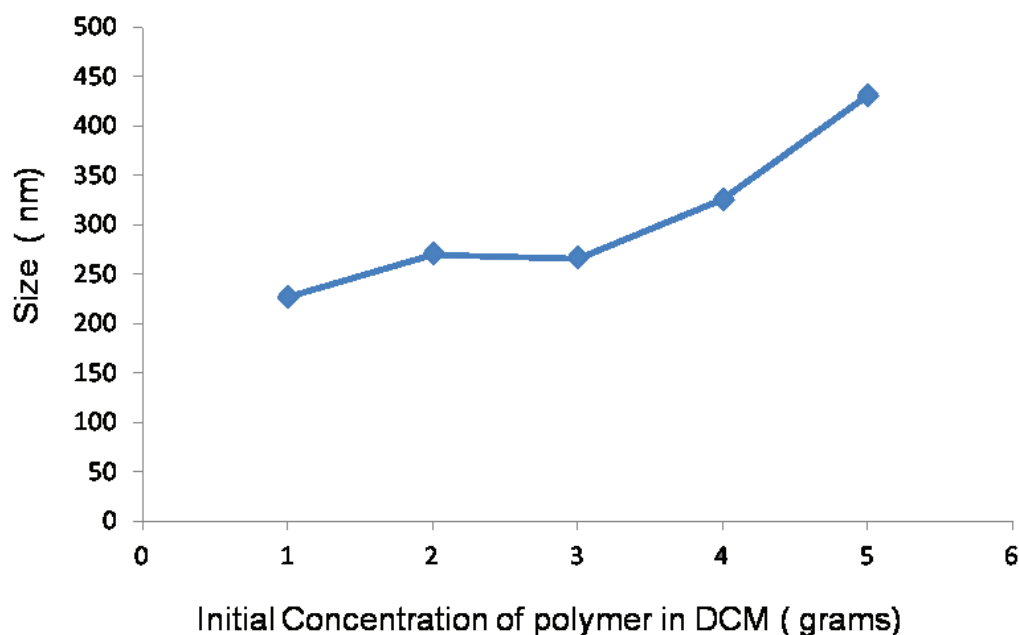


Figure: 5 Polymer concentration (in grams) effect on size of drug loaded particles prepared at 0.5 % PVA concentration and at a speed of 16,000 rpm and 21,500 rpm for 1st and 2nd emulsion respectively for 15 minutes.

3.1.4. Effect of stabilizer concentration

The type and concentration of stabilizers affect the formulation process as well as the long-term stability of double emulsions. The stability of the emulsion is important because during the evaporation of solvent, there can be a gradual decrease in the volume and subsequent increase in the viscosity of dispersed phase. This can affect the droplets size equilibrium, involving the coalescence and the agglomeration of the droplets during the early step of the solvent removal [37]. The different stabilizers were examined for their suitability to prepare stable emulsion by this method where PVA was found to be the most suitable. 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 stabilizer can be considered as one of the important factors influencing the particles size and their stability because the presence of the stabilizer in the external aqueous phase enhances the stabilization of emulsion droplets/particles against coalescence and its suitable concentration can be effective in determining the size.

PVA is used in external aqueous medium in which its concentration has a relatively negative effect. The effect of PVA concentration for decreasing sizes of the particles is already mentioned in literature [39, 40] and explained as the increase in concentration of PVA would make the external aqueous phase more viscous, hence impact of shear forces on the oily phase intensifies producing smaller droplets of emulsion.

In this work, high concentration of stabilizer leads to an increase in size of the obtained particles which can be assumed as stabilizing function of the stabilizer. It's possible that for stabilizing all the nanoparticles, amount of stabilizer used can be insufficient resulting in the tendency of some of particles to aggregate. Hence the particles with larger sizes would be produced and these findings are correlated with those of Feng et al. [41].

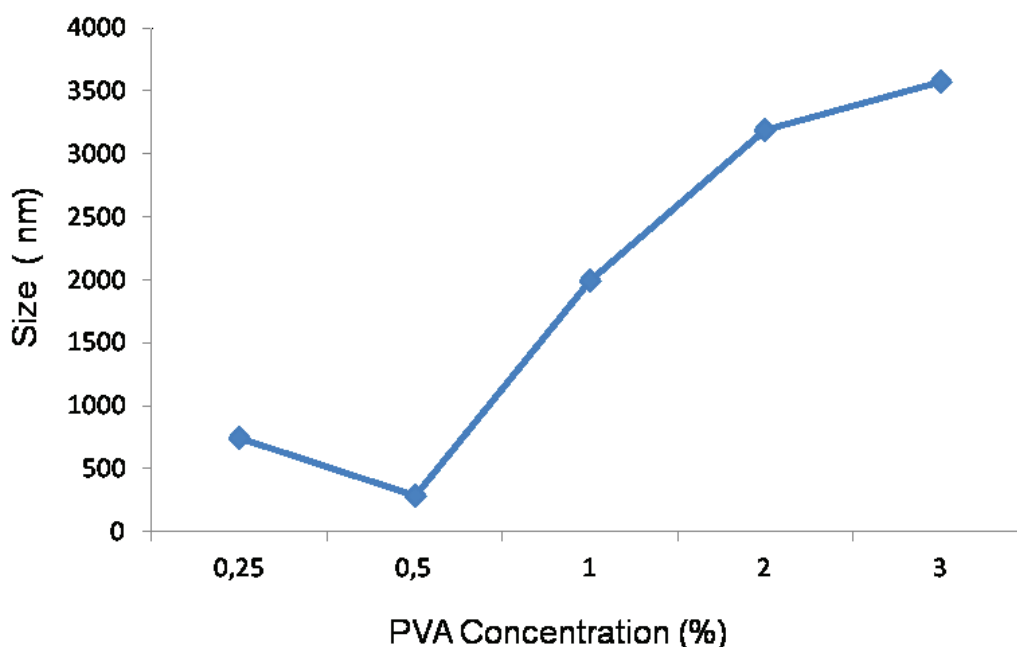


Figure: 6 Effect of PVA concentration (%) on size of drug loaded particles prepared at 25 % polymer concentration and at a speed of 16,000 rpm and 21,500 rpm for 1st and 2nd emulsion respectively for 15 minutes.

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 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.

Here, studying the effect of different parameters, there is an interesting coherence of the results in the experiments performed on the basis of same conditions. Under the conditions

of 16,000 rpm speed for 15 minutes for 1st emulsion and 21,500 rpm speed for 15 minutes for 2nd emulsion preparation with 25 % and 0.5 % concentration for polymer and stabilizer respectively. It can be seen that the average particles size ranges from 260-290 nm which proves the choice of conditions of different parameters for basic experiment was good.

3.2. Encapsulation efficiency of model drug

The encapsulation efficiency of loaded drug particles was estimated after duration of one month. The concentration of the stilbene used as active molecule comparing to the nanostructures was calculated by finding the difference between the total and free drug concentrations measured in ultra-centrifuged solution using U.V. analysis and the absorbance of the substance was at 350 nm. Twelve representative samples of nanosized particles were selected to evaluate the encapsulation efficiency at wave length 350 nm. The percent release was calculated from the following equation,

$$\text{Percent release} = \frac{\text{Total amount of drug released}}{\text{Total amount of drug loaded}} \times 100$$

The average drug released was 30 µg/ml against 202 µg/ml of drug loaded initially. The results showed that there was less than 15 % release of active drug after one month storage at room temperature for the whole samples. This shows that 85 % active drug is still encapsulated in the particles. This type of differential study for the encapsulation efficiency is also done by Guterres et al. [42].

3.3. Transmission electron microscopy (TEM) study

The transmission electron microscopy (TEM) of drug encapsulated nanoparticles was done to observe the diameter, which gave the satisfactory results. A typical micrograph taken for emulsion prepared using specific parameters (16,000 rpm for 15 minutes for 1st emulsion and 21,500 rpm for 15 minutes for 2nd emulsion) are shown in Figures 7 and 8. These figures show that the particles with size ranging from 175 nm to 350 nm are almost spherical in shape.

The presence of drug molecules encapsulated in polymer matrix was examined using transmission electron microscopy by analyzing nanoparticles prepared and kept for five months. The acquired image is shown in Figure 8 in which the presence of black dots

dispersed in the particles can easily be seen. The viewed black dots correspond to active molecules stain adequately encapsulated in the polymer particles.

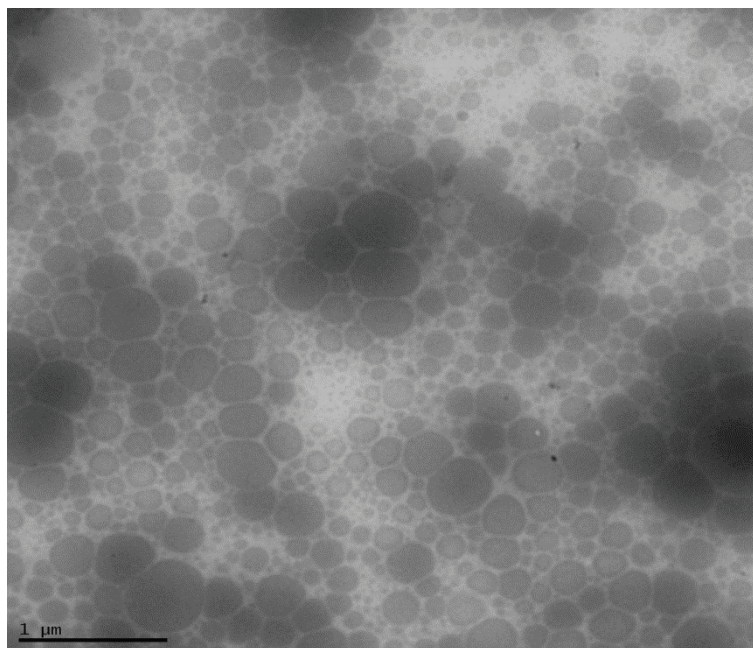


Figure: 7. TEM micrograph with the drug loaded particle distribution prepared by double emulsion evaporation method

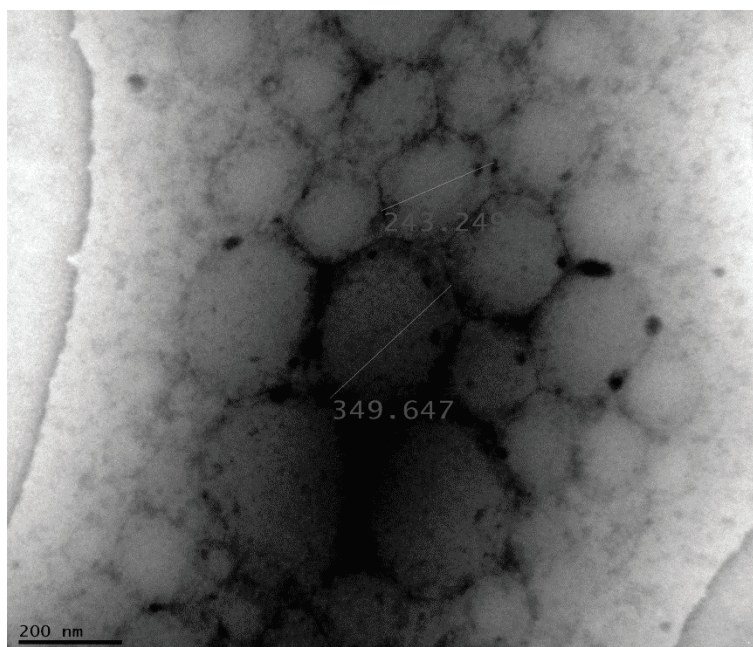


Figure: 8. TEM micrograph of the drug loaded particles showing the size of particles ≤ 350 nm.

4. 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 polycaprolactone as polymer. Poly vinyl alcohol among different stabilizers (e.g. triton-405, tween 80, poloxamer, PVP, PEG, *etc.*) was chosen as a suitable one for preparation of nanoparticles. The results proved that the double emulsion evaporation method is a good choice for the encapsulation purpose using PCL as polymer and PVA as stabilizer. 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 for each of them. The stirring time, stirring speed, concentrations of polycaprolactone and PVA were found to be considered important for nanoparticles preparation using this technique. The active molecule encapsulation rate in the prepared emulsions after a storage time of one month 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.

Acknowledgement

Thanks to Science and Technology cooperation programme between Pakistan and France and between SFERE (Société Française d'Exportation des Ressources Educatives) and HEC (Higher Education commission) of Pakistan for supporting Ph.D. studies of Mr. Naveed Ahmed.

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CHAPTER III.1. (B)

**ENCAPSULATION OF LABELLING AGENT
USING DOUBLE EMULSION PROCESS**

Résumé

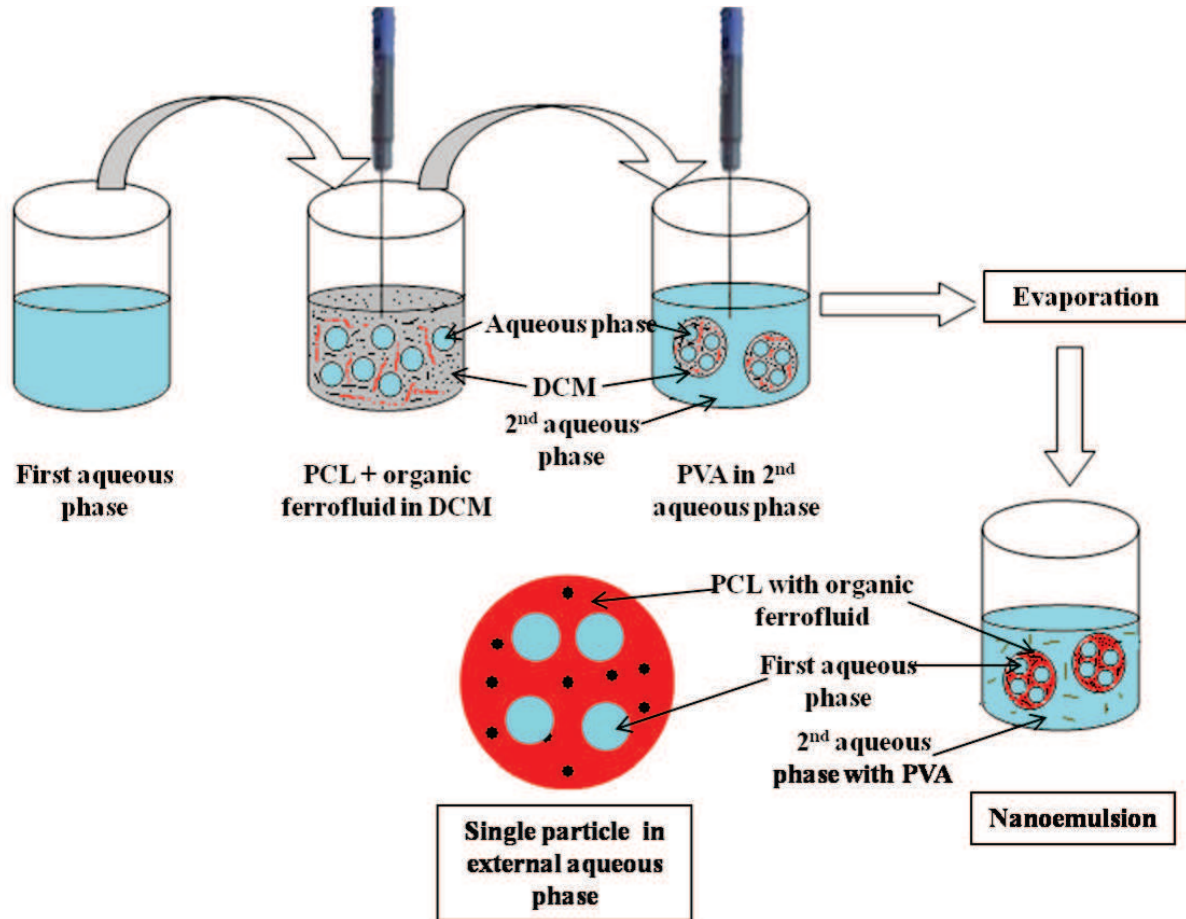
Les nanoparticules magnétiques (MNP) sont utilisées dans des domaines divers et variés et en particulier dans le domaine biomédical. Dans ce dernier domaine, les particules magnétiques sont utilisées comme support de biomolécules afin de remplacer la filtration et la centrifugation généralement incontournable dans l'analyse biomédicale *in vitro*. En ce qui concerne le biomédicale *in vivo*, les particules magnétiques sont utilisées comme agent de contraste pour faciliter le diagnostic via l'imagerie biomédicale. Elles sont également utilisées dans la thérapie physique comme l'hyperthermie et plus récemment pour la vectorisation de molécules actives. Plus récemment, une attention particulière est dédiée à la combinaison de diagnostic *in vivo* et la thérapie localisée (applications théranostics). En considérant l'importance de ce nouveau domaine de recherche en biotechnologie, le but de notre travail de recherche est l'élaboration d'une nouvelle génération de particules magnétiques en utilisant les nanoparticules d'oxyde de fer (IONPs) comme agent de contraste et contenant également un anticancéreux qui sera vectorisé. La détection de la tumeur sera diagnostiquée par l'imagerie biomédicale et sera par la suite traitée en utilisant les mêmes particules contenant l'actif spécifique. Il est ainsi possible de suivre *in vivo* la location et le séjour de ces particules à double emploi dans l'organisme.

Pour ce faire, les nanoparticules d'oxyde de fer sous forme de dispersion appelée ferrofluid sont synthétisées via la précipitation de deux sels ; sel ferrique et sel ferreux. Les nanoparticules d'oxyde de fer synthétisées sont sous forme de dispersions colloïdales stables de nanoparticules très fines de taille nanométrique et de l'ordre de 10 nm. Ces nanoparticules sont dans un premier temps préparées dans un milieu aqueux et transférées en milieu organique après une modification de surface via l'adsorption d'un tensioactif faiblement soluble dans l'eau. Cette modification permet le transfert de ces nanoparticules hydrophiles dans un solvant organique comme l'octane ou le dichlorométhane.

L'utilisation de la méthode double émulsion / évaporation de solvant a été explorée pour l'encapsulation de ferrofluid aqueux et le ferrofluid organique. Dans le cas d'utilisation d'un ferrofluid aqueux, un actif hydrophobe a été utilisé et vis-versa. Le polymère utilisé pour la formation des particules est le polycaprolactone (PCL). Ce polymère est parfaitement soluble dans le DCM, mais insoluble dans l'eau et un mélange eau/DCM.

La molécule active utilisée comme modèle est une molécule fluorescente, une dérivée du Stilbene. Une étude systématique a été réalisée en examinant un grand nombre de paramètres, la nature de ferrofluid, la quantité de nanoparticules d'oxyde de fer, la quantité

de molécule active, la vitesse de cisaillement de la première et la deuxième émulsification, le rapport eau/ DCM et en fin la nature de l'agent stabilisant ;Triton-405, tween 80, poloxamère, PVA (alcool polyvinylique) et PEG (poly éthylène glycol). Le procédé utilisé est schématisé sur la figure suivante :



Les tailles de particules de ferrofluids aqueux et organiques, les émulsions blanches, les émulsions contenant le Stilbene, le ferrofluid aqueux ou organiques ont été mesurées en utilisant la diffusion de la lumière et la microscopie électronique à transmission. Les particules finales ont moyenne comprise entre 200-400 nm et dépend principalement des vitesses cisaillements. Le caractère cationique ou anionique des particules finales a été examiné via la mesure du potentiel zêta en fonction du pH. La quantité d'oxyde de fer encapsulé est déterminée par analyse thermogravimétrie (ATG) et le taux d'encapsulation est trouvé largement supérieur à 60%. L'analyse par MET a montré la présence des nanoparticules dans la matrice polymère et la distribution de ces nanoparticules dépend de la nature de ferrofluid utilisé.

En résumé, cette nouvelle méthode de double émulsion/évaporation de solvant a permis l'obtention de particule submicronique à base de PCL comme polymère en utilisant le

PVA comme agent stabilisant. Ce procédé a montré la possibilité d'encapsuler un agent de contraste ici les nanoparticules d'oxyde de fer et également une molécule active modèle. Ce procédé est trouvé sensible à la vitesse de cisaillement de la deuxième émulsification. La formulation type permettant l'encapsulation des ferrofluids et la molécule modèle donne une taille moyenne de l'ordre de 300 nm.

General summary

Magnetic nanoparticles (MNPs) are considered highly important biomaterials due to their utilization in various areas such as drug delivery, hyperthermia, imaging mechanisms, and recently in theranostics. The later technique is based on the combination of "Therapeutics" and "Diagnostics, and it encompasses the possible advances made in subjects that includes: pharmacodiagnosics, drug discovery, molecular biology and microarray chips. Considering the importance of this new biotechnology research area, the aim of current research work is the development of hybrid particles containing an anticancer drug for therapy and iron oxide as contrast agent for the *in vivo* applications via magnetic resonance imaging (MRI). Iron oxide nanoparticles are synthesized and dispersed in suspensions called ferrofluids, which are stable colloidal dispersions of very fine nanoparticles of magnetic materials. Here, the iron oxide nanoparticles were successfully prepared in aqueous medium using co-precipitation method and transferred in organic medium. A modified double emulsion evaporation method was devised envisaging the encapsulation of active and contrast agent at same time to be used as theranostics. Among different stabilizing agents including triton-405, tween 80, poloxamer, PVA (poly vinyl alcohol) and PEG (poly ethylene glycol) screened a stable emulsion-like was obtained using PVA. PCL was used as polymer (to form the matrix) and DCM as organic solvent for the preparation of biodegradable and biocompatible nanoparticles. Drug free and Stilbene loaded nanoparticles were prepared and at same time different parameters were investigated to prepare a standard recipe for this newer modified technique. These parameters include stirring speed, stirring time, polymer and stabilizer concentration. Then using this recipe, both types of iron oxide nanoparticles were successfully encapsulated within PCL. The particle sizes of aqueous and organic ferrofluids, blank emulsions, Stilbene, aqueous and organic ferrofluid loaded emulsions were measured using DLS and TEM. The final loaded particles were found in a range of 250-340 nm. The zeta potential measurement gives an idea about the charge on the particles and indirectly measure of stability. The pH value also plays an important role in the stability of the system. It was concluded that a suitable buffer should be added for long time stability requirements. Encapsulation efficiency of the iron oxide contents were measured using TGA analysis and proved to be more than 60 %. TEM micrographs provided clear picture of the encapsulation of Stilbene derivative and iron oxide inside the nanoparticles. In short, this new double emulsion evaporation method using PCL as polymer, DCM as oily phase and PVA as stabilizing agent, proved a suitable technique for the encapsulation of active ingredients

providing a particle size of about 300 nm. It proved successful for the encapsulation of a model drug as well as contrast agent for the diagnostic purposes. Modification of related parameters of this modified double emulsion evaporation technique can allow the encapsulation of different active ingredients targeted for different applications including diagnostic applications.

Modified double emulsion process as a new route to prepare submicron biodegradable magnetic/polycaprolactone particles for *in vivo* theranostics

Naveed Ahmed, Millán Michelin-Jamois, Hatem Fessi, Abdelhamid Elaissari*

University of Lyon, F-69622, Lyon, France; University Lyon-1, Villeurbanne; CNRS, UMR 5007, LAGEP- CPE; 43 Bd 11 November 1918, F-69622 Villeurbanne, France.

*E-mail: elaissari@lagep.univ-lyon1.fr

(Published in Soft Matter, 2012, 8, 2554)

Abstract

A modified double emulsion evaporation method was devised to encapsulate active ingredients to be used for theranostic purposes. In comparison with classical double emulsion methods, the solvents used for this modified method are miscible. Aqueous and organic ferrofluids having different solid contents of iron oxide nanoparticles were prepared by the co-precipitation method. Different materials were screened for the preparation of biodegradable and biocompatible nanoparticles via this new modified double emulsion evaporation method. Factors affecting the final particle size were investigated by encapsulating a hydrophilic model drug that permitted to prepare a recipe using Polycaprolactone (PCL) as polymer, Dichloromethane (DCM) as organic solvent and Poly vinyl alcohol (PVA) as stabilizing agent. The characterization of aqueous and organic ferrofluids alone, Stilbene and iron oxide loaded nanoparticles was done by studying particle size, size distribution, zeta potential and morphology, the magnetic properties and chemical composition. All the characterization results shown that process is praise worthy for encapsulation of iron oxide nanoparticles (in both aqueous and organic medium) along with an active ingredient for theranostic purposes with size around 300 nm.

Introduction

In recent decades magnetic nanoparticles (MNPs) have been extensively used in biomedical nanotechnology due to their applications ranging from magnetic resonance imaging (MRI) [1-3], targeted drug and gene delivery,[4-5] hyperthermia, [6-7] biosensing applications, [8] cell tracking, [9] bioseparation [10] and tissue repair [11], *etc.* The magnetic nanoparticles (MNPs) are of different types based on material used for magnetic characteristics. Iron oxide nanoparticles (IONPs) are one of the most studied MNPs especially as MRI contrast agent [12-13]. The choice of IONPs among other MNPs for biomedical applications can be related to their high magnetic susceptibility, biocompatibility, stability and availability of various preparation methods [14-18]. Ferrofluid [14] is another term used for iron containing nanoparticles and considered as special nanomaterials and in particular magnetically controllable nanofluids [19]. Actually these ferrofluids are colloids of magnetic nanoparticles e.g. Fe_3O_4 , $\gamma\text{-Fe}_2\text{O}_3$, CoFe_2O_3 CoFe_2O_4 , Co, Fe or Fe-C, stably dispersed in a carrier liquid. Hence they have both magnetic and fluid properties simultaneously [20]. Different preparation methods for the ferrofluids/iron oxide particles have been developed with further techniques to modify the surface properties [21-22] of these particles according to the requirements. Mostly used methods are co-precipitation [23], sonochemical synthesis [24-

25], thermal decomposition [26], microemulsion [27] and hydrothermal synthesis [28-30], *etc.* Some biological routes have also been devised for the synthesis of the iron oxide nanoparticles [31-32]. Co-precipitation method is one of the extensively used methods and includes use of basic medium (inorganic basis) for co-precipitation of mixture of metal salts and was first discovered by Elmore [33] but is known as Massart process [34] in literature.

Polymeric nanoparticles (PNPs) attracted the researchers [35-37] attention principally for their applications in drug delivery systems. Different bioactive compounds have been successfully encapsulated within polymers and have been tested for their therapeutic activity. Many biocompatible and biodegradable polymers are preferably used for the preparation of micro and nanoparticles in medicines e.g. poly (ϵ -caprolactone) (PCL) [38], poly (D, L-lactide-co-glycolides) (PLGA) [39-40] and amino methacrylate copolymers (Eudragits RL and RS) [41-42]. This preference can be related to minimum toxic effects, no need of surgical removals as in case of implants, degradation in biological fluids and easy removal of metabolic products by normal body functions [43]. Biodegradable and biocompatible polymeric nanoparticles encapsulating different active ingredients (drugs, proteins, DNA, *etc.*) have been developed for different applications [44-47]. The iron oxide content encapsulation has also been developed using these biodegradable polymers [48-49]. Different encapsulation techniques [50-53] exist for iron oxide encapsulation as for other active ingredients. Among these techniques of active agent encapsulation, the double emulsion is considered to be the most important because it permits the encapsulation of hydrophilic and hydrophobic drug in both oily and aqueous phases [54].

The aim of this work is to prepare submicron hybrid particles bearing the dual properties of diagnosis and therapy relating to recently coined termed theranostics. Preliminary study was done encapsulating a model hydrophilic drug (Stilbene) using a new modified double emulsion evaporation method. It permitted to devise a recipe for active and contrast agent's encapsulation. Apart from preliminary study, this work is mainly divided in two parts; the first is dedicated to the preparation of aqueous iron oxide and well dispersed magnetic nanoparticles in organic phase. The second part is focused on the encapsulation of iron oxide nanoparticles dispersion (aqueous and organic) via water in oil in water modified double emulsion-evaporation process. To our knowledge, this study is the first systematic work dedicated to the encapsulation of aqueous and organic magnetic nanoparticles for *in-vivo* biomedical diagnostic and therapy.

Materials and methods

Materials

Model drug (Stilbene derivative) (CBS-X) was kindly provided by Digast (Lille, France). $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, dichloromethane (DCM) and oleic acid were purchased from Aldrich Germany. Ammonium hydroxide solution (24% in water) and nitric acid (2 M) were purchased from Fluka Analytical and Prolabo Paris respectively. The poly vinyl alcohol (PVA), used as surfactant (Mowiol® 4-88. Mw = 31 000 g/mol) and polycaprolactone (PCL) used as polymer (Mw = 14 000 g/mol) were also purchased from Aldrich Germany. All the chemical reactants were used as received.

Preparation of the iron oxide magnetic nanoparticles

The preparation of ferrofluids was generally two-step process; (i) preparation of magnetic nanoparticles and (ii) transfer of these particles in various solvents that can be either polar or non polar. Modified co-precipitation method of ferric and ferrous salts in aqueous medium was used as the starting point to prepare highly magnetic material. The method used in this study for the synthesis of ferrofluids is a modified method from Montagne et al. [55].

Preparation of aqueous iron oxide magnetic nanoparticles

In this process, first of all ferrous chloride (3.7 g) and ferric chloride (10.16 g) were dissolved in 80 ml water and then transferred in the 250 ml reactor and mechanically stirred for 40 minutes at 500 rpm. 80 ml of ammonium hydroxide (NH_4OH) (24 %) added drop by drop during a period of 100 minutes. After pouring the whole ammonium hydroxide, stirring was continued for further 2 hours. Then solution was removed from the reactor and magnetic material was separated from the supernatant using a strong magnet. A quantity of 80 ml water and 80 ml nitric acid (2 M) was added to the black magnetic material and then transferred to the reactor and stirred for 15 minutes at a speed of 500 rpm. After homogeneous dispersion, the supernatant was separated again using a strong magnet and washed using 50 ml acetone, acetone/water mixture (50:50) and water respectively. The final black magnetic material was then dispersed in 50 ml water and left overnight to allow total evaporation of remaining traces of acetone before filtration and characterization.

Preparation of organic iron oxide magnetic nanoparticles

In this process, like preparation of aqueous ferrofluids, ferrous chloride (3.7 g) and ferric chloride (10.16 g) were dissolved in 80 ml water in the 250 ml reactor under 500 rpm

mechanical stirring for 40 minutes. 80 ml of ammonium hydroxide (NH₄OH) (24 %) added (drop wise) during 100 minutes and then incubated 2 hours with continuous stirring. 8 ml of oleic acid added (drop wise) under stirring at 500 rpm. Then magnetic material was separated magnetically and washed with 50 ml water before redispersion in 30-50 ml octane. The traces of water were removed using funnel and the residual ammonia was evaporated using open reactor system at 60°C. The final nanoparticles dissolved in octane, were filtered before any characterization study.

Water in oil in water (w/o/w) emulsification process

The emulsification process was done using modified double emulsion (w/o/w) evaporation method where PCL used as polymer and DCM as oily phase. To validate this modified double emulsion technique firstly blank emulsions were prepared and secondly a hydrophilic model drug Stilbene was encapsulated to study the different parameters affecting the size and morphology of the final particles. After successful parametric study, the new double emulsion evaporation method was applied to encapsulate iron oxide nanoparticles dispersed in aqueous and organic medium. The encapsulation of Stilbene and iron oxide particles was done as follow;

Encapsulation of model hydrophilic drug

This method was developed to encapsulate a hydrophilic model drug Stilbene and to investigate different parameters affecting the size of the final drug loaded particles. Stilbene was dissolved in primary aqueous phase while PCL was dissolved in DCM serving as organic phase. Different stabilizing agents including triton-405, tween 80, poloxamer, PVA and PEG (poly ethylene glycol) were screened to choose a suitable one for this method. Various parameters including stirring speed, stirring time, stabilizer and polymer concentration were investigated for their affect on final particle sizes. To study a specific parameter, all the other factors were kept constant and final Stilbene loaded particles were characterized by diffracted light scattering (DLS) and transmission electron microscope (TEM).

Emulsification of iron oxide particles

After the iron oxide nanoparticles preparation and characterization, the next step was their emulsification within polycaprolactone. Modified double emulsion evaporation (W/O/W) technique was applied using PCL as polymer, DCM as oily phase and PVA as stabilizing agent. Two types of the ferrofluids were prepared so there will be small

modification in emulsification process for organic and aqueous ferrofluid as mentioned below but the basic technique is identical.

a) Emulsification of aqueous ferrofluids

For the emulsification process, PCL (3 g) was taken and dissolved in 12 ml of DCM. Then 1.5 ml of aqueous ferrofluid at a given solid content were introduced in this PCL/DCM solution and stirred for 15 minutes using a high energy stirring apparatus (ultra-turrax T-25) at 16.000-17.500 rpm. The prepared emulsion was poured in 150 ml PVA (0.5% wt/V) in 1 liter beaker placed in ice bath and stirred with ultra-turrax for 15 min at a speed of 21.500 rpm. The temperature rise is controlled by ice bath. Then this modified double emulsion preparation was evaporated using Buchi Rotavapor and volume of the emulsion after evaporation was measured with glass cylinder to have an idea about the quantity of the emulsion evaporated. (Original volume 163.5ml from 150 ml PVA, 12 ml DCM and 1.5 ml ferrofluid). To see the effect of evaporation on particle size and distribution in detail, 4 ml of emulsion before evaporation was taken and size was measured. A general representation of the process can be seen in the following fig.1.

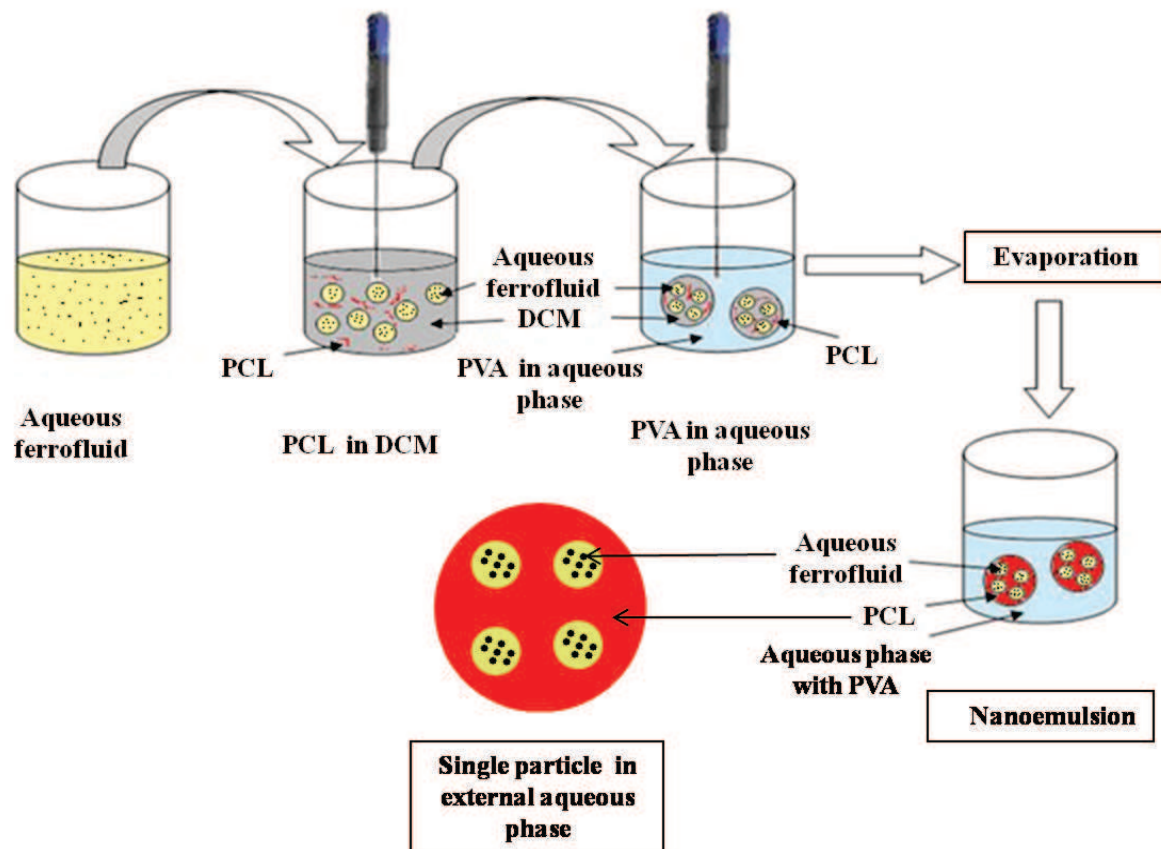


Figure: 1 Emulsification process based on modified double emulsion evaporation method using aqueous ferrofluids

b) Emulsification of organic ferrofluids

Encapsulation of the organic ferrofluid in PCL polymer matrix via new double emulsion evaporation technique was somehow different task. As oleic acid containing iron oxide nanoparticles were dispersed in organic solvent i.e., octane in the final step of the preparation, first it was tried to set a ratio between DCM and octane because the aim was to use the same ratio between oily and aqueous phase and with respect to polymer concentration also. The dried oleic acid containing iron oxide nanoparticles were directly dispersed in DCM or in octane and then mixed with DCM before mixing with PCL prepared also in DCM. The remaining encapsulation process including; stirring speed, stirring time, PCL and PVA concentrations were same as for aqueous ferrofluids encapsulation. The encapsulation of oleic acid coated iron oxide nanoparticles via modified double emulsion evaporation process is depicted in fig.2

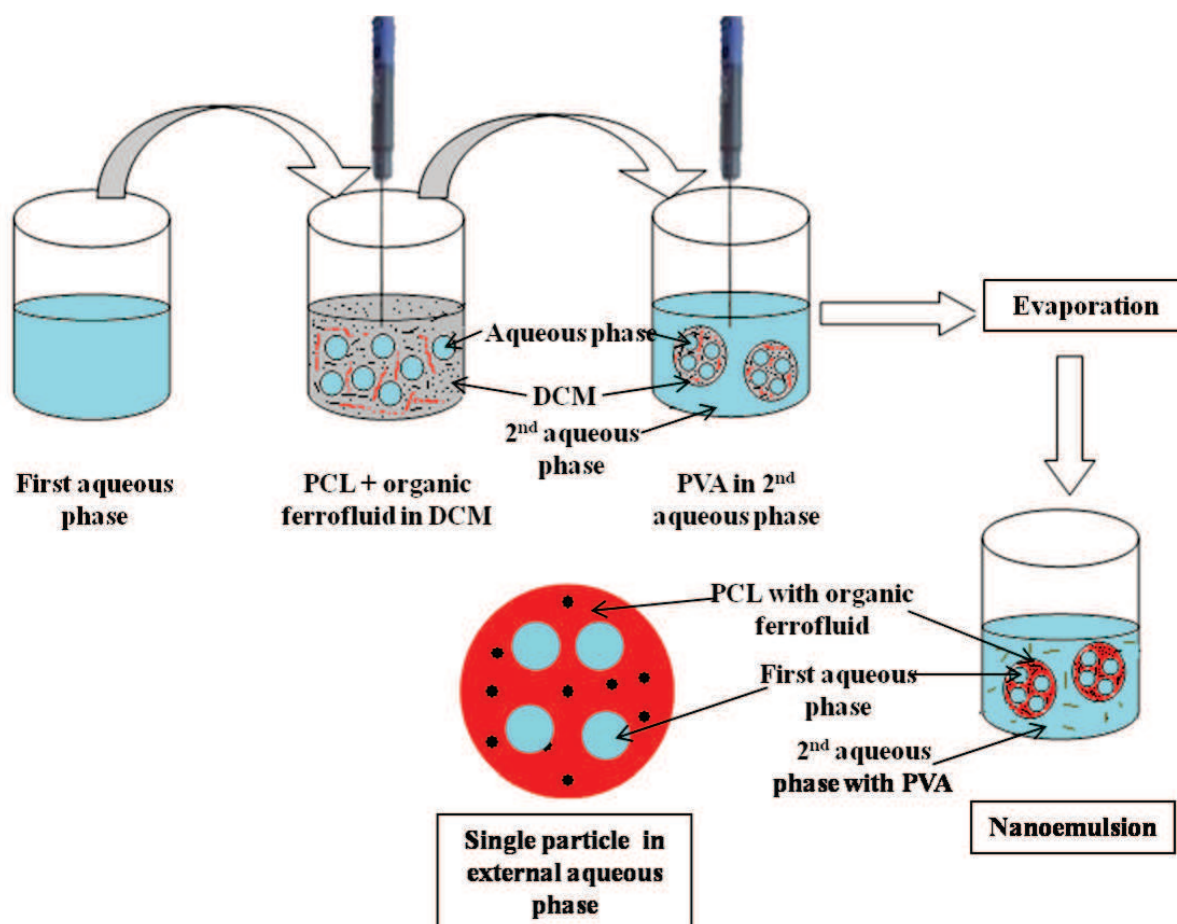


Figure: 2 Emulsification process based on modified double emulsion evaporation method using organic ferrofluids

Characterization

The colloidal characterization, the intrinsic properties and the chemical composition of the final particles were performed using various techniques.

Size measurement: Size measurement of the ferrofluids and the emulsions prepared using these ferrofluids was done by Zetasizer nanoseries “zs” of Malvern instruments (England) and DTS (nano) program was used for data evaluation. The samples were dissolved in water, 0.1mM NaCl or octane depending upon the nature. Stokes-Einstein law is used to compute the mean droplet diameter. The equation for law is; $D_H = K_B T / (3\pi\eta D)$, where D_H , K_B , T , η and D are the hydrodynamic diameter of the droplet, Boltzmann constant, temperature in Kelvin, viscosity of the medium and diffusion coefficient, respectively.

However, some samples required filtration or decantation before size measurement. For decantation the samples were placed untouched for a good time (for at least 4 days) and were decanted with the help of a pipette. In case of filtration, sample to be filtered was placed over a magnet to attract large non dispersed particles for a sufficient time. Then filtration was done by transferring the solution drop wise with a pipette into another bottle through a funnel with glass fiber in it to filter the solution. Then 2-3 drops of samples from the decanted/filtered solution were placed in a beaker containing 10-20 ml distilled water/0.1mM NaCl/octane. From this, a pipette was used to deliver the appropriate quantity into the apparatus cell for measurement.

Zeta potential measurement: There is no common technique to measure the surface charge of the minute particles in liquids. Zeta potential is an important parameter in colloids or nanoparticles, widely used in product stability studies and particle surface morphology [56]. Zeta potential measurement was based on light scattering principle and was done using the same Malvern Zetasizer adopting electrophoretic mobility technique. As organic ferrofluids were coated by oleic acid, their zeta potential was not measured and it was done only for the aqueous ferrofluids and for the final iron oxide loaded emulsions. The samples were also decanted /filtered (if necessary) and were diluted in water/0.1mM NaCl. The specific cell for zeta potential measurement was used in Malvern instrument.

Zeta potential values of aqueous ferrofluids and finally prepared emulsions were measured in different pH values to see the effect of pH. For this purpose, firstly different pH solutions were prepared using 1mM NaCl solution. The pH values were adjusted by 1mM NaOH and 1mM HCl solution using pH meter. Then samples were diluted in respective pH solution before measuring the zeta potential.

Microscopy: The transmission electron microscopy (TEM) of the iron oxide encapsulated nanoparticles were done by a Phillips CM 120 transmission electron microscope (CMEABG, University of Claude Bernard Lyon1, France) 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.

Magnetization measurement: The magnetization of the prepared emulsions was measured to see if there is sufficient magnetization in iron oxide loaded nanoparticles to be used in imaging techniques for final applications of the particles. The samples were centrifuged at 40,000 rpm for 20 minutes at a temperature of 25°C. The centrifugation was done using Optima Max-XP ultracentrifuge, Beckman Coulter made in USA. The supernatant portion was discarded and the solid contents were dried using water bath at a temperature of 65-80°C for 24-72 hours. After complete drying, the samples were like discs, hence crushed to powder using spatula. Magnetic measurements were carried out using automatic bench for magnetic measurement (ABMM) at CNRS-IRC laboratory Lyon. Magnetization of all samples was investigated by decreasing magnetic field (H) from 20,000 to -15,000 Oersted.

Encapsulation efficiency: The encapsulation efficiency determination of the final particles was a difficult task especially in case of the organic ferrofluid prepared emulsions. Thermogravimetric analysis (TGA) was used for the purpose to know about efficiency of this modified method for emulsification of iron oxide nanoparticles.

Thermogravimetric analysis: Thermogravimetric analysis (TGA) of samples of aqueous ferrofluid, organic ferrofluid, blank emulsion and the emulsions loaded with organic and aqueous ferrofluid was done to determine the amount of iron oxide inside final nanoparticles and for the comparison. The preparations of the samples for thermogravimetric analysis, all samples were centrifuged at 40,000 rpm for 20 minutes at a temperature of 25°C. Centrifugation was done using Optima Max-XP ultracentrifuge, Beckman Coulter made in USA. The supernatant liquid portion was discarded and solid contents were left for drying in a beaker with water using water bath at a temperature of 65-80°C. After complete drying, the samples were like discs so crushed to powder with spatula to be used easily for the TGA analysis. The analysis was carried out on a TA 2950 (TA Instruments USA). All the samples were analyzed in alumina crucibles within a temperature range of 20-800°C (heating rate 10°C/min) in a nitrogen environment (Flux of 5 ml/min).

Results and discussion

Ferrofluids characterization

Zeta potential of aqueous ferrofluid at different pH was measured in order to point out the colloidal stability domain of such nanoparticles and the effect of pH on the surface charge density. The results showed that as the pH value increases from pH 3 to 7, zeta potential value in between +45 to +40 mV is almost constant. The ferrofluids prepared by co precipitation method are ionic ferrofluids and exhibit the electrostatic interactions coming from the surface charges carried by oxygen atoms of the oxide. The iron oxide particles are amphoteric being negatively charged in basic medium and positively charged in acidic medium. Surface charges on the oxygen atoms of oxide produced due to nitric acid addition at final stage of preparation providing positive charges. Above pH 7, the zeta potential decreases drastically and becomes negative above pH 9.5. The organic ferrofluid cannot be examined in terms of zeta potential due to the organic medium. This trend of aqueous ferrofluids against different pH values is explained in form of a graph in fig. 3.

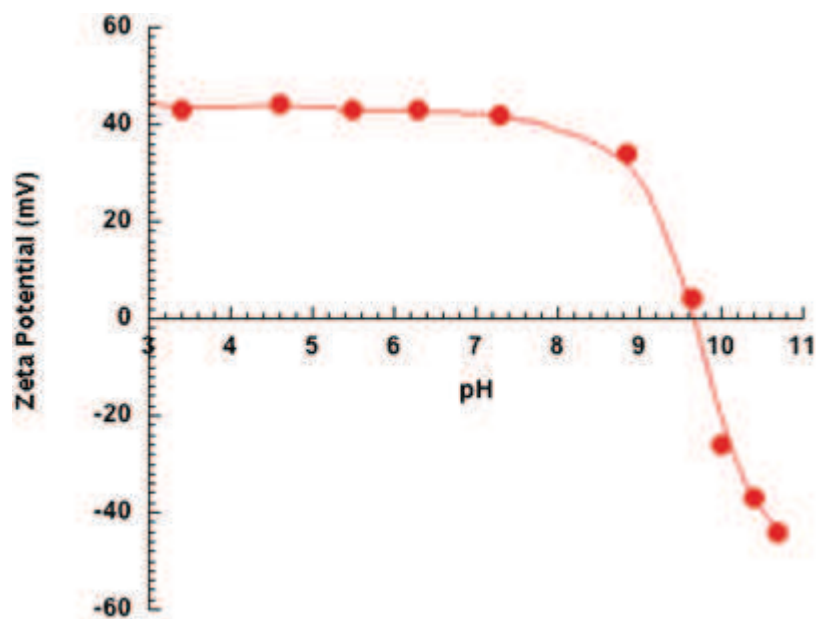


Figure: 3 Zeta potential measurements versus pH of the iron oxide nanoparticles dispersed in aqueous medium

The particle size and size distribution of the prepared ferrofluids were investigated using light scattering technique and the obtained results are reported in fig.4. The size distribution of the prepared aqueous ferrofluid and organic ferrofluid show that they are

narrowly size distributed. The average hydrodynamic size was found to be around 10 nm for organic iron oxide nanoparticles and close to 8 nm in case of aqueous ferrofluid. Similar values have already reported. It is interesting to notice that the organic ferrofluid exhibits well-defined peak, whereas, the aqueous one exhibits the presence of some aggregated particles bearing average hydrodynamic size around 20 nm (fig. 4). The sharp peak observed in the case of organic ferrofluid attributed to well dispersion of iron oxide in organic medium while aggregation of particles in aqueous ferrofluid explains some particles without sufficient nitric acid. However, the percentage of aggregated particles shown them few in number, otherwise presence of adsorbed nitric acid maintained the particles stabilization.

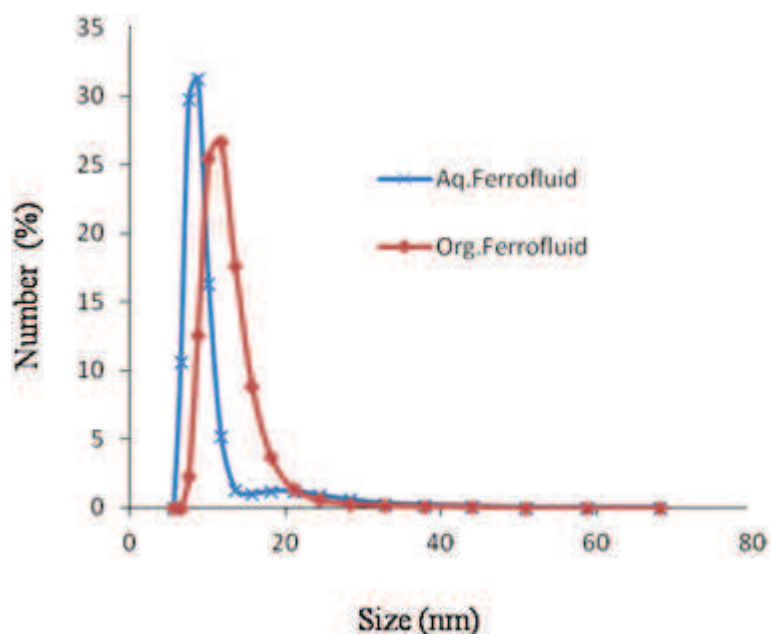


Figure: 4 Particle size and size distribution of aqueous and organic ferrofluid

Elaboration of free and drug containing nanoparticles via modified double emulsification- solvent evaporation process

The aim of the work was to prepare submicron hybrid particles bearing the dual properties of diagnosis and therapy relating to recently coined termed theranostics. The hybrid particles contain two ingredients: an active agent and a contrast agent for diagnostic purpose via imaging techniques. A new multiple emulsion (w/o/w) evaporation method was devised to encapsulate both ingredients. Primarily submicron particles of a hydrophilic model drug (Stilbene) are prepared using PCL as polymer. Different stabilizing agents including triton-405, tween 80, poloxamer, PVA (poly vinyl alcohol) and PEG (poly ethylene glycol) were screened, and a stable emulsion was obtained using PVA (0.5%). Drug loaded particles were characterized and different parameters affecting size of the particles investigated to obtain

most suitable conditions. These parameters include stirring time, stirring speed, polymer and stabilizer concentration. In this study, high energy stirring apparatus, ultra-turrax T-25, was used for dispersion of one phase into another. The effect of stirring time for both emulsification steps was estimated while all other parameters including stirring speed, polymer concentration, stabilizer concentration and stirring time for reciprocal emulsification were kept constant. During first emulsification different time intervals from 5-25 minutes were used to investigate the time effect on final particle morphology keeping all other parameters constant. It was found that 10-20 minutes stirring time produced the final particles ranging from 300 nm to 400 nm however, 15 minutes were concluded as suitable time for first emulsification with other constant parameters, producing less than 300 nm final particles. Increasing or decreasing the stirring time for first emulsification (w/o) in given conditions increased the particle size as shown in fig. 5. In a similar way, time intervals from 5-25 minutes were used to investigate the effect of second stirring time on final particles morphology keeping all other parameters constant. Interestingly, the hydrodynamic diameter measurements of final particles revealed 10-20 minutes as suitable time period for second step of emulsification but the minimum particle size was achieved at 15 minutes. This results because less than 10 minutes is not a sufficient time for well fragmentation of the particles and more than 20 minutes is too long time causing re-aggregation of the particles in presence of foam resulting due to stabilizing agent. If we see the time intervals from 10-20 minutes, it gives a very good trend of decreasing the particles size after 10 minutes till 15 minutes and increasing again from 15 minutes as shown in the graph in figure 5. In less than 10 minutes, the energy supplied via ultra-turrax (T-25) was not sufficient for complete fragmentation while above 20 minutes, the foam increases causing increase in size and with increasing time. However, 15 minutes time interval gave suitable results for second emulsification. Hence, 15 minutes stirring time was concluded suitable for both emulsification steps (w/o and w/o/w).

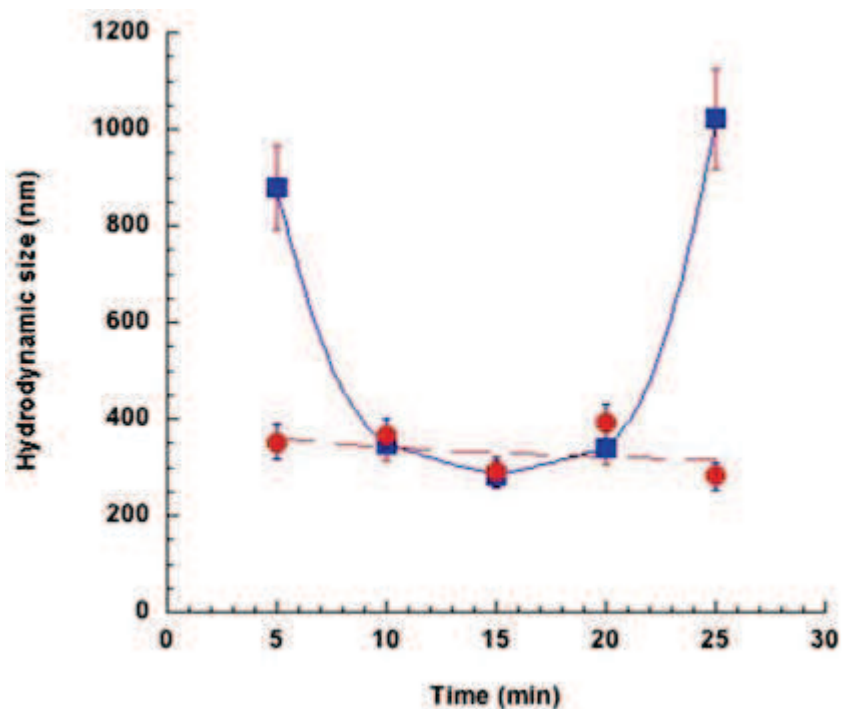


Figure: 5 Stirring time (min) vs. final particle size (nm) ● first emulsification ■ second emulsification

The effect of stirring speed on morphology of the final drug loaded submicron particles was investigated for both emulsification steps while all other parameters including stirring speed, polymer concentration, stabilizer concentration and stirring time for reciprocal emulsification were kept constant. During first emulsification, different stirring speeds ranging from 13,000 rpm to 24,000 rpm were studied on the basis of final particle size. Results explained a significant effect of first emulsion stirring speed on size of the final particles as shown in fig. 6. Speed at 16,000 rpm and 24,000 rpm produced the nanoparticles with minimum size ranging from 285-298 nm. Stirring speed of 16,000 rpm was the best choice because 24,000 rpm gave heating problems that affected the particles morphology.

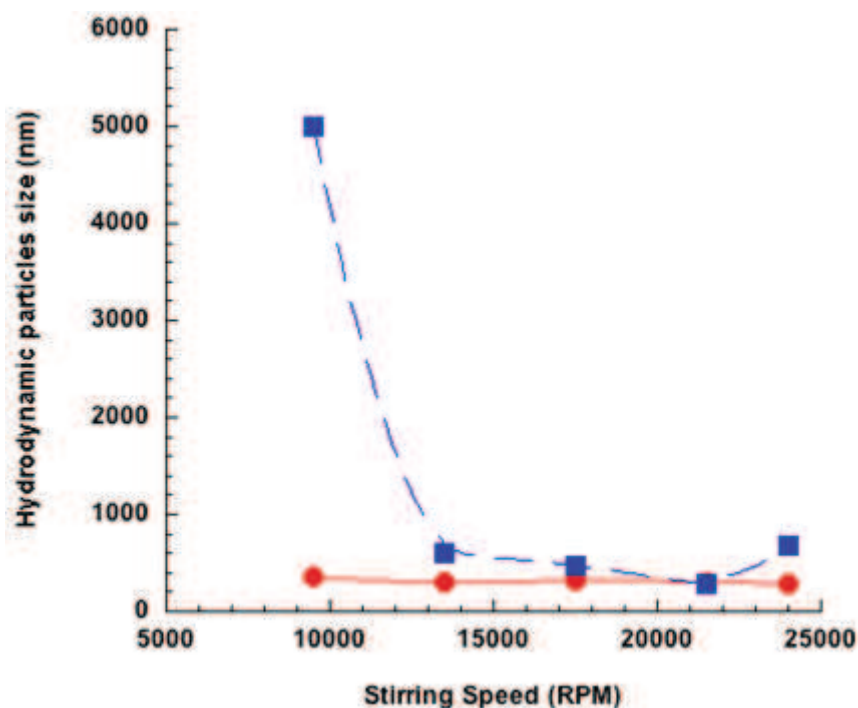


Figure: 6 Stirring speed (rpm) vs. final particle size (nm) (red circles) first emulsification (blue squares) second emulsification

In same way, stirring speed for second emulsification was studied ranging from 9,500 rpm to 24,000 rpm keeping other parameters constant. It was found greatly important to control the final particles size during emulsification. The particles produced at a stirring speed upto 13,000 rpm were in micrometers as shown in fig. 6. With increasing the stirring speed the size decreases and at 21,500 rpm optimal results were obtained and this is what generally observed in case of miniemulsion also. This can be explained in another way as low stirring speed provides less energy being insufficient for complete fragmentation and high speed serve the prupose providing sufficient energy required. Hence, it was found that for submicron drug loaded particles; stirring speed is an important factor to be considered both for first and second emulsification. Under given conditions of the modified double emulsification evaporation technique, the suitable stirring speed for first and second emulsification was found at 16000 rpm and 21,500 rpm respectively.

The concentration of polymer, polycaprolactone (PCL) in this study, is important factor to control the final particle size. Keeping all other parameters constant, different PCL concentrations from 8-41% with respect to oily phase (DCM) were studied. It was observed that 25 % (mass/volume) PCL concentration to DCM produced suitable results in terms of particle size and the obtained results are described in fig. 7.

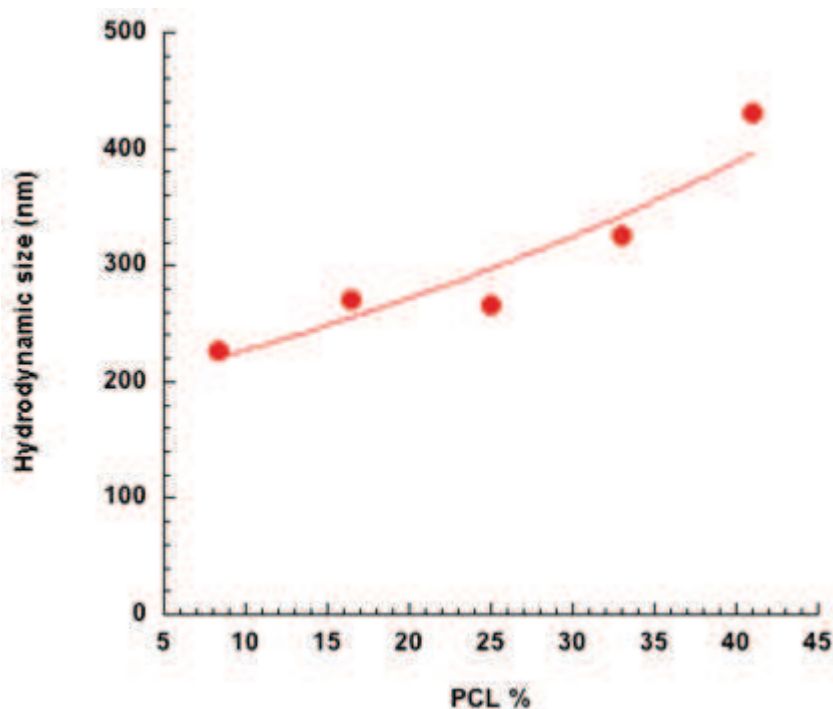


Figure: 7 PCL percentage vs. final particle size

The stability of the emulsions is based on effectiveness of the stabilizing agent in terms of concentration. PVA was screened and used as stabilizer in second emulsification step (continuous aqueous phase) for this study and effect of its concentration on final particle size was investigated. Different concentrations of PVA ranging from 0.25% to 3% were studied while all other parameters were constant. Increased concentration of PVA proved inutile for this work and decreased concentration was unable to stabilize the emulsion being insufficient to prevent aggregation. The obtained results revealed 0.5% PVA concentration as suitable one for this modified method as shown in fig.8. The emulsions prepared at this concentration were stable for a long time. At increased concentration (above 2%) of PVA, the curve showing increase in particle size became flat explaining no more effect on size. However, this higher concentration caused the increased foam in the preparation.

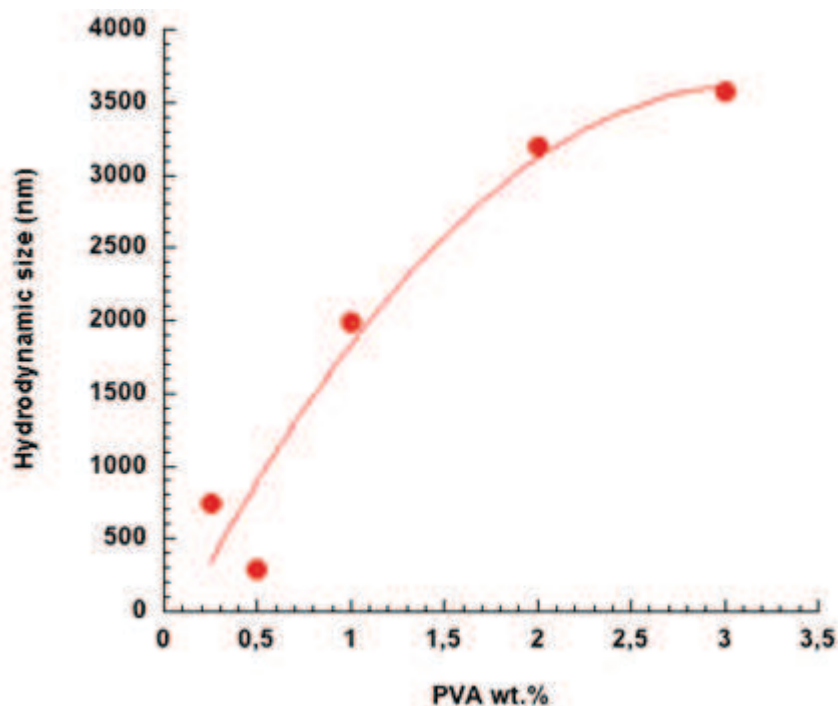


Figure: 8 Different PVA concentrations (%) vs. final particle size (nm)

Studying all the parameters affecting morphology of the drug loaded particles permitted to devise a recipe for the encapsulation of iron oxide. It has been concluded to prepare primary emulsion (w/o) applying stirring at speed of 16,000-17,500 rpm for 15 minutes using 25% PCL to oily phase while for secondary emulsion (w/o/w) stirring for 15 min at speed of 21,500 rpm using 0.5% PVA as stabilizing agent. After this preliminary study, systematic study was then investigated and the obtained iron oxide containing PCL particles are characterized and the results are adequately compared.

Ferrofluid encapsulation via modified double emulsification-solvent evaporation process

The encapsulation of water dispersed iron oxide nanoparticles (aqueous ferrofluid), was first performed as a function of iron oxide content and the final dispersion were found to exhibit the presence of settled aggregated magnetic particles for high solid content. This can be attributed to the limitation of the process in terms of iron oxide/PCL ratio. In fact, using aqueous ferrofluid, the first emulsification step leads to water containing iron oxide nanoparticles in oil containing PCL and the second step leads to aqueous ferrofluid in oil in water and due to high homogenization speed, iron oxide nanoparticles are transferred into aqueous phase.

Regarding the encapsulation of iron oxide nanoparticles stabilized with oleic acid and dispersed in octane (and diluted in DCM), the obtained water in oil in water dispersion exhibits the presence of black oil layer at air water interface. This can be attributed to the effect of octane, which is not miscible with water and then expelled from the polymer matrix. In order to avoid such problem, octane was totally discarded and the prepared iron oxide coated with oleic acid and dispersed in octane was dried and then dispersed in DCM followed by filtration step.

Size distribution

The distributions of the prepared emulsions (blank or loaded) are narrow size distributed. The average hydrodynamic size was found to be around 225 nm for iron oxide free PCL particles. In case of polymer particles loaded with iron oxide contents, the average size of the final particles appeared to be 260-270 nm. Important to note is that the prepared final emulsions exhibit well defined peaks explaining their stability. Figure 9 represents the size distribution of a blank emulsion, of an emulsion with aqueous ferrofluid and of an emulsion with organic ferrofluid. These size distribution graphs were drawn after taking data from Malvern Zetasizer that was used for size measurement of all the samples in this work.

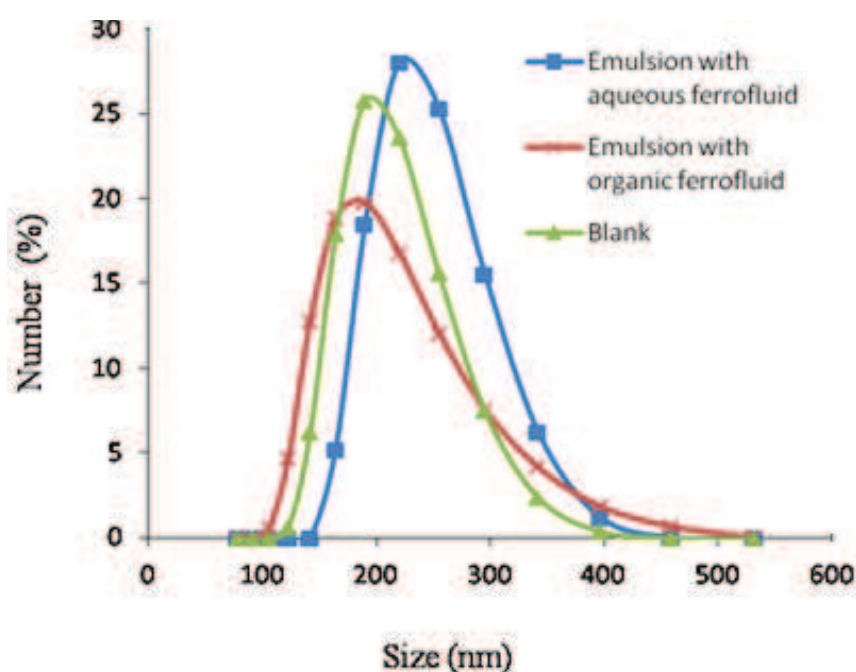


Figure: 9 Graph showing size distribution of blank emulsion, an emulsion of aqueous ferrofluid and of an emulsion of organic ferrofluid

Effect of iron oxide quantity on the magnetic particles size

Different emulsions were prepared using different aqueous ferrofluids and the results obtained are described as a graph. In this study all the emulsion were prepared by exactly the same method and all parameters were kept constant except the solid contents of the ferrofluid used. First of all a blank emulsion was prepared without encapsulating iron oxide and then iron oxide particles in aqueous medium having solid contents percentage from 0.5 % to 5 % were encapsulated in a series to see the effect of iron oxide quantity in encapsulated particles. The size measurement was done using dynamic light scattering (DLS) and the results indicated that there is no significance effect on the final hydrodynamic average particle size of increase in iron oxide particles quantity. When the non-encapsulated iron oxide was removed, the effect of iron oxide solid content was totally negligible (Fig. 10).

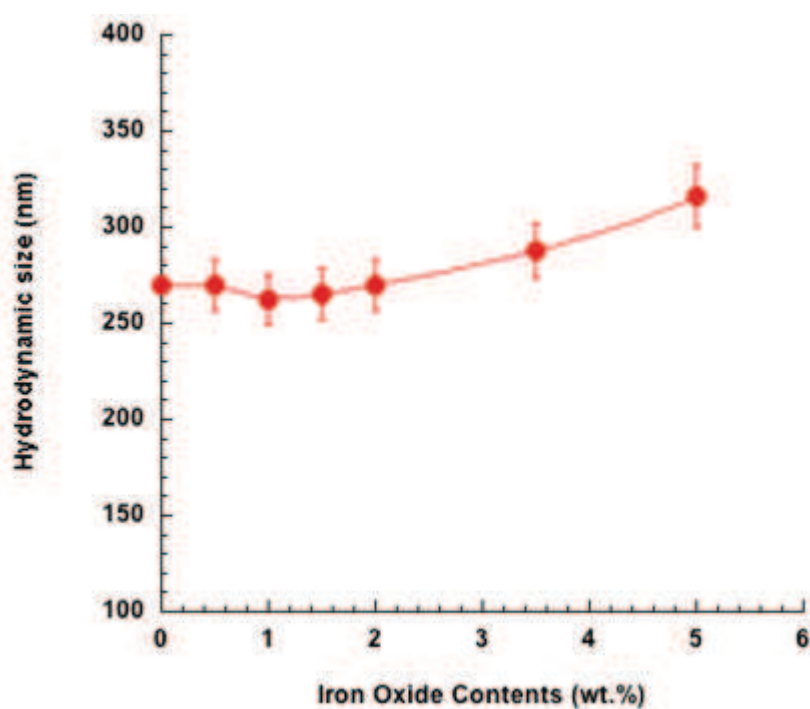


Figure: 10 Hydrodynamic particles size of the final hybrid particles versus solid content of the aqueous ferrofluid used

In case of iron oxide particles in organic medium, the encapsulation process posed a little problem of miscibility of octane and DCM. As mentioned in the materials and methods section, the ferrofluids were dispersed in octane in final step of their preparation; the octane solution of ferrofluids was mixed with DCM solution without changing the final volume of oily phase (12 ml). The iron oxide was well encapsulated and a good emulsion was obtained but with an additional layer of octane having more concentration of the iron oxide. The result

was the same for both octane/DCM ratios used. Then dried iron oxide particles were dissolved in DCM along with polymer and encapsulated accordingly. The method worked well without an additional layer of octane. However, for all cases of organic ferrofluid encapsulation (either using octane or without octane), higher amount of iron oxide nanoparticles than a specific quantity, non encapsulated iron oxide particles were observed in both emulsification and evaporation steps along with settlement of excess of iron oxide during storage. This represents that above said modified method works well until a specific quantity of iron oxide and when this limit surpasses, further encapsulation is difficult. However, according to aim of the study the iron oxide encapsulation is for the MRI purposes and that requires a small quantity of magnetic contents for detection so the method works in an efficient way to serve the purpose. To encapsulate a higher quantity of iron oxide, the method can be modified accordingly e.g., it can be encapsulated with increasing the volumes/quantities involved and finally the emulsion can be concentrated or the increase in polymer amount alone can serve the purpose, although it may result in small increase in size of final loaded particles.

Zeta potential measurement

The zeta potential of the prepared PCL based particles was investigated as a function of pH. The obtained zeta potential values are negative irrespective pH (ranging from pH 3 to 10) and the presence of magnetic nanoparticles. The negative zeta potential can be attributed to the possible hydrolysis of PCL, which leads to hydroxyl and carboxylic groups.

This effect of the nitric acid treatment is also visible when these iron oxide particles in aqueous medium were encapsulated by modified double emulsion technique. As the solid contents value of iron oxide particles increases, the negative charge on the particles decreases and after a specific solid content value, it becomes positive. The reached positive zeta potential can be attributed to the presence of high positive iron oxide nanoparticles in the medium or on PCL based particles. This change in zeta potential can be seen by the following Fig.11. In case of the encapsulation of organic iron oxide nanoparticles, the effect of solid content was found to affect the encapsulation efficiency and the final dispersion aspect. However, the tendency to affect the zeta potential of the final particles was same as that of aqueous ferrofluids. Although the zeta potential values are smaller but they are sufficient to stabilize the system for a certain time, however, if the settling/aggregation of the particles occur, can be removed by small agitation e.g., just by shaking or putting the emulsion bottle on the roller for 2-5 minutes.

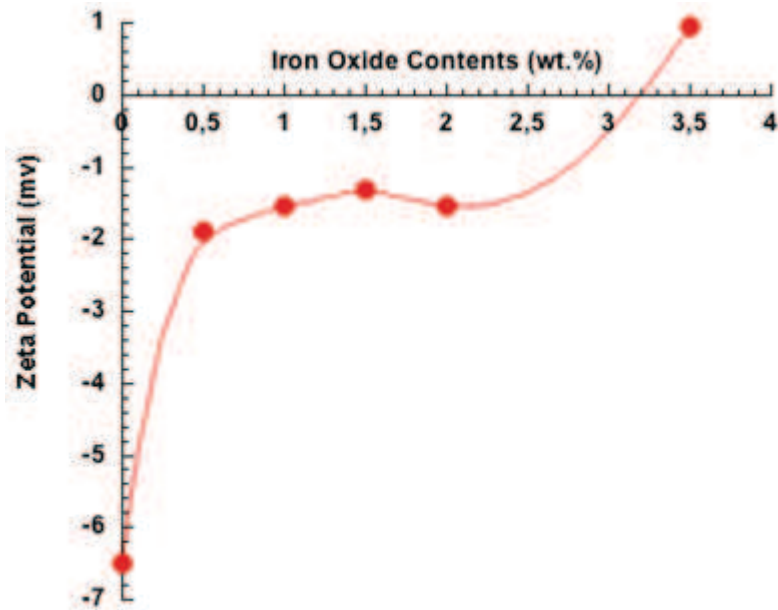


Figure: 11 Zeta Potential values of the final hybrid particles versus solid content of the aqueous ferrofluid

Magnetization results

The magnetization measurement of aqueous and organic ferrofluid loaded nanoparticles was done to show the susceptibility of particles in an applied magnetic field. The results are depicted in the following fig.12. It can be seen, the use of aqueous or organic ferrofluid has no specific effect on the superparamagnetic properties of the final particles. Both types of the particles are detected with an equal ease and can be used for diagnostic purposes. In addition, the saturation magnetization (emu/g) was also found to be above 2, which explains the possible detection of the final particles via magnetization measurement such as MRI. The difference in plateau values can be attributed to the difference in the iron oxide amount used in each process only. It is also interesting to notice that the prepared iron oxide nanoparticles are superparamagnetic in nature as already reported [55].

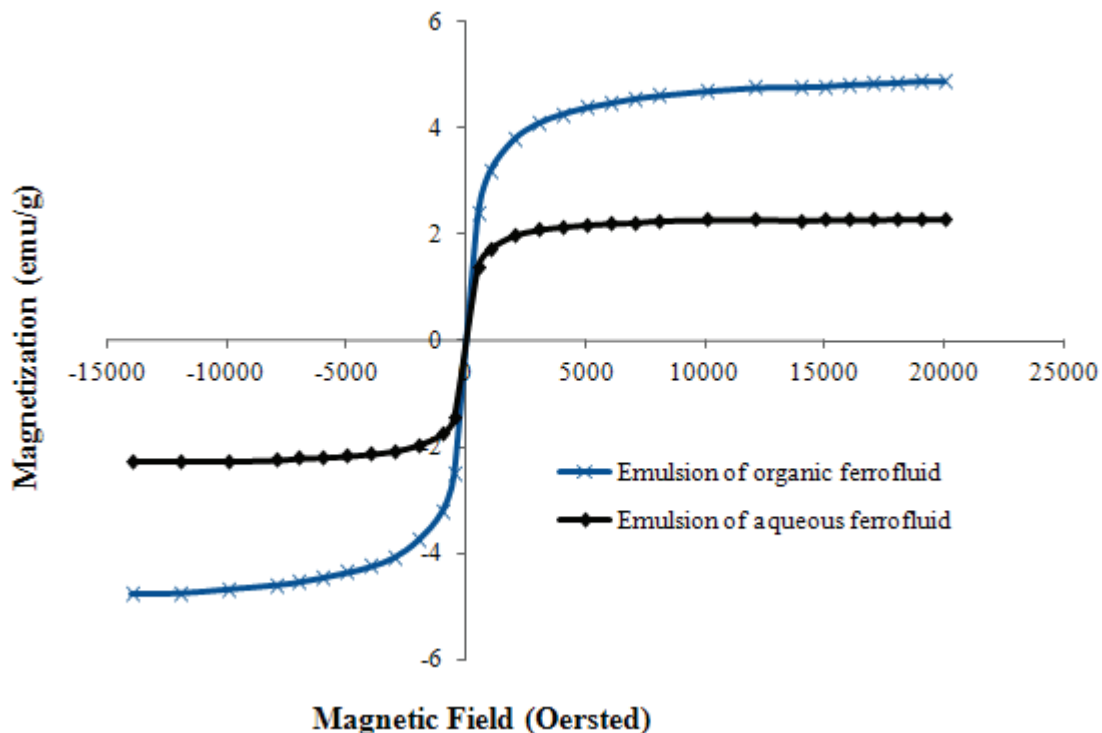


Figure: 12 Magnetization power against magnetic field for the nanoparticles prepared by aqueous and organic ferrofluids

Morphological study

The morphological study of the PCL submicron particles was done using transmission electron microscopic technique. The transmission electron microscopy (TEM) of all the samples prepared were done to analyze the difference on the morphology of different particles. Samples of the blank emulsions gave a satisfactory image about the particle morphology without encapsulation of any ingredient. The TEM images of stilbene loaded nanoparticles also sufficiently explained the morphology supporting the idea to use stilbene encapsulation for parameters study as shown in fig. 13(a). However, in some TEM micrographs the drug particles were found outside the polymeric shell showing the passage of aqueous drug particles from interior aqueous phase to the exterior aqueous phase. Then the next step was the TEM study of iron oxide loaded nanoparticles. Typical micrographs were taken for emulsions prepared using iron oxide in aqueous medium and in organic medium. Both types of emulsions were well analyzed using TEM to compare the results especially morphological point of view. The obtained images show the presence of black dots dispersed in the particles. The viewed black dots correspond to iron oxide particles adequately encapsulated inside the polymer or on the surface of polymeric shell inside the particles.

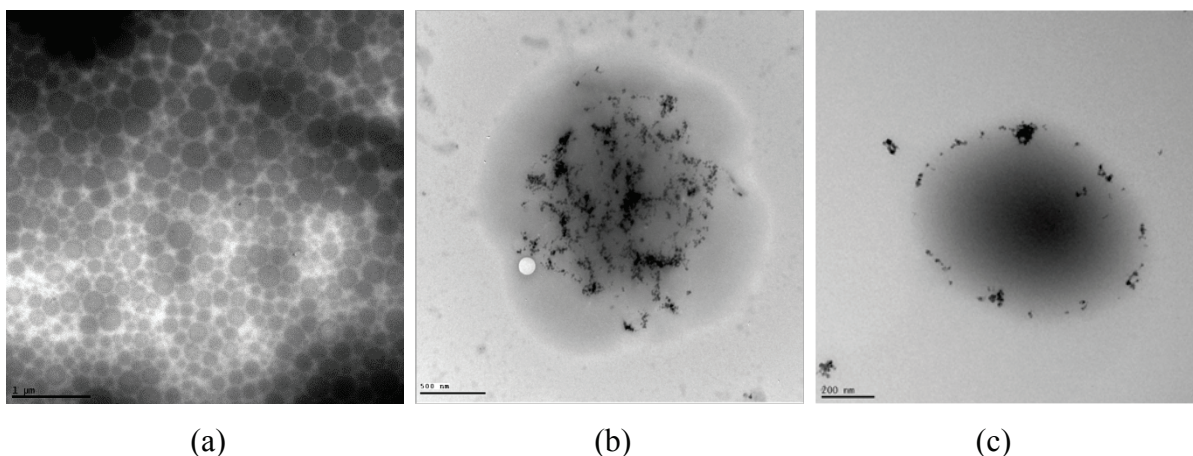


Figure: 13 (a) Active molecule hydrophilic drug nanoparticles prepared via double emulsification process. (b) Encapsulation of iron oxide particles in aqueous medium iron oxide particles are well dispersed inside the polymer. (c) Encapsulation of iron oxide particles in aqueous medium where iron oxide particles on surface of polymer

It can be seen from the fig. 13 (b) and (c) that iron oxide particles are present on the surface as well as inside the particles. It explain that iron oxide nanoparticles in aqueous medium tend to disperse towards the second aqueous phase of the emulsion by crossing the polymeric shell of the particle. So in this case the iron oxide nanoparticles are present evenly in the nanoparticles. Such type of phenomena is explained by Florence et al. [57] in factors responsible for emulsion deterioration but in this case the emulsion remained stable for more than 100 days. It is considered as passage of a portion of interior aqueous phase towards exterior aqueous phase or can be rupture of some of w/o particles during second emulsification step having extensive shearing. These results are somehow identical to those seen in Stilbene encapsulation. Fig. 13 (b) represents a close view of the particle showing that inside the polymeric shell, the iron oxide contents are well dispersed representing the presence in first aqueous phase of the emulsion.

On the other hand in case of organic ferrofluid encapsulation, the iron oxide content tried to remain inside the polymeric shell showing the hydrophobicity and attached to the polymeric matrix because both iron oxide and polymer were soluble in organic phase *i-e.*, DCM. This phenomenon of iron oxide restriction inside the polymer can be clearly observed from fig. 14 (a) and (b). It can also be seen from fig. (a) that iron oxide contents followed the polymer tried to remain inside the polymeric shell.

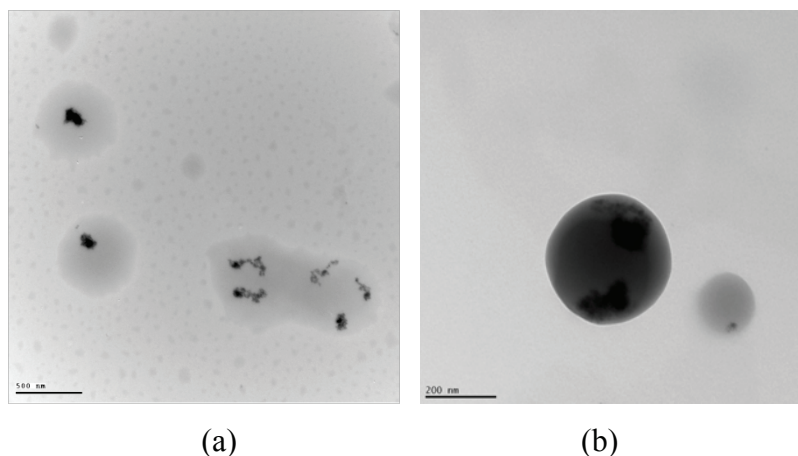


Figure: 14 (a) Encapsulation of iron oxide particles in organic medium with 500 nm magnification. (b) Encapsulation of iron oxide particles in organic medium with different 200 nm magnification.

These TEM micrographs also give an idea about the size of the final nanoparticles. They are well in nano range although not 100% spherical in shape. The microscopic study of the iron oxide containing nanoparticles revealed the difference between the aqueous and organic ferrofluid encapsulation process. It explains that in case of aqueous ferrofluid encapsulation, they tend to move towards the second aqueous phase and present both on the surface and interior of the particles. However, in organic ferrofluid encapsulation, the magnetic contents remained inside the polymeric shell being hydrophobic.

Chemical composition

The encapsulation efficiency of the method was determined by thermogravimetric analysis (TGA) and is demonstrated in terms of a graph. The graph clearly demonstrates the curves of weight loss of different emulsions prepared by modified double emulsion evaporation technique encapsulating the different quantity in terms of solid contents. For the sake of comparison a blank preparation was done without introducing iron oxide nanoparticles and is passed through this analysis. Also pure iron oxide nanoparticles dispersed in aqueous medium were analyzed by this method to have a good comparison curves.

Fig. 15 graph (a) represents a curve, marked as the aqueous ferrofluid, is of pure iron oxide nanoparticles in aqueous medium showing more than 5% weight loss, explaining the presence of some organic impurities or to the non dried sample. On the other hand, curve marked as blank is of iron oxide free polymer particles preparation and gives total weight loss explaining the complete calcinations of the particles.

The curve corresponding to emulsion prepared using aqueous ferrofluid (9% (w/v) solid contents) shows the presence of more than 5% w/w iron oxide contents, which explains the encapsulation of iron oxide nanoparticles.

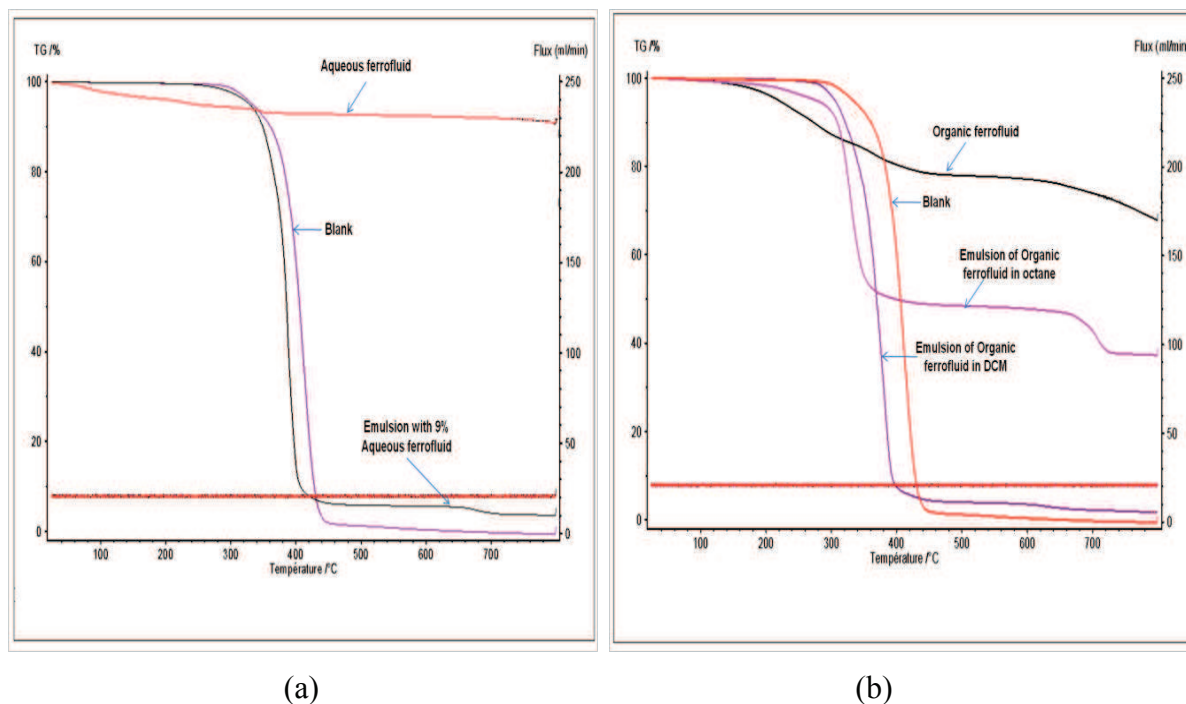


Figure: 15 (a) TGA weight loss curves for aqueous ferrofluid, blank and of nanocapsules prepared using 9 % aqueous ferrofluid. (b) TGA weight loss curves for organic ferrofluid, blank, nanocapsules prepared using organic ferrofluid (i.e. iron oxide in octane and diluted in DCM before encapsulation).

On the other hand figure 15 (b) represents the comparison of the TGA results for organic ferrofluid, blank and the hybrid particles prepared using organic ferrofluids (both with and without octane). The difference of TGA curves for aqueous ferrofluid in fig. 15 (a) and for organic ferrofluid in fig. 15 (b), under the same experimental and measurement conditions, is because of the treatment of organic ferrofluid with oleic acid in last step of preparation. The minute quantity of the iron oxide contents representation in graphs is due to the low dosage of iron oxide contents, according to the requirements for final applications.

Conclusion

Iron oxide nanoparticles are synthesized and dispersed in suspensions called ferrofluids, which are stable colloidal dispersions of very fine nanoparticles of magnetic materials. Here these ferrofluids were successfully prepared in aqueous and organic medium using co-precipitation method. A modified double emulsion evaporation method was devised envisaging the encapsulation of active and contrast agent at same time to be used as

theranostics. Among different stabilizing agents including triton-405, tween 80, poloxamer, PVA (poly vinyl alcohol) and PEG (poly ethylene glycol) screened a stable emulsion-like was obtained using PVA. PCL was used as polymer (to form the matrix) and DCM as organic solvent for the preparation of biodegradable and biocompatible nanoparticles. Drug free and Stilbene loaded nanoparticles were prepared and at same time different parameters were investigated to prepare a standard recipe for encapsulation of iron oxide nanoparticles. These parameters include stirring speed, stirring time, polymer and stabilizer concentration. Both types of the iron oxide nanoparticles were successfully encapsulated within PCL. The particle sizes of the aqueous and organic ferrofluids, blank emulsions, Stilbene, aqueous and organic ferrofluid loaded emulsions were measured using DLS and TEM. The final loaded particles were found in a range of 250-340 nm. The zeta potential measurement gives an idea about the charge on the particles and indirectly measure of stability. The pH value also plays an important role in the stability of the system. It was concluded that a suitable buffer should be added for long time stability requirements. Encapsulation efficiency of the iron oxide contents were measured using TGA analysis and proved to be more than 60%. TEM micrographs provided clear picture of the encapsulation of Stilbene and iron oxide inside the nanoparticles. In short, this new double emulsion evaporation method using PCL as polymer, DCM as oily phase and PVA as stabilizing agent, proved a suitable technique for the encapsulation of active ingredients providing a particle size of about 300 nm. It proved successful for the encapsulation of a model drug as well as contrast agent for the diagnostic purposes. Modification of the related parameters of this new double emulsion evaporation technique allows the encapsulation of the different active ingredients targeting for different applications including diagnostic applications.

Acknowledgment

We are thankful to Mr. Ahmad Bitar for TEM analysis of magnetic particles samples. We are also thankful to Science and Technology cooperation program between Pakistan and France and between SFERE (Société Française d'Exportation des Ressources Educatives) and Higher Education commission (HEC) of Pakistan for supporting Ph.D. studies of Mr. Naveed Ahmed.

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III.2.EVALUATION OF NANOPARTICLES

CHAPTER III.2. (A)

EVALUATION OF NANOPARTICLES *IN VITRO*

***In vitro* MRI of hybrid (iron oxide/poly caprolactone) magnetic nanoparticles prepared via modified double emulsion evaporation mechanism**

Naveed Ahmed a, Nasir. Mahmood Ahmad b, Hatem Fessi a and Abdelhamid Elaissari a
aUniversity of Lyon, F-69622, Lyon, France; University Lyon-1, Villeurbanne; CNRS, UMR
5007, LAGEP- CPE; 43 bd 11 Novembre 1918, F-69622 Villeurbanne, France.

b School of Chemical and Materials Engineering (SCME), National University of Sciences
and Technology (NUST), NUST H-12 Campus, Islamabad-44000, Pakistan

* Corresponding author: Phone: +33-472431841, Fax: +33-472431682

E-mail: elaissari@lagep.univ-lyon1.fr

(In preparation for Submission)

Résumé

Les particules hybrides magnétiques sont largement utilisées dans le domaine biomédical. Dans le domaine *in vivo*, ces particules sont utilisées à des fins diagnostiques et en particulier dans l'imagerie biomédicale (IRM). Les particules magnétiques hybrides précédemment élaborées et parfaitement caractérisées en termes de taille, distribution de taille, potentiel zêta et composition chimique sont utilisées pour une première évaluation en IRM.

En effet, ces particules hybrides ont été analysées pour mesurer l'effet de la présence d'oxyde de fer comme agent de contraste via la mesure de la relaxation T_1 et T_2 . Une comparaison a été réalisée entre les particules préparées et un produit commercial, le gadolinium. La tendance générale des résultats montre que plus des particules élaborées sont riches en oxyde de fer, plus le contraste est important par rapport au produit commercial le gadolinium utilisé ici comme référence.

Abstract

Hybrid magnetic particles are being applied in biomedical field with various aims. One of the targets is use of magnetic particles for diagnostic purposes particularly in imaging mechanisms. Here, new type of magnetic particles are being reported for use in magnetic resonance imaging (MRI). Iron oxide nanoparticles (IONPs) were prepared using a modified co-precipitation method. Hybrid magnetic nanoparticles were prepared encapsulating iron oxide nanoparticles in Poly caprolactone (PCL) via modified double emulsion evaporation technique. The characterization of IONPs and hybrid nanoparticles were done using parameters of size, zeta potential, microscopy and thermogravimetric analysis. The prepared particles were then analyzed for T1 and T2 weighted enhancement of contrast in MRI in vitro. A comparison of prepared particles was done with commercially available Gadolinium for the contrast efficiency in MRI. Results showed the particles with nanosize range and good morphology along with supereparamagnetic character. The enhancement of the contrast via IONPs and comparison with Gadolinium commercial products in MRI was found successful.

Introduction

The rapid progress in the nanoparticles preparation and increased innovations in the development and applications of nanoparticles is result of some attractive features related to them. These features can be exploited in different ways depending upon the need why nanoparticle technology is being applied. In Pharmaceutical field, small size is explored for drug delivery purposes and giving rise to the investigation in pharmacodynamics and pharmacokinetic implications. Pharmaceutical technology tools are in use for preparation of nanoparticles to be applied for applications that can be drug delivery, protein delivery, diagnostic purposes or therapeutic applications. Recently researchers are working on the nanoparticle systems having both types of therapeutic and diagnostic moieties so they can be used for dual functions simultaneously. Nonetheless the important thing to be kept in mind is the biodegradability of the materials used. For instance in drug delivery case, these nanoparticles can permit the binding of the drug to specific receptor along with influencing activity of that receptor [1]. Similarly in case of diagnosis nano biotechnology is applied in imaging mechanisms where different nanoparticles of various contrast agents are being prepared and applied. Magnetic nanoparticles have special focus for the imaging because of having magnetic characteristics hence sure possibility to be used in magnetic resonance imaging (MRI) that is a non invasive diagnostic technique. Magnetic nanoparticles are applied

for other purposes also including ribonucleic acid separation (DNA and RNA) [2], enzyme purification [3], drug targeting [4] and hyperthermia [5]. Restricting the discussion to magnetic nanoparticles, nanoparticles of containing contrast agents are prepared for MRI including Gadolinium, Manganese, Iron oxide, *etc.* Nonetheless, for other imaging mechanisms other contrast agents are also in use like iodinated compounds for X-rays. Gadolinium and Iron oxide nanoparticles are frequently used as contrast agents in MRI and various preparations have been commercialized after approval from FDA although there are some limitations [6].

For preparation of nanoparticles as contrast agent for MRI purposes various organic, inorganic and hybrid materials are in use to have the most efficient contrast agents through various chemical and pharmaceutical preparation techniques. A specific preparation technique is chosen keeping in view the final applications including *in vivo* and *in vitro* environment. For instance, *in vivo* applications the limitations increases and choice reduces while in case of *in vitro* some factors does not apply increasing the choice for method and materials to be used in preparation. Iron oxide nanoparticles (IONPs) are attractive as contrast agent having paramagnetic and superparamagnetic characteristics. They are prepared in various fashions depending upon the need, applications and may be on starting materials too. Further are techniques for modifying their characteristics including solvent evaporation, emulsion preparations, solvent diffusion, emulsion polymerization, microemulsion polymerization, suspension polymerization, dispersion polymerization and list continues. Chemical co-precipitation is the mostly used mechanism and then phase transfer is involved for iron oxide nanoparticles [7]. Other above mentioned techniques are for the encapsulation or polymerization of IONPs. One such method is emulsion evaporation and coupling multiple emulsions with evaporation produced a suitable mechanism for the encapsulation of both types of IONPs with same ease. Modified double emulsion evaporation method is involved in use of polymer for creating a matrix around IONPs and dispersion of system in water eliminates the solubility problems.

For studies of internal organs, MRI reached the highest resolution in images in a fastest acquisition times. Since the quality of the MRI images depends on the main parameters like that of the proton spin density, the nuclear spin–lattice relaxation time T_1 and the spin-spin relaxation time T_2 . In view of this, there is a high interest in the development of new contrast agents (CA), which are capable to improve the image contrast by increasing (locally) the nuclear relaxation rates [8-10].

This work is in relation with previously published work from our group [11, 12]. Here, iron oxide nanoparticles were prepared using co-precipitation method and then were emulsified using oil in water emulsion mechanism where IONPs dispersed in octane were used as oily phase. Characterization of IONPs alone, emulsified and polymerized was done using different techniques. Finally these nanoparticles were analyzed for *in vitro* T₁ and T₂ weighted MRI and compared with commercially available Gadolinium (Gadovist). Satisfactory results were obtained justifying our method and preparation technique as well as potential of using the developed IONS as contrast agents.

Materials and methods

Materials

FeCl₂·4H₂O, FeCl₃·6H₂O, dichloromethane (DCM), oleic acid, poly vinyl alcohol (PVA), used as surfactant (Mowiol® 4-88. Mw = 31 000 g/mol) and polycaprolactone (PCL) used as polymer (Mw = 14 000 g/mol) were purchased from Aldrich Germany. Ammonium hydroxide solution (24% in water) and nitric acid (2 M) were bought from Fluka Analytical and Prolabo Paris respectively. Gadolinium (Gadovist®), a Bayer product was donated/provided by hospital. Deionized water used in all experiments was from Milli-Q system. All the chemical reactants were used as such.

Methods

Preparation of iron oxide nanoparticle via co-precipitation method

IONPs were prepared using a modified method of co-precipitation technique mentioned by Montagne et al. [7]. The exact method followed is same as explained in our earlier publication [12]. Both ferrous and ferric chloride were dissolved in water and then stirred before the precipitation in a basic medium provided by ammonia. After a specific time of stirring, nitric acid or oleic acid was added to have the particles dispersible in water or in an organic solvent after washing step. Actually, for preparation of IONPs by this modified method, primarily ferrous chloride 3.7g and ferric chloride 10.16 g were dissolved in 80 ml water and transferred in the 250 ml reactor. Stirring was done for 40 minutes at a speed of 300-500 rpm. Then over a period of 80-100 minutes, 80 ml ammonium hydroxide (NH₄OH) (24 %) was added drop by drop with continuous stirring at a speed of 300-500 rpm. Stirring was continued for next 2 hours.

Depending upon the final preparation of the IONPs, the method was modified. For aqueous ferrofluids, completing the 2 hours stirring after the addition of ammonia; the preparation was then removed from reactor and separated with help of magnetic. The liquid portion is discarded and black iron oxide lump was dissolved in 80 ml water. 80 ml Nitric acid (2M) was also added. The whole solution was again put in reactor and stirred for 15 minutes at a speed of 300-500 rpm to prepare a homogeneous dispersion. Finally this dispersion was removed from reactor and magnetically separated. The liquid portion was discarded and magnetic material was washed using acetone, acetone/water mixture and at the end dispersed in 50 ml water and left in open air for complete evaporation of acetone.

On the other hand for the preparation of organic ferrofluids, 2 h stirring was done after the addition of ammonia. Then 8 ml oleic acid was added dropwise with help of a pipette and stirring was continued at a speed of 300-500 rpm even for 15 minutes more after complete addition of oleic acid. The black magnetic material was removed from reactor, separated magnetically, washed with water and dispersed in 30-50 ml acetone. The water traces present in the dispersion were removed with help of a separating funnel and the ammonia traces were evaporated attaching water bath with reactor at 60°C. The final nanoparticles were separated from acetone magnetically and dispersed in octane.

Preparation of iron oxide /poly caprolactone nanoparticles

To prepare iron oxide/polymer particles, multiple emulsion (double emulsion) evaporation method was chosen as described in our previous published work [11, 12]. Primarily W/O/W double emulsion was prepared using dichloromethane (DCM) as organic/oily phase and then its evaporation was done using rotavapor. In detail for preparation of a blank nanoemulsion, 3 g of poly caprolactone (PCL) was dissolved in 12 ml DCM. Then 1.5 ml of distilled water was added to this PCL/DCM solution and stirring was done for 15 minutes at speed of 16,000-17,500 rpm without using any surfactant. The primary emulsion was then transferred to 150 ml 0.5 % polyvinyl alcohol (PVA) solution and stirring was done at speed of 21,500 rpm for 15 minutes. PVA served as surfactant in this emulsification step. Finally evaporation of DCM was carried out under pressure at 32-35°C using Buchirotavapor followed by characterization of the preparation.

To prepare the nanoemulsion using IONPs dispersed in aqueous media, the first aqueous phase was replaced by aqueous ferrofluid with a specific solid contents and rest of the process was continued in similar way as that for blank emulsion. Whereas for

encapsulation of organic ferrofluid (IONPs dispersed in organic solvent), it was dried after dispersion in octane in the final step of preparation. The dried ferrofluid is then dispersed in 4 ml DCM while 3 g PCL was dissolved in 8 ml DCM and both were mixed later. Then this DCM solution was put on roller for 3-5 minutes for complete dissolution in DCM. Rest of the method is same as that for preparation of blank emulsion.

Size and zeta potential measurement

The hydrodynamic particles size (Dh) of the colloidal dispersions is determined by dynamic light scattering on a Zetasizer nanoseries “zs” instrument from Malvern at room temperature and in 10⁻³ M NaCl concentration at 25°C. The mean hydrodynamic diameter is calculated by using the Stokes–Einstein equation, each measurement was performed 4/5 times and then the average was taken.

Zeta potential measurement

Electrophoretic mobility (μ_e) measurements of the dispersions are carried out on a Zetasizer nanoseries “zs” instrument (from Malvern) as a function of pH at 25 °C in 10⁻³ Mol/L NaCl solution. Each reported value is the average of 4/5 measurements. Electrophoretic mobility is converted into zeta potential by using Smoluchowski’s equation by the instrument itself.

Transmission electron microscopy (TEM)

The transmission electron microscopies (TEM) on the iron oxide encapsulated nanoparticles were done by a Philips CM120 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 to see the particles in micrograph.

Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) of samples of aqueous ferrofluid, organic ferrofluid, blank emulsion and the emulsions loaded with organic and aqueous ferrofluid was done to have an idea about the amount of the iron oxide inside final nanoparticles and for the comparison. The preparations of the samples for thermogravimetric analysis, all samples were centrifuged at 40,000 rpm for 20-25 minutes at a temperature of 25°C. Centrifugation was

done using Optima Max-XP ultracentrifuge, Beckman Coulter made in USA. The supernatant liquid was thrown and the solid contents were left for drying in a beaker with water using water bath at a temperature of 65-80°C. After complete drying, the samples were like discs so crushed to powder with spatula to be used easily for the TGA analysis. The analysis was carried out on a TA 2950 (TA instruments USA). All the samples were analyzed in alumina crucibles within a temperature range of 20-800°C (heating rate 10°C/min) in a nitrogen environment (Flux of 5 ml/min).

Magnetization

The magnetization characteristic of the prepared emulsions was measured to see if there is sufficient magnetization in iron oxide loaded nanoparticles to be used in imaging techniques for final applications of the particles. The samples were centrifuged at 40,000 rpm for 20-25 minutes at a temperature of 25°C. The centrifugation was done using same Optima Max-XP ultracentrifuge, Beckman Coulter made in USA. Then the supernatant liquid was thrown and the solid contents were dried using water bath at a temperature of 65-80°C. After complete drying, the samples were like discs, hence crushed to powder using spatula. Magnetic measurements were carried out using automatic bench for magnetic measurement (ABMM) at CNRS-IRC laboratory Lyon. Magnetization of all samples was investigated by decreasing magnetic field (H) from 20,000 to -15,000 Oersted.

Magnetic resonance imaging (MRI)

T_1 and T_2 enhancement of signals were measured in the MRI clinical instrument from Toshiba, Japan, using 1.5 tesla magnetic strength. Specific concentration of the samples was dissolved in 10 ml of deionized water. Glass sample vials containing accurately weighed amount of MNPs were placed inside the homogeneous magnetic field to obtain the MRI images clinical 1.5 T superconducting magnet (Toshiba Vantage Titan 1.5T MRI machine, Japan) and featuring a 71 cm aperture with a 55 x 55 x 50 cm FOV. The host software was used for data acquisition, and visualization, analysis of the obtained images of the sample vials. For all the concentrations, the transverse relaxation time (T_2) was measured from the coronal images (Thickness = 4 mm) at various echo times (TE) from 15 ms to 164 ms with a repetition time (TR) of 5,000 ms. Similarly, the T_1 relaxation time was measured by varying TR between 25 ms to 825 ms while keeping TE constant at 12 ms. After acquiring the MRI images of the samples, the intensities of the image were measured within manually-drawn

regions of interest (ROIs) for each of the samples. Mean values of the intensity were taken to compare the relative intensity count of each sample. In control experiments, commercially available Gd-based contrast agent (Gadovist®) and water were used to compare the contrast capability of the present work MNP. The samples were placed inside the MRI machine in such a way that top most vial is of water only and then there are different samples of various concentrations, and then at the end in last row in the middle of two samples or at the bottom a single small water vials was used to locate the images.

Results and discussion

Particles characterization

Size, size distribution and zeta potential measurement

Light scattering technique was used in determination of size and size distribution of the each type of the particles including particles in aqueous and organic medium and final hybrid particles after emulsification of iron oxide nanoparticles. The analysis was done using 1 mM NaCl solution in case of aqueous ferrofluid, blank and IONPs loaded emulsions while in case of organic ferrofluids, measurement was done using the dispersion solvent. The hydrodynamic size was found to be from 10-50 nm for the different types of ferrofluids. The hydrodynamic size and size distribution of the ferrofluid found via dynamic Light scattering (DLS) is given in figure below. The diagram describes aggregation in hydrodynamic size of the organic ferrofluid showing some aggregates as explained in last published paper [12].

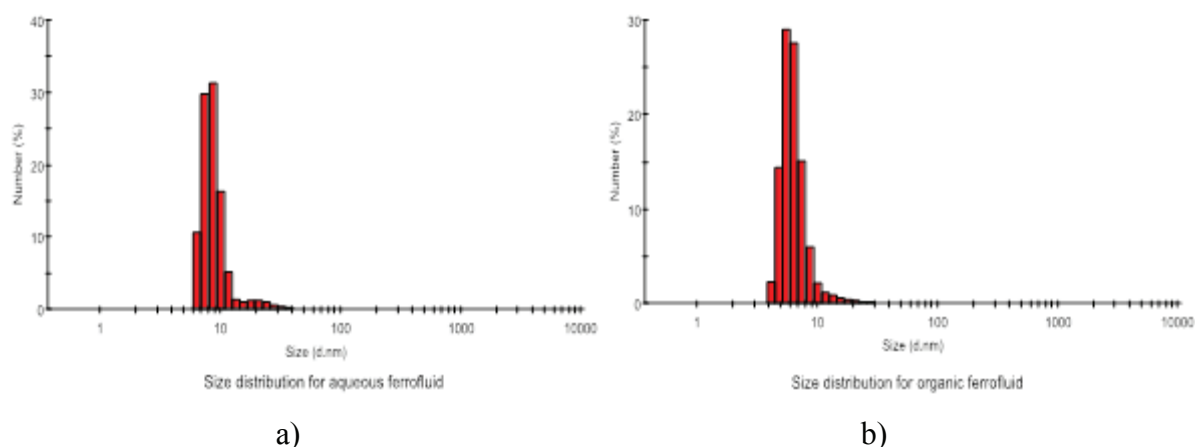


Figure: 1 Hydrodynamic diameter of a) aqueous ferrofluid and b) organic ferrofluid

Zeta potential values for aqueous ferrofluid were measured using same Malvern zeta sizer and it was found the resulting IONPS are cationic in charge causing the stability of the particles. The zeta potential values were found in range of + 35 to +40 mV. However a zeta potential study was also done in different pH values and zeta potential values obtained in descending order with increase of pH as mentioned in earlier article [12]. This explains the ionic ferrofluids were obtained and the particles show electrostatic interactions resulting from the surface charges due to oxygen atoms.

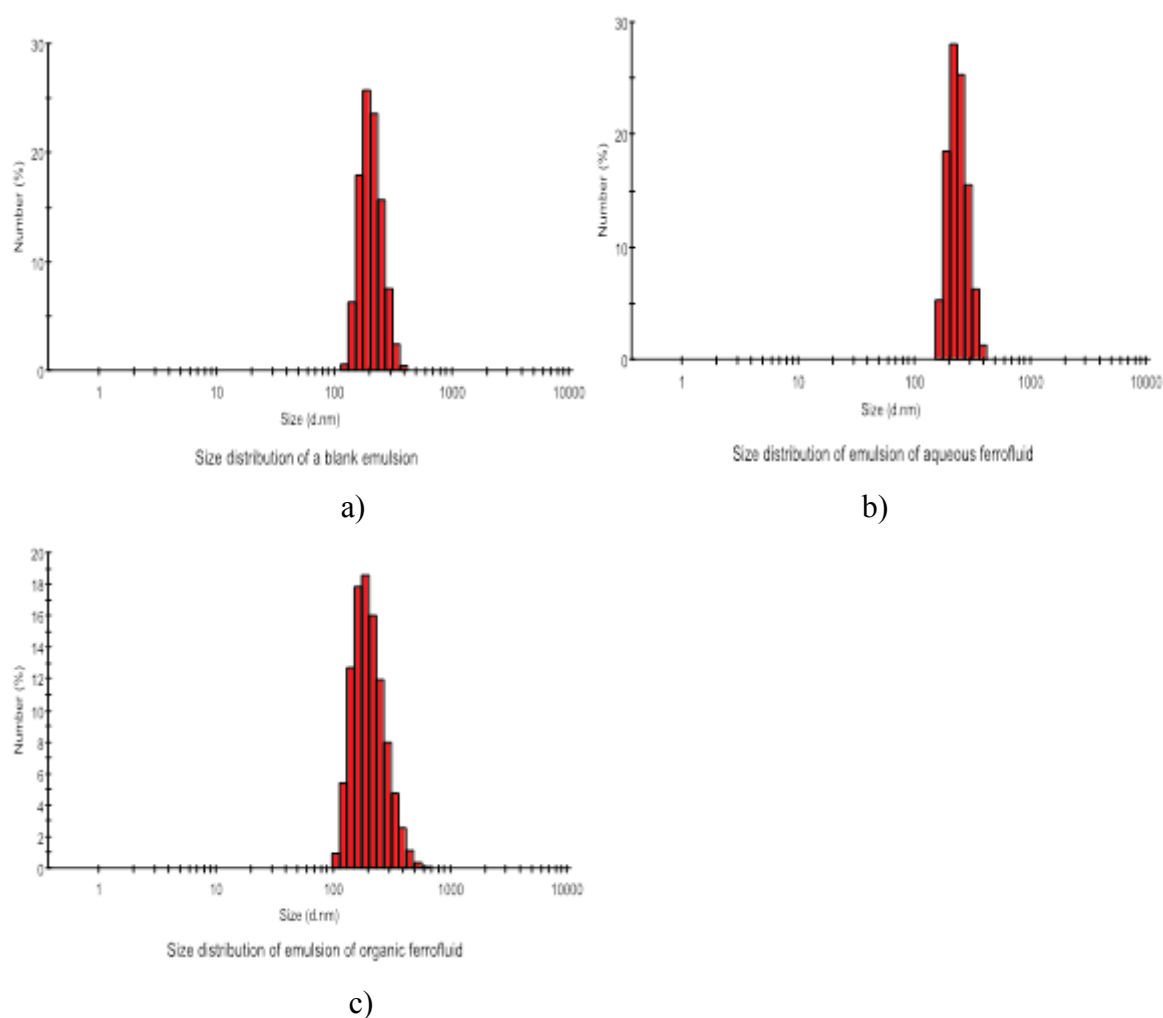


Figure: 2 Hydrodynamic diameter of nanoparticles a) blank b) loaded with aqueous ferrofluid and c) loaded with organic ferrofluid

In a similar way the hydrodynamic size and zeta potential of the emulsions prepared with or without loading of IONPs were measured using same Zetasizer. The size and zeta potential measurement was done using 1 mM NaCl solution. The particle size of blank was found to be 230-270 nm while in case of the emulsions loading with IONPs dispersed in both

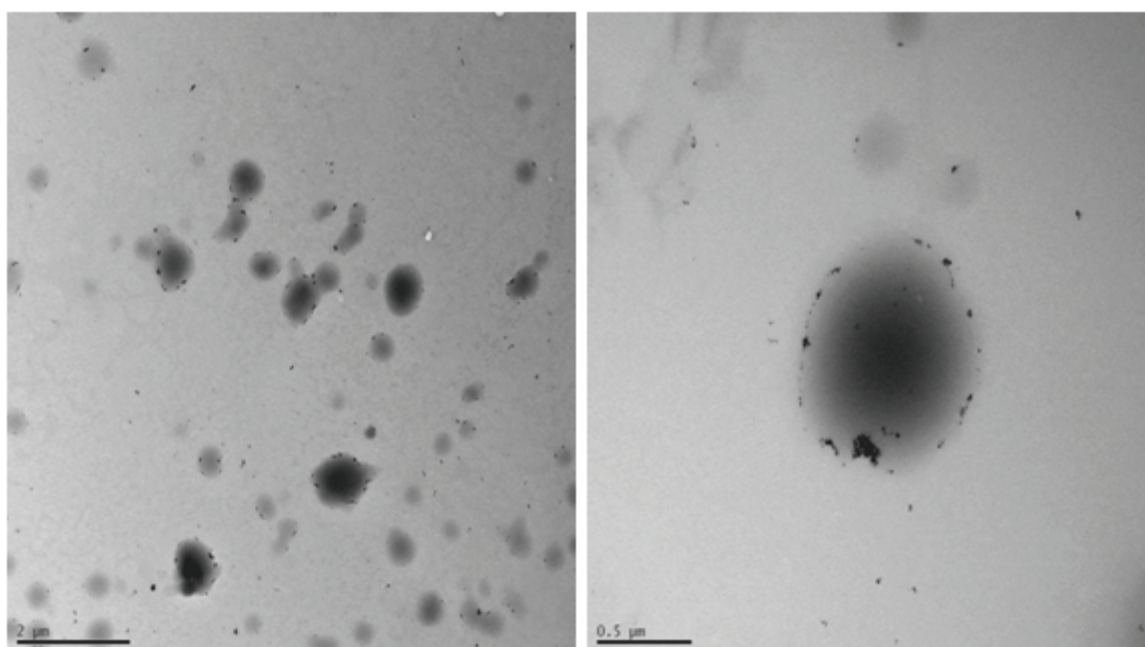
media were found in the range of 250-350 nm. It has been also found that the increasing the iron oxide contents had no specific effect on the final particle size of the emulsions. This may be because of the retention of the same volume introduced with differentiation of solid contents in both cases. In case of aqueous ferrofluids the IONPs were introduced in first aqueous phase and the volume remained constant in all cases to 1.5 ml with respect to 12 ml of the organic phase. Similarly in case of organic ferrofluid, various amounts of iron oxide particles were dissolved in fixed volume of oily phase and the rest of process worked in similar way. In short, the iron oxide contents have no important effect on final particle size of the emulsions obtained. This may occurs because the method limits a certain quantity of iron oxide loading and after that it does not work. Hence in this limit, an obvious result observed that iron oxide contents have no effect on size. Hydrodynamic size and size distribution of the emulsions is shown in above shown figure.

Zeta potential was also studied of the blank, aqueous ferrofluid and organic ferrofluid loaded emulsions. The zeta potential was found in negative range that may be result of the hydrolysis of polymer during emulsification. In a similar way, no big difference was found in case of zeta potential of the emulsions due to loading of iron oxide nanoparticles while a smaller effect is observed in case of aqueous ferrofluid loading in Polymeric particles. This effect results in the transfer of zeta potential values towards positive axis and it may attributed to the effect of nitric acid that has been used in the final preparation of aqueous ferrofluids and it has been well explained in recently published article [12].

Transmission electron microscopy (TEM) of IONPs/PCL particles

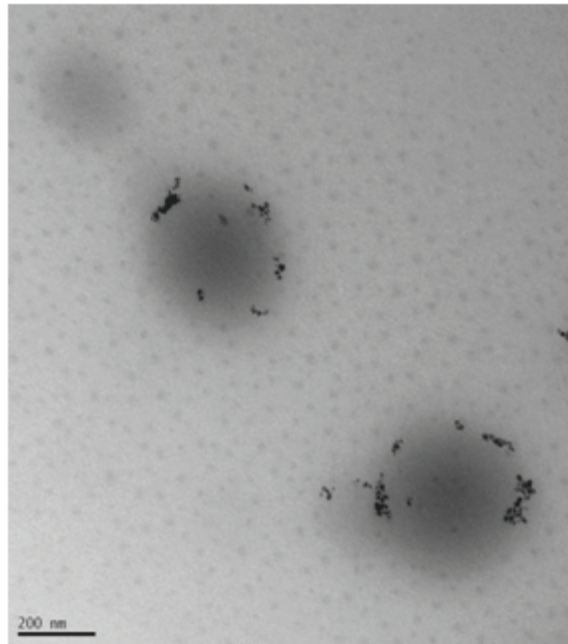
Transmission electron microscopy was done for the analysis of morphology of the obtained particles. The second aim was the observation of difference of morphology by modified double emulsion evaporation technique. The morphology of blank emulsions provided insight of the particles that found satisfactory. The emulsions loaded with aqueous ferrofluid were found also in expected size range of 250-350 nm. Nonetheless, other interesting aspects came into knowledge about the morphology and the mechanism of the encapsulation of double emulsion evaporation technique. It has been found that the iron oxide nanoparticles tried to escape from the internal aqueous phase to the external aqueous phase passing through oily phase. Figures shown below explain the nature of this passage and the block dots in the images correspond to iron oxide nanoparticles. The iron oxide nanoparticles soluble in water are coming out of the polymer matrix and hence found outside the polymer

matrix as well as inside and on surface of the polymer. The first hypothesis that can be described as the cause of this passage is high energy stirring during second emulsification. This energy may cause the rupture of some of particles formed during first emulsification step hence allowing the IONPs moving from interior aqueous phase to the exterior aqueous phase and result in the even distribution of the IONPs in emulsion. Secondly it can be proposed that the transfer of IONPs from interior to exterior is due to the unavailability of true oil in the oily phase as DCM is not true oil. Because of this unavailability of the true oily phase the technique is termed as modified double emulsion or it can be said as pseudo double emulsion. However these results are somehow expectable also because iron oxide nanoparticles are water soluble and at same time this can be considered as the proof for good solubility of the prepared iron oxide nanoparticles in water.



(a)

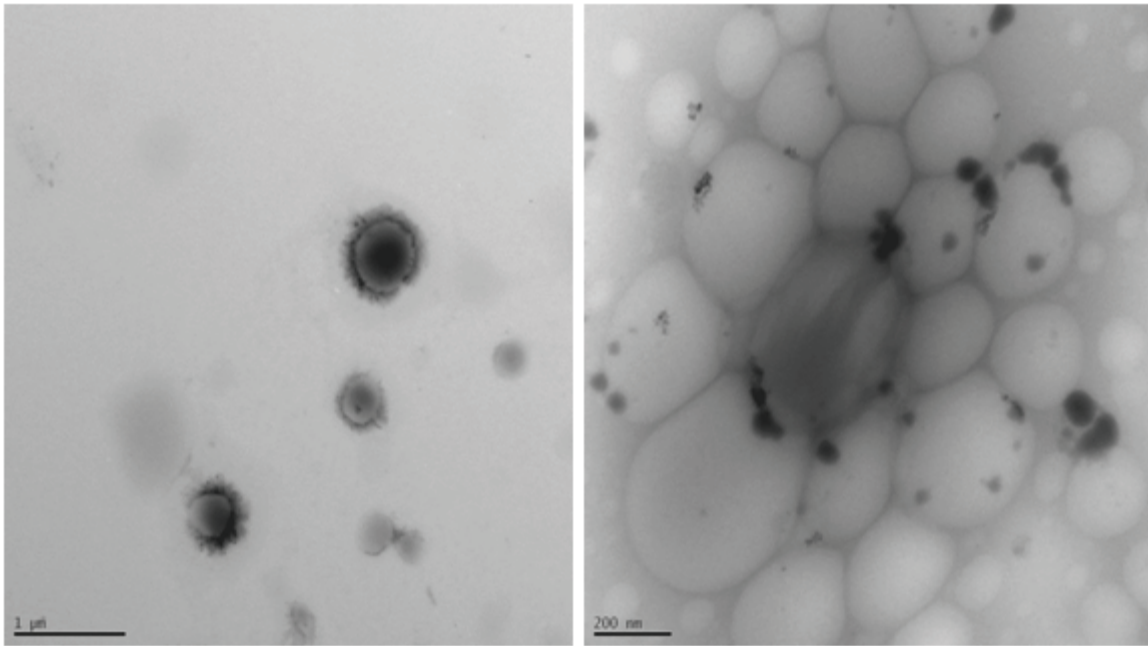
(b)



(c)

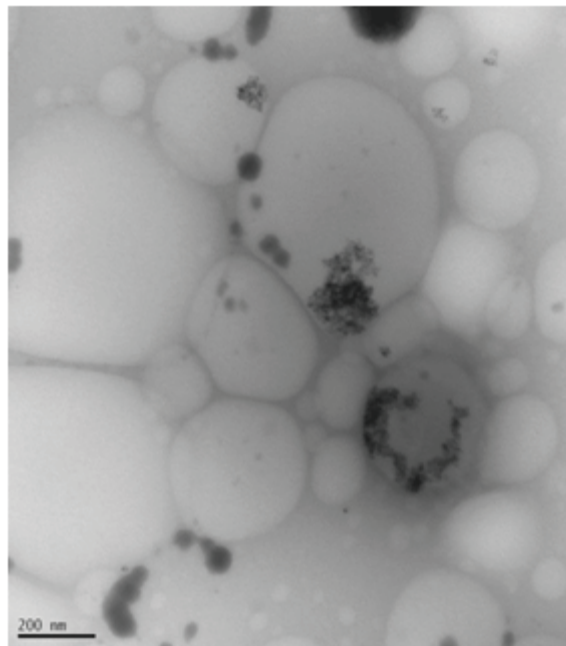
Figure: 3 TEM micrographs of nanoparticles loaded with iron oxide in aqueous medium

Encapsulation of organic ferrofluid provided somehow different results and different approach is observed. In this case the iron oxide nanoparticles were found limited inside the polymer matrix. Figures below explain the organic ferrofluid restrictions to the polymer. This happened because of the hydrophobic nature. Another important aspect that can be observed in these micrographs is the presence of black dots corresponding to iron oxide particles in one place inside polymer.



(a)

(b)



(c)

Figure: 4 TEM micrographs of nanoparticles loaded with iron oxide in organic medium

Simply the investigations in TEM helped to understand the morphology of both types of iron oxide inside the emulsion and their behavior with respect to the polymer. It described the difference between emulsions of two types of IONPs. Finally it also revealed those particles are in nanosize range although they are not 100 % spherical.

Thermogravimetric analysis (TGA)

The encapsulation efficiency and chemical composition can be well estimated by TGA. Thermogravimetric analysis was done on pure iron oxide particles and emulsified particles along with blank emulsions also to be used as reference. Interesting results were obtained which allowed calculation of the encapsulation efficiency. In case of aqueous ferrofluids, the encapsulation efficiency found to be 66% while in case of organic ferrofluids, its also compromising but somehow lower than that in case of aqueous ferrofluids. The results are explained well in last published article [12].

Magnetization

The magnetization study was done on all the particles including aqueous and organic ferrofluids and the emulsions loaded with these ferrofluids. It was to analyze the susceptibility of the particles when a magnetic field is applied. Results revealed the superparamagnetic characteristics of the particles and the nature of the ferrofluid did not affect the magnetic characteristic of the particles. However, the saturation magnetization values were also found near or above 2 showing that the amount of iron oxide loaded in particles is sufficient for the detection in Magnetic resonance imaging. Results are similar to that of earlier reported by same group [12]. The following figure containing graph explains the hysteresis curve of magnetization.

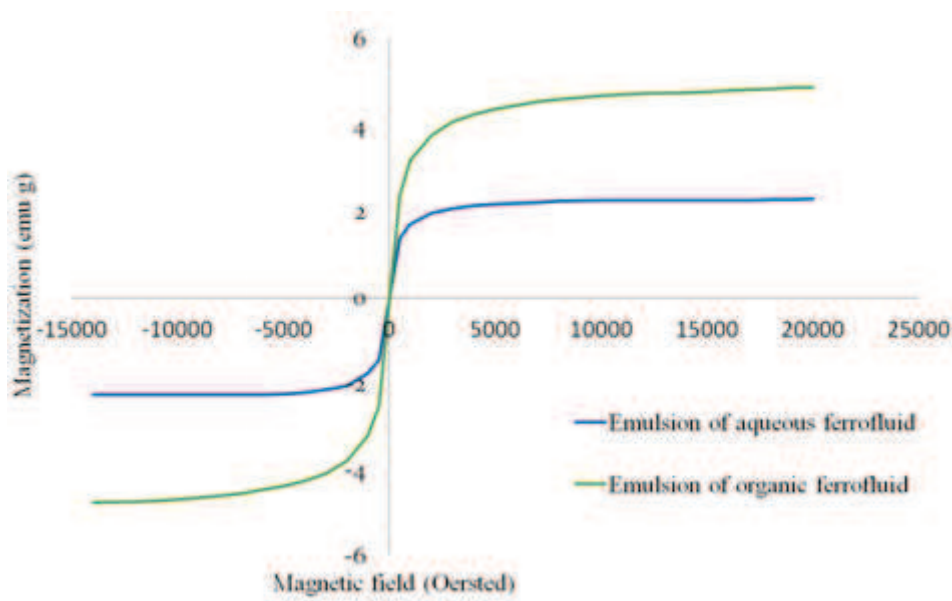


Figure: 5 Magnetization results for the nanoparticles loaded with aqueous and organic ferrofluids

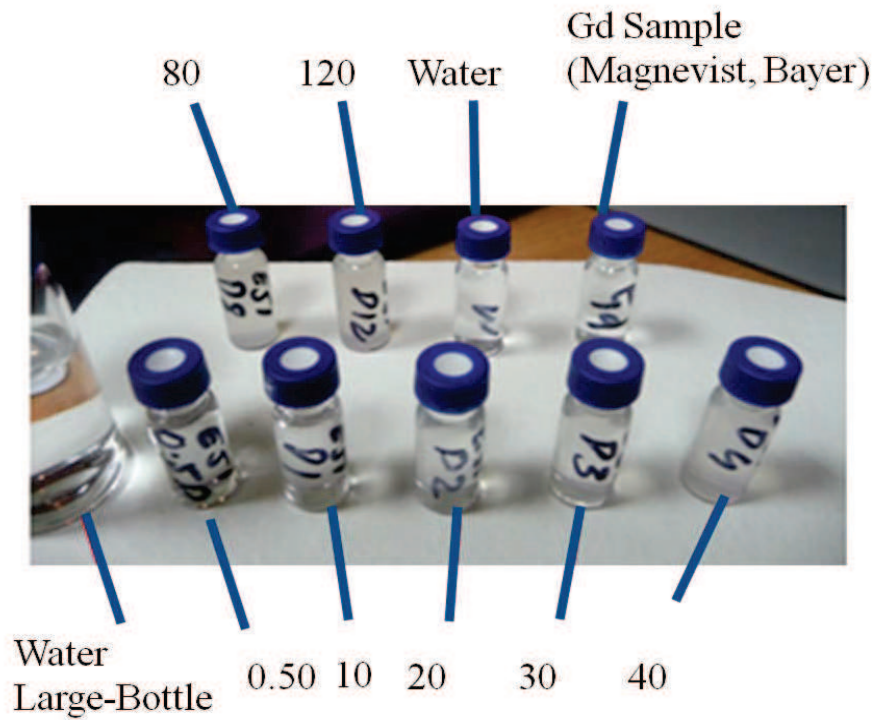
Magnetic resonance imaging (MRI)

The MRI characteristics of the prepared ferrofluids were investigated at various concentrations. For this purpose, initial experimental work involved acquiring MRI of one of the sample designated as E-51 (Preparation loaded with 75 mg of dried organic ferrofluid). Various concentrations of E-51 were tested in order to determine their contractibility via T_1 sequence and T_2 sequence. Table 1 summarizes the concentrations of E-51 employed for the MRI studies.

Table 1: E-51 Samples description and solution preparation.

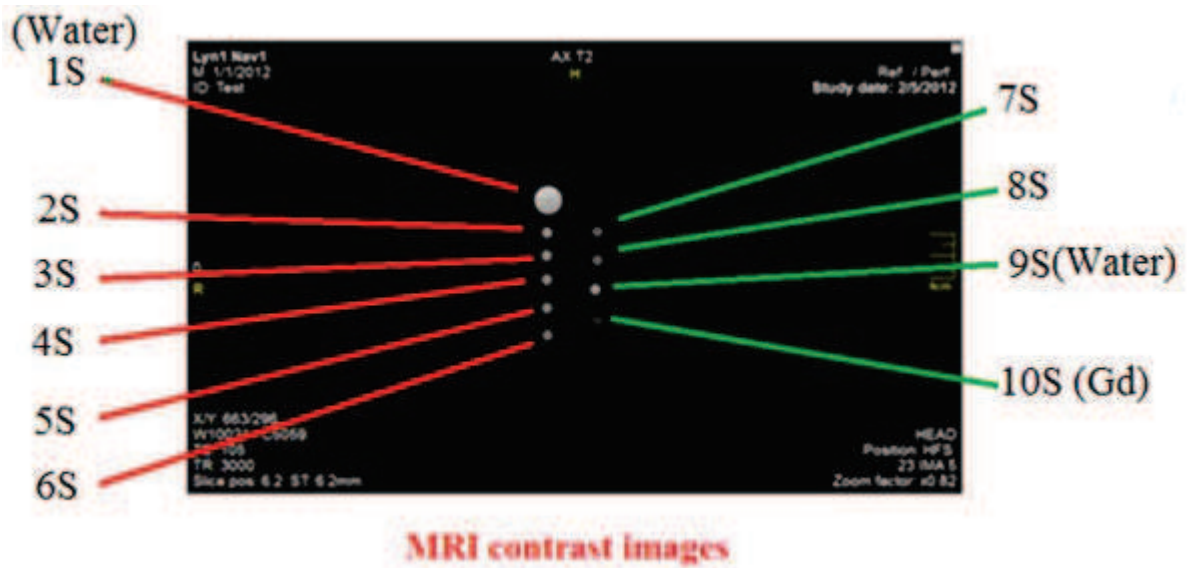
Sample Series	E-51 or Gd in 2.1106 g H ₂ O	Sample Series	E-51 or Gd in 2.1106 g H ₂ O
1S-W	10 g (only water in large vial)	7S-E-51 (8 D)	0.081 g
2S-E-51 (0.5D)	0.0053 g	8S-E-51 (12 D)	0.1223
3S-E-51 (1 D)	0.0136 g	9S-W	2.1106 g (only water in smaller vial)
4S-E-51 (2 D)	0.0210 g	10S-Gd Gd based	0.2 ml
5S-E-51 (3 D)	0.0310 g		
6S-E-51 (4 D)	0.0501 g		

Figure 6 (i) and (ii) respectively represent the actual placement of samples inside the MRI machine, and their MRI images. All concentrations are designated in Figure (6) for clarity. A relatively large samples vial containing only water and smaller sample vial containing only dilute solution of Gd were also employed in order to compare their contrasting capability with the ferrofluid. The MRI images taken for both the T_1 and T_2 sequences are presented in Figure 7 (i) and 7 (ii), respectively.



Samples Placement in MRI Machine

(i)



(ii)

Figure: 6 (i) The actual placement of samples inside the MRI machine, and (ii) their MRI images in T2 sequence. See Table 1 for sample information.

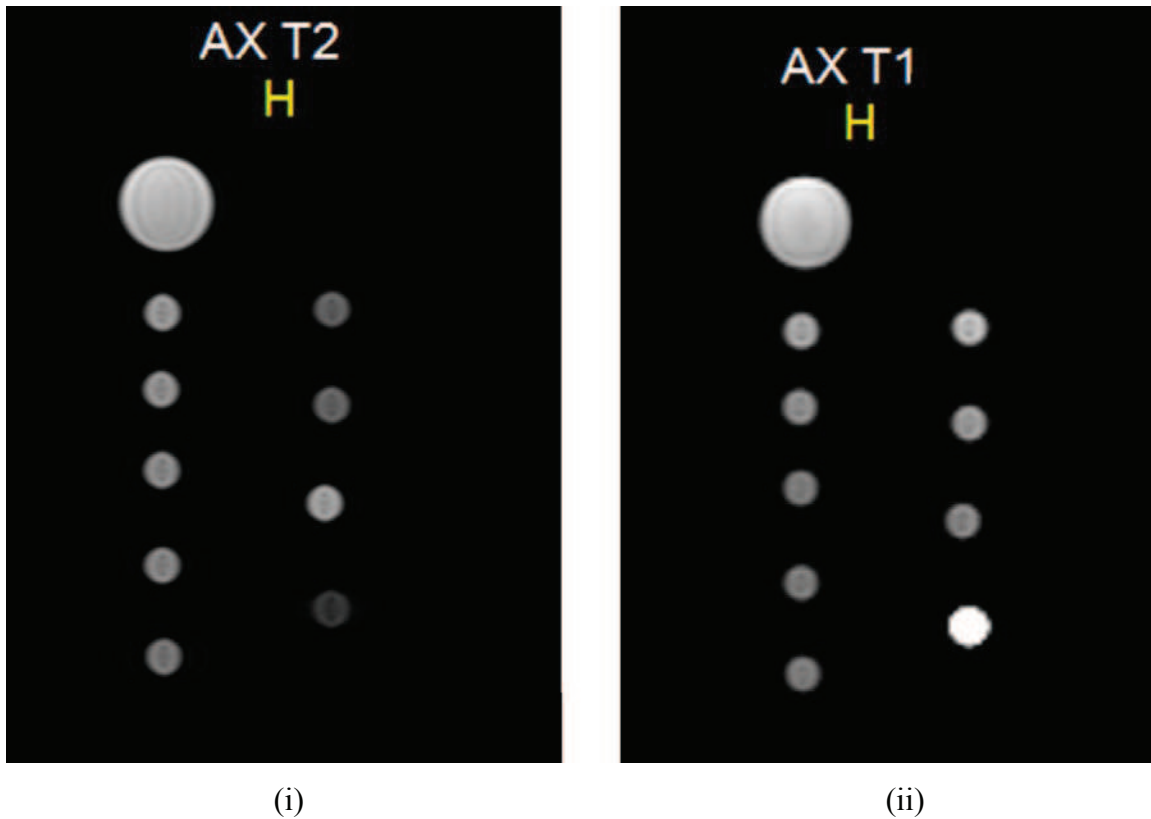


Figure: 7 T_1 and T_2 weighted sequences MRI images of the ferrolfluids E-51 at different concentrations. For comparison purpose MRI of water (small and large vials) and Gd are also presented. See Figure 6 for designation of various MRI contrast images.

The average intensity data was obtained from the MR images and mean intensity within ROI in intensities of pixels was used. Figure 8 represents the comparison of average intensity of various samples in case of T_2 and T_1 MRI images. There are several interesting observations evident from the T_1 and T_2 MRI images and Figure 8. For examples, it is clearly observed that Gd is a better positive T_1 contrast agent, while E-51 based on ferrolfluids is a better negative T_2 contrast agent, and variation of concentration do not have significant influence in the variations of T_1 intensity. In addition, with increase in the concentrations of the E-51 in the water sample, the T_2 negative contrastability of E-51 increased with the increase in concentration of ferrolfluids and thus in good agreement with the reported literature [14-16]. Furthermore, Gd exhibited more positive contrast in T_1 relative to T_2 , which is again in good agreement with already established MRI facts.

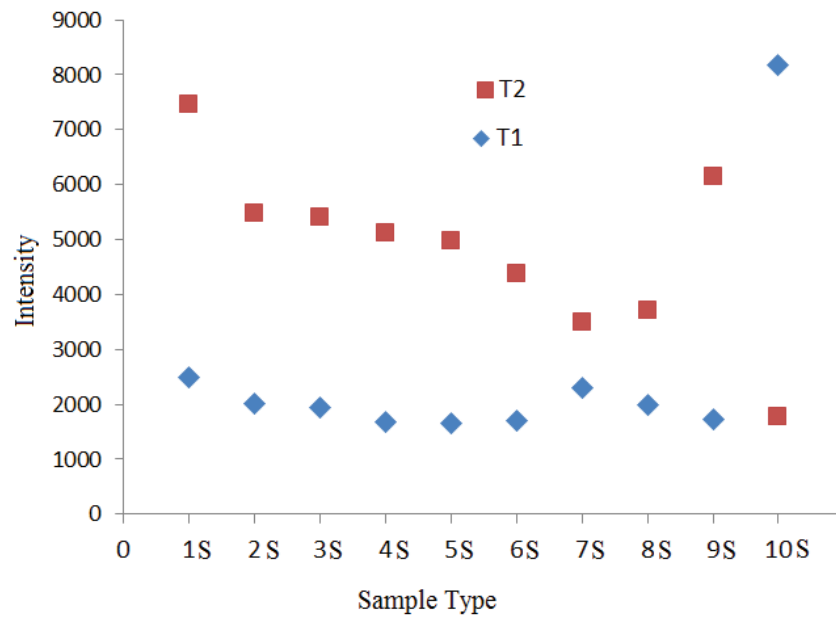


Figure: 8 T_2 ((TE105); TR3000) and T_1 (TE=12; TR: 540) intensity measurements for various samples of E-51, water and Gd (See Table 1 for Sample information). Intensity data obtained from MR images and mean intensity within ROI in intensities of pixels was used.

In view of the interesting results of the T_2 and T_1 for the samples of E-51 and its comparison with water and Gd, further work was carried out to analyze additional ferrofluids samples to understand their specific characteristics relevant to their MRI contrast ability. For this purpose, various concentrations of samples were prepared in water as summarized in Table 2.

Table 2: Concentration of solutions of Gd and prepared MNP employed for MRI. All concentrations are in 10 ml water. Concentrations are given in separate tables in series format, as these were placed inside the MRI machine as such.

Sample Name	Nature of Sample	Concentrations used	
		Conc. 1	Conc. 2
Gd	Commercialized Gadolinium sample from Gadovist®	0.20 ml	0.40 ml
		0.60 ml	0.80 ml
		0.0210 g	0.0530 g
		0.0983 g	0.2014 g
E 46	Preparation loaded with 48 mg of dried organic ferrofluid	0.0197 g	0.0439 g
		0.0995 g	0.2096 g
		0.0122 g	0.0418 g
		0.1069 g	0.2027 g
E 23	Preparation loaded with 250 mg of dried organic ferrofluid	0.0123 g	0.0511 g
		0.1053 g	0.1945 g
		0.0124 g	0.0501 g
		0.1213 g	0.2095 g
E 8	Preparation loaded with aqueous ferrofluid having 9 % solid contents of iron oxide	0.0136 g	0.0575 g
		0.1218 g	0.2050 g
		0.0190 g	0.0562 g
		0.1053 g	0.2155 g
EXP 10	Aqueous ferrofluid with 1.58 % solid contents of iron oxide	0.0175 g	0.0636 g
		0.1055 g	0.2013 g
		0.0172 g	0.0451 g
		0.1092 g	0.1923 g
E 4	Preparation loaded with aqueous ferrofluid having 5 % solid contents of iron oxide	No iron oxide was added	
		No iron oxide was added	
		No iron oxide was added	
		No iron oxide was added	
E 51	Preparation loaded with 75 mg of dried organic ferrofluid	Aqueous ferrofluid with 1 % solid contents of iron oxide	
		Aqueous ferrofluid with 1 % solid contents of iron oxide	
		Aqueous ferrofluid with 1 % solid contents of iron oxide	
		Aqueous ferrofluid with 1 % solid contents of iron oxide	
Blank 2	No iron oxide was added	No iron oxide was added	
		No iron oxide was added	
		No iron oxide was added	
		No iron oxide was added	
EXP 2	Aqueous ferrofluid with 1 % solid contents of iron oxide	No iron oxide was added	
		No iron oxide was added	
		No iron oxide was added	
		No iron oxide was added	
Blank 1	No iron oxide was added	No iron oxide was added	
		No iron oxide was added	
		No iron oxide was added	
		No iron oxide was added	

Figures 9-10 represent the representative magnetic resonance imaging (MRI) characteristics of MNPs. The various concentrations of the sample were used and the graphs were made on the basis of ROI intensity acquired. The mean intensity was drawn against TE values used in the case of T₂ sequence. There are several important observations evident from the figures. The T₂ weighted intensity of water was affected significantly by the MNPs relative to the control Gd sample and water samples. As the MNP concentration was increased

in the aqueous solution, in general the signal intensity decreased in agreement with reported results [14-16]. This indicates the good potential of the developed magnetic materials as negative contrast agents. It is also noted from the MRI images that the top most concentration (Conc. 4) and bottom concentration (Conc. 1) are not clearly visible as compare to the sample concentrations (Conc. 2 and Conc. 3) placed in the middle. This anomalous observation is attributed to the sample vials for Conc, 1 and Conc. 4, which were placed not exactly in the range of the effect of the MRI coil. Due to this reason, their MRI contrastability cannot be correctly attributed to their concentrations and characteristics. Keeping in view the above mentioned problems, focus of the discussion has been on the data recorded from the middle concentrations, i.e., Conc. 2 and Conc. 3. For the sake of simplicity and clarification, hence the MRI contrast and intensity for the Conc. 1 and Conc. 4 have been ignored in the current discussion.

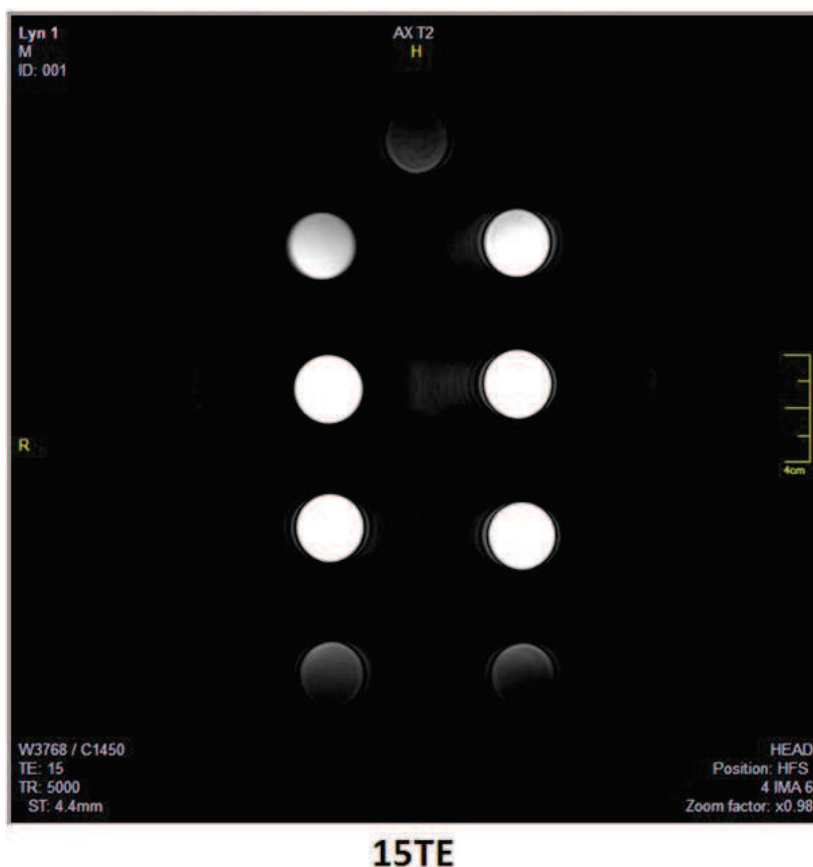


Figure: 9 T₂ image of Gd and hybrid particles loaded with organic ferrofluids (E-46) with TE: 15 and TR: 5000.

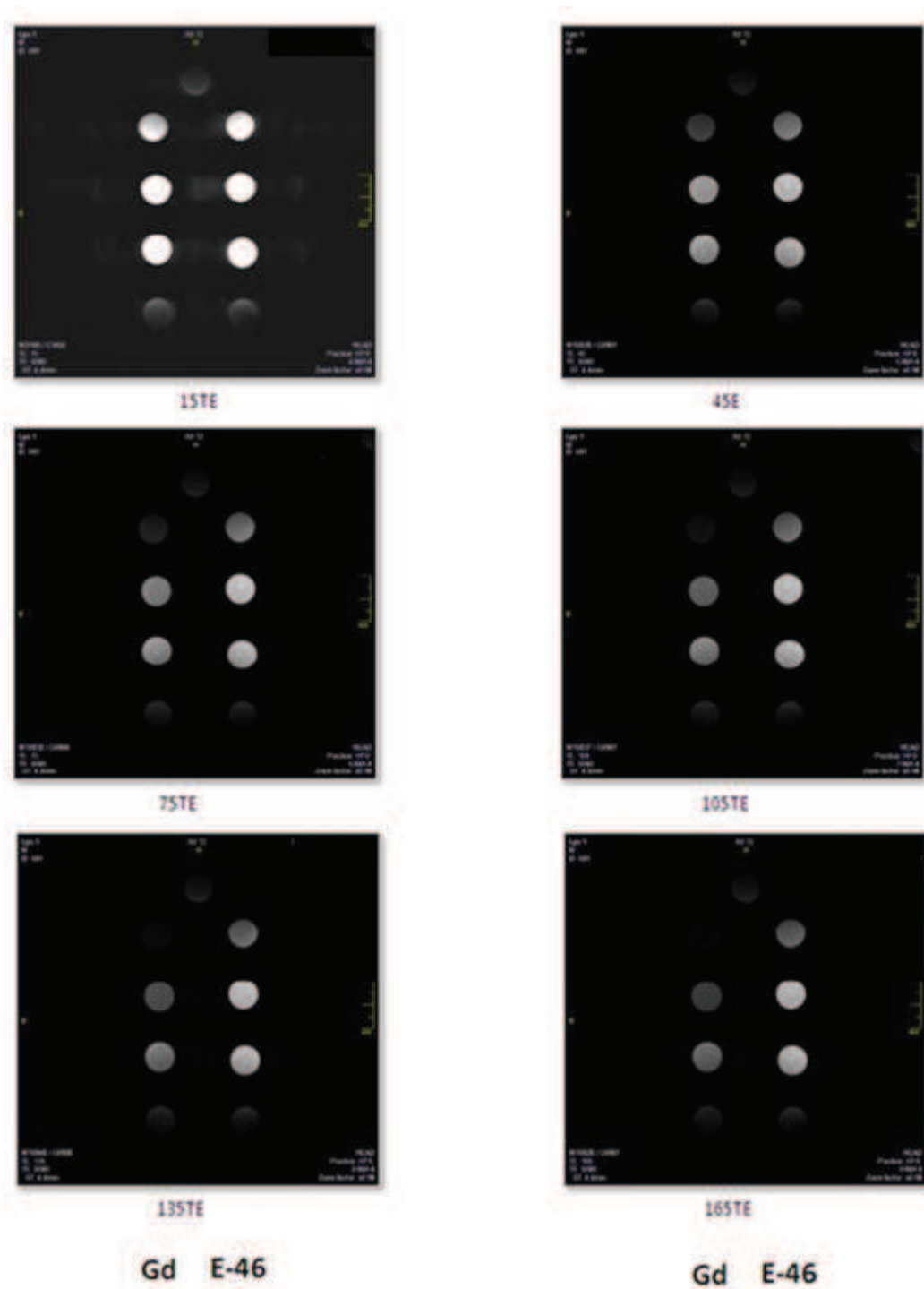


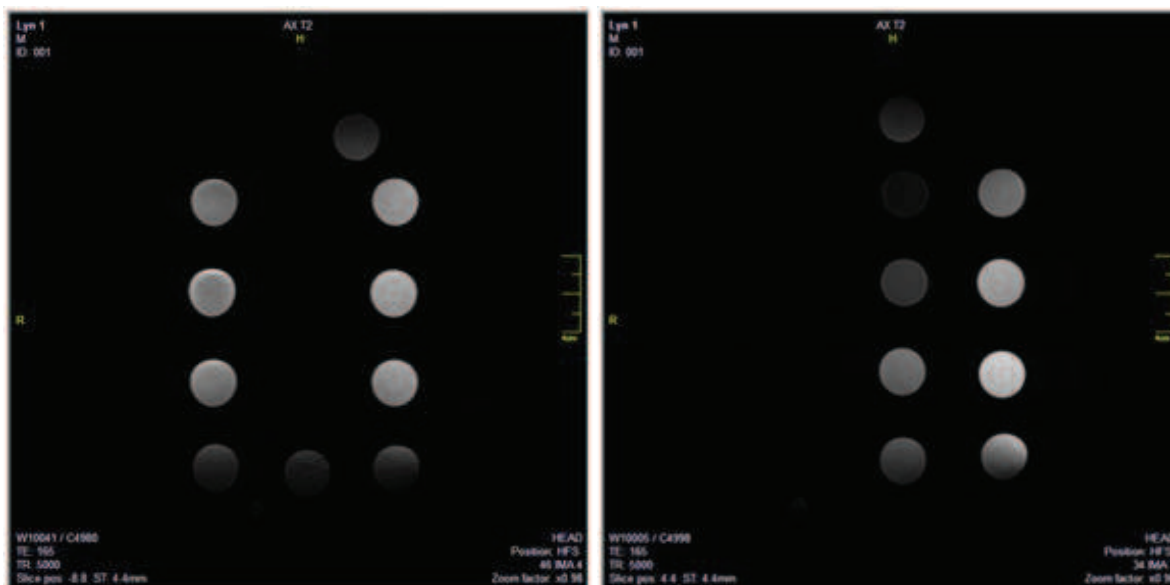
Figure: 10 T_2 -weighted images for Gd and hybrid particles loaded with organic ferrofluids (E-46) at various TE values and at TR: 5000. MRI images were acquired at different concentrations: starting from lower part of the MR image with sequence of Conc. 1, Conc.2, Conc. 3, and Conc.4. (See Table 2 for further details)

Figure 10 indicates that MRI intensity decrease with TE values. In addition, increase in concentration of MNP is generating higher negative contrast. Both of these observations

are in good agreement with literature [17]. In addition, this observation also suggests the enhancement of T_2 relaxation rate with concentration. Since, the T_2 relaxation process occurs because of the exchange of energy between protons in water molecules. In the presence of an externally applied magnetic field, MNP nanoparticles create inhomogeneity in the magnetic field affecting the microenvironment that results in dephasing of the magnetic moments of protons and hence T_2 shortening. The higher T_2 intensity values of prepared MNPs relative to Gd suggests a better contrast property of these formulations in the case of T_2 sequence, and could be more sensitive as an MRI contrast agents (CAs). This might be attributed to the ability of MNPs to induce more local inhomogeneity in the magnetic field than Gd. At high magnetic field, the local increase in magnetic field is related to the saturation magnetization of the superparamagnetic contrast agents. Greater local magnetic field inhomogeneity creates more contrast and hence greater T_2 relaxivity. It appears that combination with poly caprolactone (PCL) in these formulation (PCL coating) cause the increase in hydrophilic character that allows closer proximity of the contrast agent to water molecules, leading to shortening of the spin-lattice relaxation time. This has been observed that coatings types and thickness have pronounced effect on the contrast of the MNPs. For example, certain coatings of hydrophobic polymer can causes formulation to be less hydrophilic than dextran-coated based, and hence has a reduced hydration effect, thus causing reduced proximity of water molecules to the iron-oxide core of MNPs. [17-21].

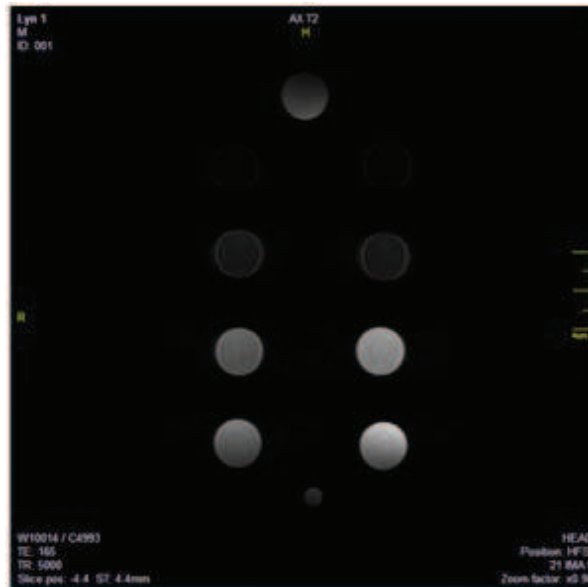
As in the case of E-46, T_2 -weighted images of the remaining samples were also acquired and representative MRI images are presented in Figure 11 From the acquired MRI images, intensity (T_2 Sequence) variation with TE for Gd and prepared ferrofluids in water at different concentrations (data as mean intensity within ROI in intensities of pixels) were also estimated. The results of the average intensity are shown in Figure 12 for various samples. There are important observations evident form the MRI images and intensity values. For example, the enhancement of contrast in T_2 sequence was observed in Figure 11 (i) and (ii) by comparing the Blank1 and Blank 2 with the EXP 10 and EXP 2. It should be noted that Blank 1 and Blank 2 are the emulsions that contain no iron oxide amount, while the EXP 2 and EXP 10 are the pure iron oxide nanoparticles in aqueous media (aqueous ferrofluids) and even E-4 and E-51 are different also in their contents. The details are also mentioned in Table 2. The Blank 1 and Blank 2 samples were observed as bright or semi bright, while the samples rich with iron oxide contents shown purely black contrast and the pixel boundaries are impossible to define. The higher iron contents are responsible for the enhancement of contrast. The

difference among different circles in this image is due to the various concentrations of samples used for the MRI. In a similar way, a comparison is possible for the preparations loaded with iron oxide contents in organic media. Taking the example of E-23 and E-46, Table 2 mentions the difference of the iron oxide contents in both samples. E-23 is highly enriched with iron oxide nanoparticles as compare to that of E-46 with 1:5 ratio exists. Hence considering different concentrations of E 23, the enhancement of contrast is visible even in case of lowest concentration used. A similar observation is noted for other samples containing Fe_2O_3 such as E-46, E-23 and E-8. This observation is also evident from the Intensity vs. TE graphs shown in Figure 12 (ii), (iii) and (iv). If one compares the intensity variation with TE for E 46 and E 23 in case of concentration 3, then it is evident that at similar TE, E-23 exhibit significantly higher negative contrast relative to E-46 because of the presence of higher iron oxide contents. Thus the relatively higher Fe_2O_3 concentrations generate more negative contrast with lower intensity values, which can be again attributed to the presence of higher values of iron oxide in the prepared samples. Similar results are observed in the case of E-8 samples (see Figure 12 (iii)), where higher negative contrast are noted at higher concentration values. Furthermore, it is difficult to directly compare E-8 (aqueous ferrofluids) with E-46 or E-23 (organic ferrofluids) because of the differences in terms of iron contents as well as different nature of these colloids.



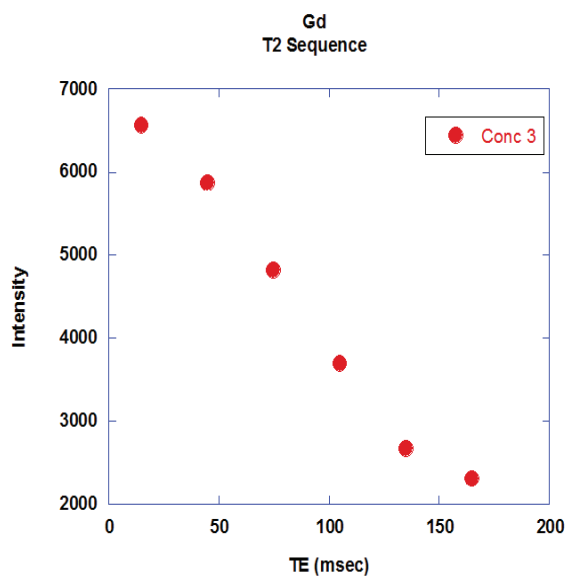
(i) T_2 : (Blank 2; Exp 2; Blank1)

(ii) T_2 for (Exp 10; E-4; E-51)

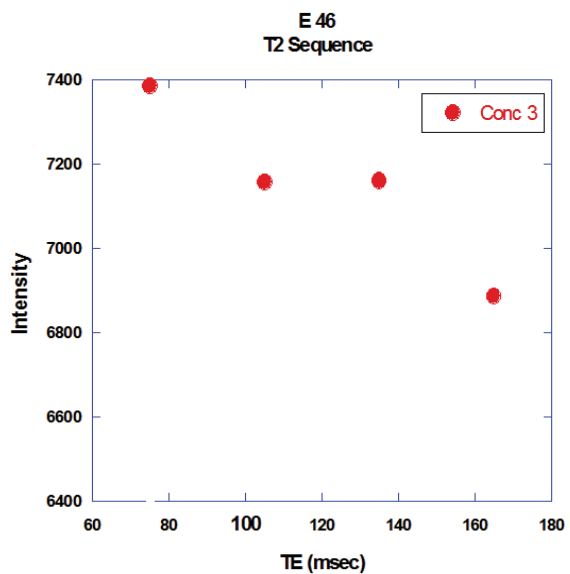


(iii) T₂ Images (E-23; E-8)

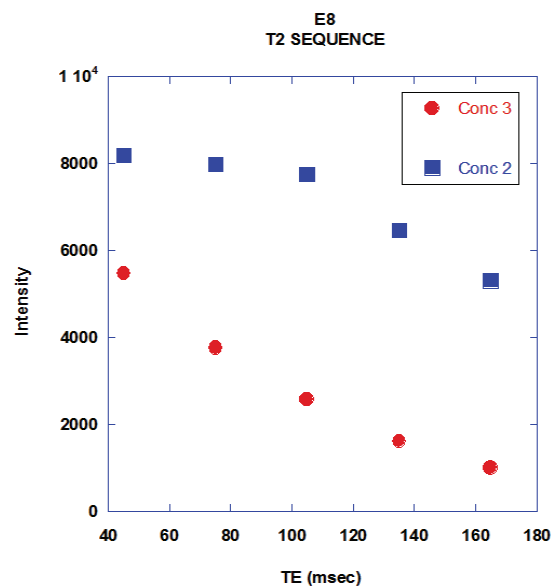
Figure: 11 T₂ magnetic resonance imaging (MRI) properties of various MNPs recorded in water at various concentrations at 25 °C; i) T₂ contrast for blank emulsion (Blank2) aqueous ferrofluid (Exp2) and another blank (Blank1) ii) T₂ for aqueous ferrofluids (Exp 10) hybrid particles loaded with aqueous (E-4) and organic ferrofluids (E-51) iii) T₂ contrast images for nanoparticles loaded with organic (E-23) and aqueous ferrofluids (E-8)



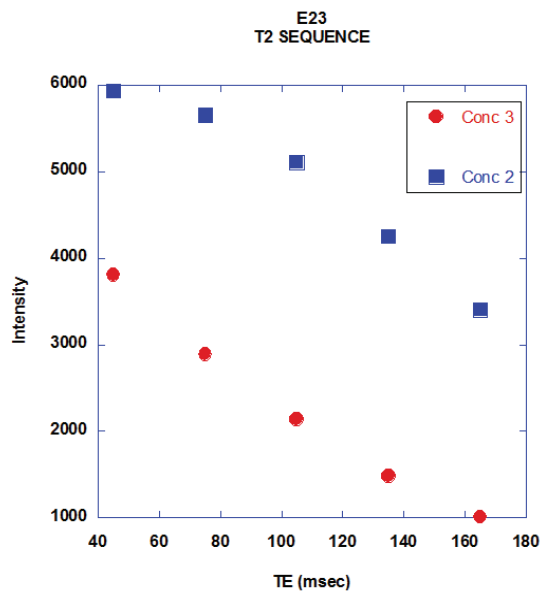
(i)



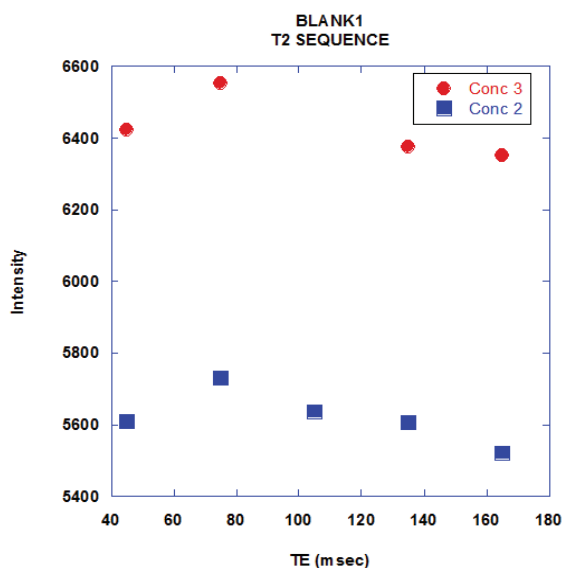
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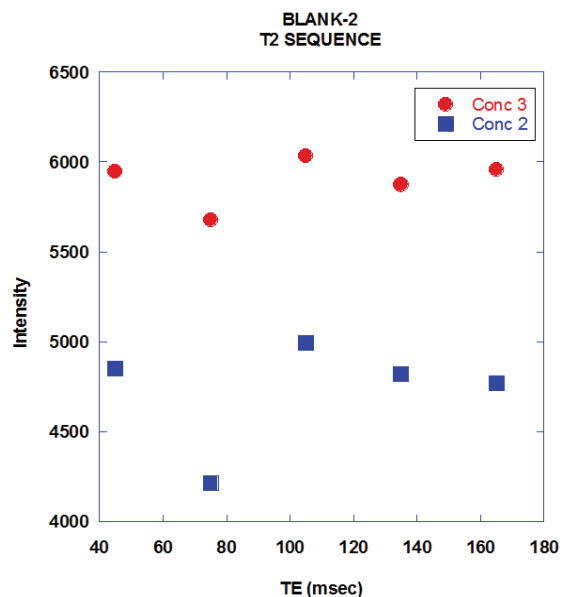
(iii)



(iv)



(v)



(vi)

Figure: 12 Intensity variations with TE for different concentrations of Gd (T_2 Sequence) in water at different concentrations (data as mean intensity within ROI in intensities of pixels). T_2 relaxation analysis curves of Gd and MNPs in water at different TE and at different concentrations (data obtained from MR images and mean intensity within ROI in intensities of pixels was used). The Sample IDs are given at the top of each graph and are explained in the Table 1 in detail.

Although major focus of the present work was to investigate the imaging capability of the prepared ferrofluid in terms of T_2 enhancement of contrast since these are commonly known for this purpose. Nonetheless, recently there is interesting work reported regarding the

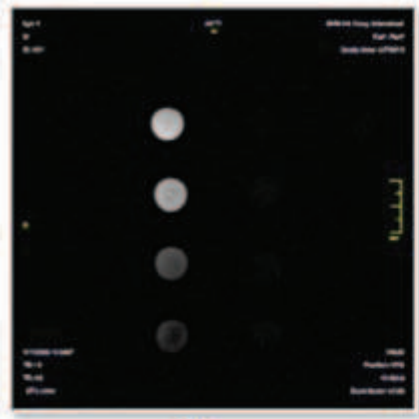
T_1 contrastability based on Fe_2O_3 [22-23] and therefore we have also tested the prepared ferrofluids for their T_1 characteristics. The presence of paramagnetic ions near the tissue shortens the T_1 relaxation time. Especially the transition and lanthanide metal ions with a large number of unpaired electrons of the type of Gd^{3+} , Fe^{3+} , and Mn^{2+} can show effective relaxation. The signal-increasing imaging effect is related to T_1 contrast agents and enhanced T_1 relaxation. The major advantage of T_1 contrast agents as compare to T_2 is positive imaging by signal enhancing. This can maximize the anatomic imaging with high spatial resolution. Their bright signal can be distinguished clearly from other pathogenic or biological conditions.

Over the last 20 years, most nanoparticulate contrast agents have been T_2 contrast agents using iron oxide nanoparticles. However these magnetic nanoparticle-based agents have several disadvantages which give a signal-decreasing effect and limit their extensive clinical applications since these are negative imaging agents. The resulting dark signal could be confused with other pathogenic conditions, and renders images of lower contrast than T_1 contrasted images. Because of such and other reasons, the most extensively and clinically used MRI contrast agents are based on Gd complex based T_1 agents rather than Fe_2O_3 nanoparticles. However, due to the toxicity of heavy metal ions, the conventional contrast agents are in the form of ionic complexes with chelating ligands, which are thermodynamically and kinetically stable and less toxic. There is, however, no biochemistry based on gadolinium (III) ion in natural humans. In spite of their fewer unpaired electrons and lower magnetic moments, manganese (II), iron (III), and copper (II) ions could be alternative candidates.

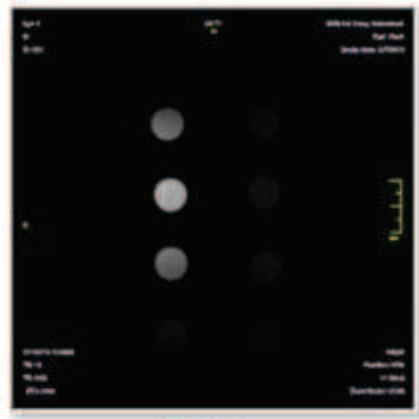
Recently, intensive research has been devoted to developing new T_1 contrast agents that overcome the above-mentioned drawbacks of Gd^{3+} ion- and metal ion-based T_1 contrast agents and SPIO-based T_2 contrast agents. For example, a recent study involved systematic development of a potential MR T_1 contrast agent based on very small PEGylated iron oxide nanoparticles [23]. The results suggest that to form a T_1 contrast agent out of iron oxide a core size of approximately 5 nm has to be used. Adjustment of the size of the crystalline core provided suitable relaxometric properties. The developed contrast agent exhibited the lowest r_2/r_1 ratio (2.4) at 1.41 T reported so far for PEGylated iron oxide nanoparticles as well as a r_1 relaxivity ($7.3 \text{ mM}^{-1} \text{ s}^{-1}$) that is two times higher compared to that of Magnevist as a typical T_1 contrast agent based on gadolinium as a clinical standard.

In view of above discussion and continue research interest, the prepared ferrofluids were also employed to investigate for their T_1 weighted images. Figures 13 and 14 represent the representative MR images and intensity variation with TR. T_1 relaxation process also requires close proximity of the hydrogen atoms to the contrast agent. In the present formulations, coatings of PCL seems to decrease the T_1 relaxivity of MNPs, perhaps because the nature of the coatings effecting the hydrophilicity of MNPs. Thus, It appears that coating used in prepared MNPs, have influenced the imaging capabilities as it is known that more hydrophilic coatings allows closer proximity of the contrast agent to water molecules, leading to shortening of the spin-lattice relaxation time. If coating contains hydrophilic or hydrophobic chains, then such specific coatings can causes an effect on degree of hydration and hence influence the MRI imaging capability of the MNP.

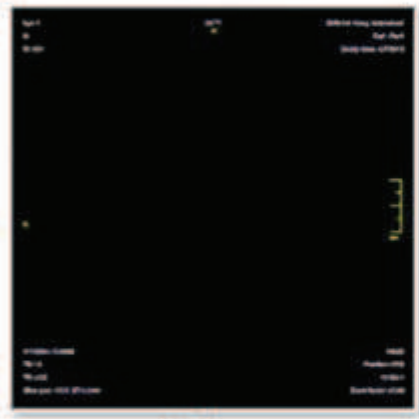
From the comparison of MRI images of Gd with that of MNPs formulation in the figures given below, it can be noticed the distinct capabilities of the MNP to generate contrast and thus can be discussed with the aid of reported literature [21-23]. Since, the paramagnetic CAs of the complexes of gadolinium has the predominant effect at low doses in T_1 shortening (and R1 enhancement). Thus, organs taking up such agents will become bright (positive agent) in a T_1 -weighted MRI sequence. On the other hand, superparamagnetic SPION influence signal intensity mainly by shortening the relaxation times to produce the darkening (negative agent) of the contrast-enhanced tissue [21]. From the MRI images given in Figure 13 (i) and (ii), it is evident that Gd exhibited positive contrastability in case of T_1 sequence. In the case of iron oxide colloids, T_1 weighed images of the prepared ferrofluids suggest some interesting observations. Furthermore, representation of the intensity vs. TR suggests the enhancement of signal in the case of Gd relative to E 46 sample tto suggest the higher positive contrast-ability of Gd. Figure 14 also represent the T_1 images of the prepared ferrofluids. Because of the different sizes, charges, amount of Fe_2O_3 , it is not possible to discuss the comparative T_1 contrastability of the prepared ferrofluid. However, it seems that in general, the investigated concentrations of the sample do not seem to significantly influence the degree of contrast. Furthermore, it is observed that a sufficient amount of Fe_2O_3 is required to exhibit a certain extent of MRI contrast. For example, as compare to Exp 2, Blank 1 and Blank 2 do not show bright contrast. Blank 1 and Blank 2 do not contain any Fe_2O_3 and thus it is possible that presence of Fe_2O_3 have influenced the T_1 contrastability in the investigated samples.



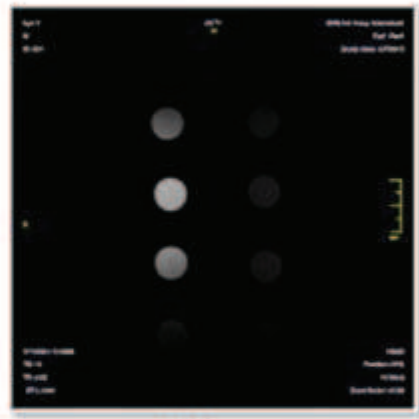
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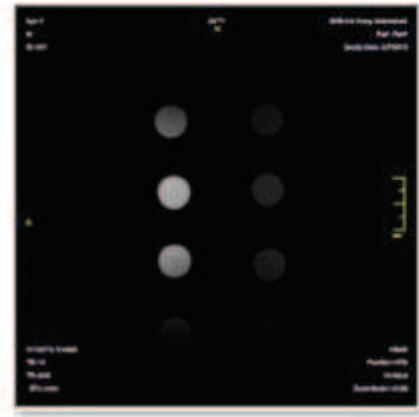
225TR



425TR

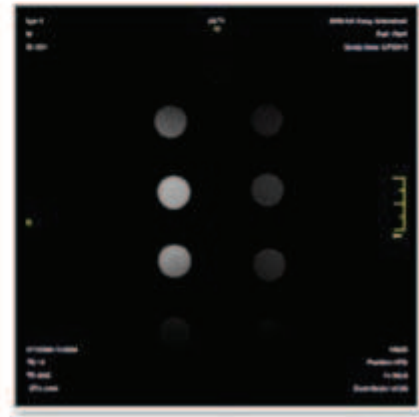


425TR



625TR

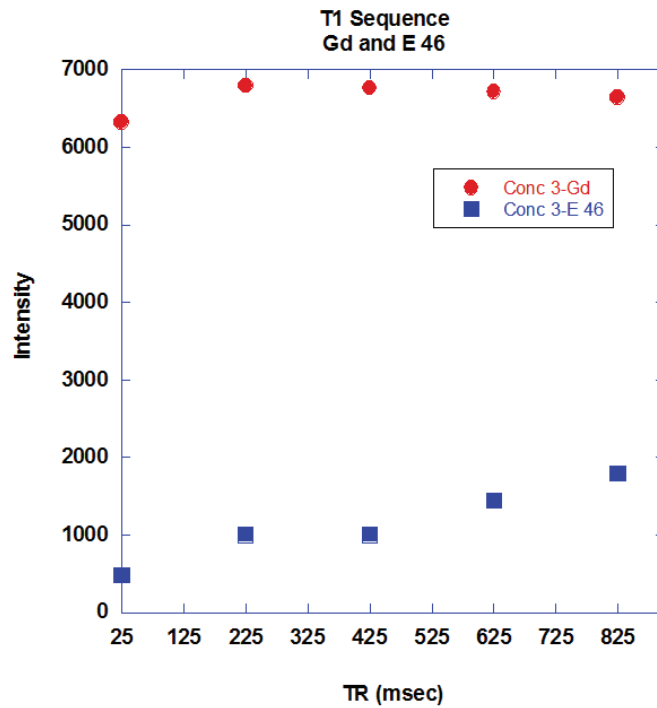
Gd E-46



825TR

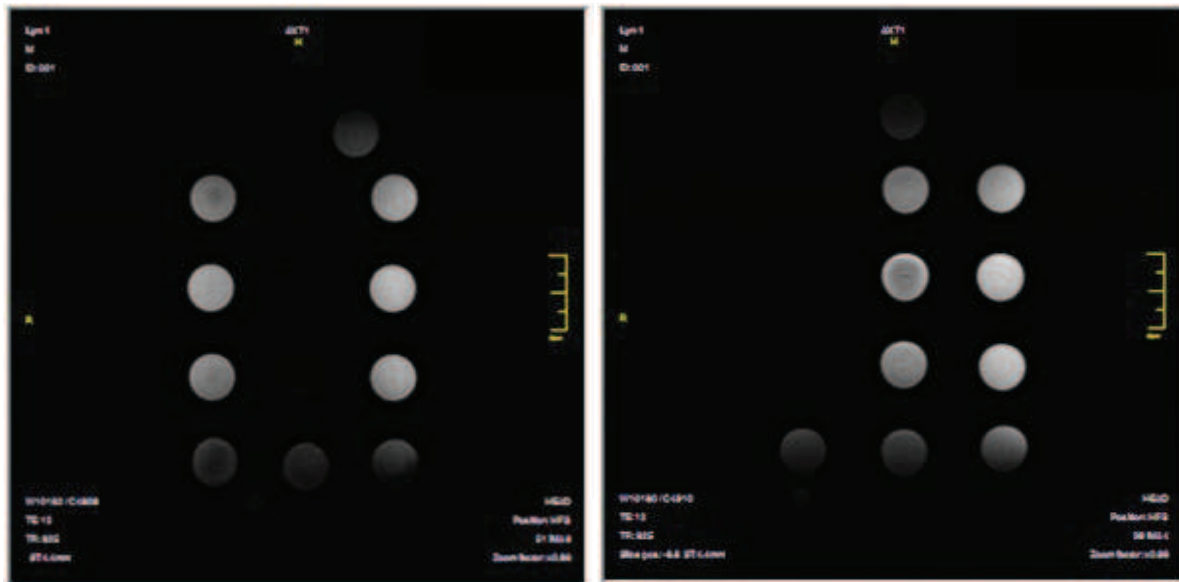
Gd E-46

(i)



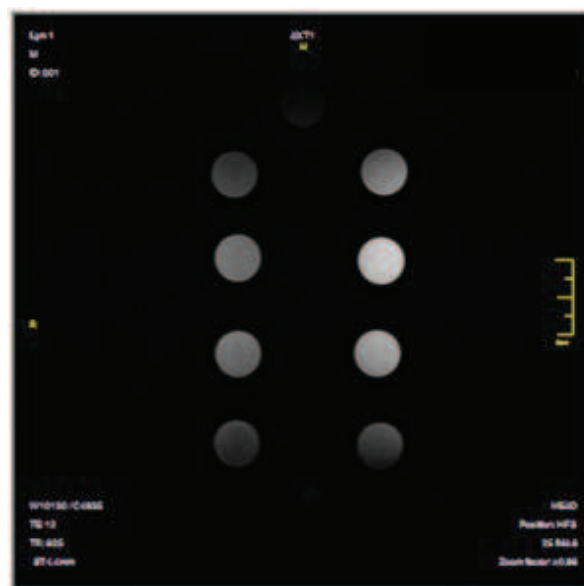
(ii)

Figure 13: Magnetic resonance imaging properties of MNPs (E-46): (i) T_1 weighted images for Gd and hybrid particles loaded with organic ferrofluids (E-46) and (ii) T_1 relaxation analysis curves of MNPs in water at different TR and at different iron concentrations (data as mean intensity within ROI in intensities of pixels).



(i) T_1 (Blank2-Exp2-Blank1)

(ii) T_1 for for (Exp 10), (E-4) and (E-51)



(iii) T₁ Images E-23 and E-8

Figure 14: T₁ magnetic resonance imaging properties of various MNPs recorded in water at various concentrations at 25 °C; i) T₁ contrast for blank emulsion (Blank2) aqueous ferrofluid (Exp2) and another blank (Blank1) ii) T₁ for aqueous ferrofluids (Exp 10) hybrid particles loaded with aqueous (E-4) and organic ferrofluids (E-51) iii) T₁ contrast images for nanoparticles loaded with organic (E-23) and aqueous ferrofluids (E-8).

The MRI images and intensity data of the prepared ferrofluids suggest that these can be helpful to develop more efficient contrast agent for diagnostic purpose. Apart from MRI images to perform diagnostics, the prepared ferrofluids can also be explored for various others applications. For example, these could be helpful to minimize the clearance of MNP. Normally such system development is done via coating on the MNPs to be used for the drug delivery purposes. However, since the prepared MNPs are encapsulated within PCL, it is hopeful that PCL brought the stealth characteristics to these particles from the reticuloendothelial system (RES) [24-26]. The slower clearance rate will result in the long circulation of the MNPs in the body thus MNPs will be able to impart their characteristics for drug delivery and assistance in imaging. In future, more works is planned to compare the various ratios of PCL used for coating and its effect. A comparison of PCL coating with other coatings/surfactants can be made to alter the circulation time of MNPs. Another important therapeutics characteristics possess by the MNPs is the induction of hyperthermia using an alternating magnetic field and thus enhancing the efficacy of the anticancerous [27-28]. The

MNPs are responsible for hyperthermia effect as they heat on applying magnetic field and can kill the cancerous cells at a temperature above 40°C. However, there are the studies that hyperthermia could also sensitize the tumor cells for therapeutic effect of anticancer [29-30]. Another use of the magnetic properties is the tumor targeting with help of external magnetic field where the MNP loaded with drug could be brought on specific area for the concentration of the drug and ultimately higher therapeutic efficacy. The different characteristics of the cancerous cells from normal body cells could be studied to monitor the effect of anticancer active ingredients. All above mentioned applications of the developed novel and hybrid magnetic particles prepared using a modified co-precipitation method will able to further stimulate the exploration in the areas of biomedical diagnostic and therapy and assist in leading solution to cure and prevent fatal diseases like cancer.

Conclusion

Nanoparticles are applied in biomedical field for the drug delivery and diagnostic purposes. Magnetic nanoparticles helped in the improvement of diagnosis *in vitro* and *in vivo*. This work is the part of project for the preparation of theranostic agents where hybrid iron oxide nanoparticles were prepared. Primarily iron oxide nanoparticles were prepared and then dispersed in both aqueous and organic media. These IONPs were loaded to the polymeric nanoparticles via a modified double emulsion technique. IONPs and the final nanoparticles were well characterized for the hydrodynamic size, size distribution, morphology, chemical composition and their superparamagnetic characteristics. Both types of nanoparticles were analyzed for *in vitro* MRI in comparing with water used as control. The IONPs were found to have a hydrodynamic size of below 30 nm while the final iron oxide loaded nanoparticles were found to be in range of 250-350 nm. Analysis for MRI *in vitro* revealed these nanoparticles as suitable for use as contrast agent. T₁ and T₂ enhancement of contrast was observed and found that iron oxide nanoparticles and hybrid particle are sufficiently detectable. The TE values were drawn against the intensity obtained and successful results were obtained.

These successful results were sufficient for the advancement of project towards the co-encapsulation of an active ingredient with iron oxide as quantity of iron oxide loaded is sufficiently effective for diagnostic purposes using MRI.

Acknowledgment

We are thankful to Science and Technology cooperation programme between Pakistan & France and HEC (Higher Education commission) of Pakistan for supporting Ph.D. studies of Mr. Naveed Ahmed.

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CHAPTER III.2. (B)

EVALUATION OF NANOPARTICLES IN VIVO

**Magnetic nanoemulsion for detection of minimum iron oxide quantity for
in vivo magnetic resonance imaging**

Naveed Ahmed, Chiraz Jaafar-Maalej, Mohamed M. Eissa, Hatem Fessi and Abdelhamid
Elaissari

Université de Lyon, F- 69622, Lyon, France; Université Lyon 1, Villeurbanne, CNRS, UMR-
5007, Laboratoire d'Automatique et de Génie des Procédés

* Corresponding author: Phone: +33-472431841, Fax: +33-472431682

E-mail: elaissari@lagep.univ-lyon1.fr

(Submitted to Journal of Biomedical Nanotechnology)

Résumé

De nos jours, les techniques d'imagerie sont largement appliquées pour le diagnostic de pathologies diverses. Ces techniques font appel à de nombreux agents de contraste et avec une évolution continue dans le domaine de la bio-nanotechnologie et les avancées sont nombreuses à ce niveau. Parmi les agents de contraste utilisés, les nanoparticules d'oxyde de fer (IONPs) ont été développées par de nombreuses équipes scientifiques et ont été appliquées avec grand succès à l'imagerie par résonance magnétique (IRM) *in vivo*.

Lors de cette étude *in vivo*, une émulsion magnétique commerciale a été testée chez des rats après avoir été caractérisé en termes de taille des particules et la teneur en oxyde de fer. Cette émulsion magnétique a été diluée et administré par voie intraveineuse à des rats Sprague-Dawley conformément aux directives européennes pour les soins et l'utilisation des animaux de laboratoire. Des images ont été enregistrées par μ IRM (Pharmascan de Bruker pour la recherche en IRM).

Les particules utilisées ont une taille hydrodynamique de l'ordre de 200 nm contenant 60% massique d'oxyde de fer. Des essais préliminaires ont permis de démontrer qu'une concentration de 0,05% de ces particules magnétiques commerciale était détectable par μ IRM. Les images ainsi obtenues montrent la distribution *in vivo* de ces particules au sein des organes comme le foie, la rate et les reins. Ces premiers résultats sont encourageants pour envisager d'autres applications.

Abstract

Nowadays, bio-imaging techniques are widely applied for the diagnosis of various diseased/tumoral tissues in the body using different contrast agents. Accordingly, the advancement in bio-nanotechnology research is enhanced in this regard. Among contrast agents used, superparamagnetic iron oxide nanoparticles were developed by many researchers and applied for *in vivo* magnetic resonance imaging (MRI). In this study, a new oil-in-water magnetic emulsion was used as contrast agent in MRI, after being characterized in terms of particle size, iron oxide content, magnetic properties and colloidal stability using dynamic light scattering (DLS), TGA, vibrating sample magnetometer (VSM) and zeta potential measurement techniques, respectively. The hydrodynamic size and magnetic content of the magnetic colloidal particles were found to be 200 nm and 75 wt %, respectively. In addition, the used magnetic emulsion possesses superparamagnetic properties and high colloidal stability in aqueous medium. Then, the magnetic emulsion was highly diluted and administered intravenously to the Sprague dawley rats to be tested as contrast agent for *in vivo* MRI. In this preliminary study, MRI images showed significant enhancement in contrast, especially for T₂ (relaxation time) contrast enhancement, indicating the distribution of magnetic colloidal nanoparticles within organs, like liver, spleen and kidneys of the Sprague dawley rats. In addition, it was found that 500µL of the highly diluted magnetic emulsion (0.05wt %) was found adequate for MRI analysis. This seems to be useful for further investigations especially in theranostic applications of magnetic emulsion.

Key words

Magnetic emulsion, iron oxide nanoparticles, contrast agent, magnetic resonance imaging, rats.

Introduction

Early diagnosis is helpful in well management of any disease and consequently for rapid recovery of the patient. In this context, modern diagnostic techniques, which based on imaging modalities, are widely used including computed tomography (CT) scan, magnetic resonance imaging (MRI), X-ray, positron emission tomography (PET), etc. MRI has got a great deal of importance among all of them. With recent development of nanotechnology and its applications in the theranostic field, new horizons have been explored in diagnostic

modalities as well as therapeutic aspects [1]. In this context, the application of nanomaterials in diagnosis increased the worth of imaging techniques.

Considering the case of MRI, different contrast agents bearing magnetic properties are being used like gadolinium, manganese oxide, gold, or iron oxide, which could facilitate the visualization of the specific organs and increase the visibility of anomalies allowing rapid diagnosis of the disease [2]. In recent decade, magnetic iron oxide nanoparticles (IONPs) are one of the best choices to be applied as contrast agent in MRI, and especially in enhancement of contrast in T_2 (relaxation time) imaging sequences [3-5]. The superiority of IONPs is emanating from their biodegradability, biocompatibility, ease of preparation and surface modification. In addition, their superparamagnetic characteristics are another attracting feature, which can be exploited for targeting the active drug-bearing magnetic nanoparticles, under the effect of an external magnetic field to the specific infected organs or tissues inside the body. Consequently, this leads to increasing drug efficacy and reducing their side effect. To achieve this target, control of the surface chemistry of IONPs is required in order to enhance their colloidal stability, biocompatibility, and in some cases low biomolecules adsorption [6]. In this context, magnetic iron oxide nanoparticles are surface modified using oleic acid, dextran, polyacrylic acid, polymaleic anhydride, etc, for *in vivo* applications [7].

In the applications of bionanotechnology, nanoparticles encapsulating these contrast agents have been prepared and applied. These nanoparticles (NPs) with narrow size distribution allow greater area of exposure, hence giving enhanced contrast. However, to reduce the various limitations of contrast agents in imaging, different pharmaceutical techniques have been involved for the modification of these contrast agents keeping their magnetic properties and reducing their side effects. The surface modification of NPs is one of these techniques that helped to achieve this target. Surface modification can be obtained by using various polymerization techniques, emulsification mechanisms or by using several other pharmaceutical methods [8-10].

Recently, several magnetic iron oxide based products are already commercialized as contrast agents for MRI. However, some of them have been stopped due to some limitations [11] related to the toxicity of iron oxide particles. In this regard, some reports on the toxicity of iron oxide particles [12] and on the materials used for the modification of their surfaces [13-15] have been published.

In our concept, the toxicity can be attributed to the presence of other materials rather than iron oxide like octane and oleic acid, which are used during the preparation of magnetic

iron oxide colloidal particles. For instance, during the preparation of o/w magnetite emulsion, the iron oxide nanoparticles usually stabilized with oleic acid, followed by dispersion in octane and then in water containing a stabilizing agent [16]. However, the octane solvent is evaporated under reduced pressure, but in most cases, some traces of octane still exist in the final o/w magnetic emulsion in addition to the presence of oleic acid that can be responsible of toxicity.

For *in vivo* MRI diagnostic application, and regarding to aforementioned toxicological reports, the best option is to use minimum quantity of the magnetic iron oxide emulsified particles. To accomplish such purpose, a need of a feasibility study was felt.

Hence, the objective of this research is to evaluate a new generation of emulsified magnetic iron oxide nanoparticles as contrast agent for *in vivo* MRI. The nature of the emulsified magnetic particles in this research is different from other studies in that this o/w magnetic emulsion has very low octane residual (less than 10mg/g dried iron oxide) to minimize or to lower the toxicity of these particles. In this context, a commercially prepared o/w magnetic emulsion was fully characterized before its use in this regard. In order to investigate the feasibility of using such types of particles and to estimate the minimum detectable quantity to be used for *in vivo* MRI, a highly diluted sample of the magnetic emulsion was prepared and administered intravenously to Sprague dawely rats, as animal specimens, which have already been used in various MRI studies [17, 18]. The effect of magnetic emulsion on the T_1 and T_2 (relaxation times) contrast enhancement was also discussed.

Experimental section

Materials

The used o/w magnetic emulsion was provided from Ademtech (Bordeaux, France). It consists of magnetite nanoparticles stabilized with oleic acid, followed by dispersion in octane and then in water using sodium dodecyl sulfate (SDS) surfactant as a stabilizing agent. Its octane content is less than 10mg/g of dried iron oxide nanoparticles. More information is already reported by Montagne et al. [16]. NaCl, NaOH and HCl (35% w/v) solution were purchased from Aldrich Company. Isoflurane gas used for rat anesthesia was from Laboratoire Belamont, (Boulogne Billancourt, France). Milli-Q water was used in all experiments as the dispersion medium.

Characterization of magnetic emulsion

Before characterization of the o/w magnetic emulsion, it was washed three times by Milli-Q water with the help of an external magnet to get rid of the non-magnetic part originated from the excess SDS stabilizing agent. Figure 1 shows schematic presentation of the o/w magnetic emulsion.

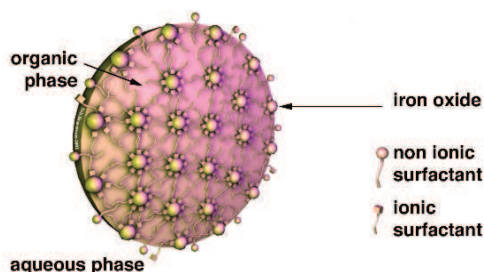


Figure: 1 Schematic presentation for o/w magnetic emulsion

Particle size measurements

Hydrodynamic size (D_h) of magnetic emulsion droplets was measured by dynamic light scattering technique using Malvern Zetasizer 3000 HS instrument. The measurements were performed at 25°C by diluting a drop of the magnetic emulsion in 1×10^{-3} M NaCl solution. The reported value of D_h was the average of 30 measurements.

Transmission electron microscopy (TEM)

TEM (Phillips CM120) was used to investigate the morphology and microstructure of the magnetic emulsion particles. Briefly, a drop of highly water diluted sample was deposited onto a carbon-coated copper grid, and then it was left to dry at room temperature overnight before TEM analysis.

Thermal gravimetric analysis (TGA)

Magnetite content of the magnetic emulsion was determined by thermal gravimetric analysis using TGA (NETZSCH TG209) analyzer. The measurements were carried out under nitrogen atmosphere from ambient temperature up to 600°C with heating rate 20°C/min.

Zeta potential measurements

Zeta potential (ζ) of the magnetic colloidal particles was investigated as a function of pH of the dispersion media. The measurements were carried out at 25°C using Malvern Zetasizer (NanoZS2000) by diluting a drop of the magnetic emulsion in 1×10^{-3} M NaCl solution at a specific pH value. The reported value of ζ was the average of 90 measurements.

Magnetic properties

Saturation magnetization (Ms) and magnetic behavior of the dried magnetic emulsion were investigated using vibrating sample magnetometer (VSM). Ms of all dried particles was investigated by decreasing the applied magnetic field (H) from +20000 to -20000 Oersted, at room temperature.

Magnetic resonance imaging (MRI)

Sprague-dawley rats (200-250 g) were used for MRI analysis. All animal experiments were conducted according to a protocol approved by the ethics committee of Claude Bernard University Lyon1, and in conformity with the European guidelines for the care and use of laboratory animals.

The anesthesia protocol was conducted with an approved system (TEM Sega, Lormont, France). First, the rats were placed in an anesthesia induction box using a mixture of 3% isoflurane gas and air containing 30 % oxygen administered at 600ml/min rate. The animals were then placed in a supine position on a plastic bed (Bruker Biospec Animal Handling Systems) and maintained under anesthesia during the whole MRI protocol at 1.5 to 2.5% isoflurane dose delivered via a dedicated cone mask. The body temperature was maintained at 37°C via the integrated flowing warm water circuit integrated within the dedicated bed. A respiratory sensor was placed on the abdomen to continuously monitor the respiration rate displayed using a triggering device (ECG Trigger Unit HR V2.0, Rapid Biomedical, Würzburg, Germany).

In vivo MRI acquisitions were performed on a Bruker 7-Tesla Biospec horizontal bore system (Bruker, Bruker Biospin MRI GbmH, Germany) equipped with 400 mT/m gradient set and interfaced to a Bruker Paravision 5.1 console. A Bruker linear-volume coil (outer diameter = 112 mm and inner diameter = 72 mm) was used for MRI acquisitions under an emission/reception configuration.

A quick spin-echo localizer with three orthogonal orientations and 10 cm field of view was first used allowing calculation of fixed spatial coordinates for following scans. Then, a low-resolution T₂-weighted triggered scan acquired in coronal view was used to accurately set-up the geometry of the following high-resolution MR images.

Next, high-resolution T₂-weighted spin-echo images were obtained in axial orientation based on a fat suppressed (FS) Rapid Acquisition with Relaxation Enhanced (RARE) sequence with the following parameters: repetition time (TR) 3000 ms, echo time (TE) 38.1 ms, and RARE factor = 8.

Last, high-resolution T_1 -weighted gradient-echo images based on a fat suppressed (FS) Fast Low Angle SHot (FLASH) sequence were acquired with the following parameters: repetition time (TR) 800 ms, and echo time (TE) 3.36 ms.

Both sequences were respiration triggered using the triggering device with identical geometry (slice thickness = 2 mm, field of view (FOV) of $8 \times 8 \text{ cm}^2$, matrix size of 256×256 , resulting in an in-plane resolution of $312 \times 312 \mu\text{m}^2$).

Results and discussion

Magnetic emulsion characterization

The mean hydrodynamic size (D_h) of the used magnetic colloidal particles was determined by DLS technique and it was found to be 250 nm with polydispersity index (PDI) 0.06. The solid content was found to be 10 wt% by using moisture analyzer. Furthermore, morphology of the (o/w) magnetic emulsion particles was investigated using TEM, as shown in Figure (2). The TEM image shows spherical morphology and low size distribution of the (o/w) magnetic emulsion droplets, as evidenced by DLS measurements.

Magnetic content in the (o/w) magnetic emulsion was determined by using TGA technique. As seen in Figure (3), TGA thermogram of iron oxide magnetic emulsion shows two main inflections; a very moderate one (4 % weight loss) between 25 and 200 °C, which attributed to release of moisture entrapped by the emulsified iron oxide nanoparticles, and a larger one (20 % weight loss) between 220 and 500 °C due to the decomposition of organic layer (oleic acid and SDS surfactants)-treated iron oxide nanoparticles. This means that about 76 %wt of the magnetic iron oxide nanoparticles are coated with organic material.

The chemical structure, physical properties and the amount of the used inorganic “magnetic” material in the final particles are the driven parameters controlling their physical separation under magnetic field. Then, the magnetic properties of used oil in water (o/w) magnetic emulsion were analyzed and results are shown in Figure (4). The used magnetic emulsion showed superparamagnetic behavior with no remanence upon removing the magnetic field. The specific saturation magnetization (M_s) of the magnetic emulsion was found to be 43.85emu/g. More interestingly, if we assume that the used iron oxide is magnetite (saturation magnetization is 60 emu g^{-1}) [19], then the amount of magnetic material in the used magnetic emulsion is 73 wt. % and 27 wt. % organic layer (oleic acid and SDS surfactants). This result is in conformity with that obtained by TGA analysis.

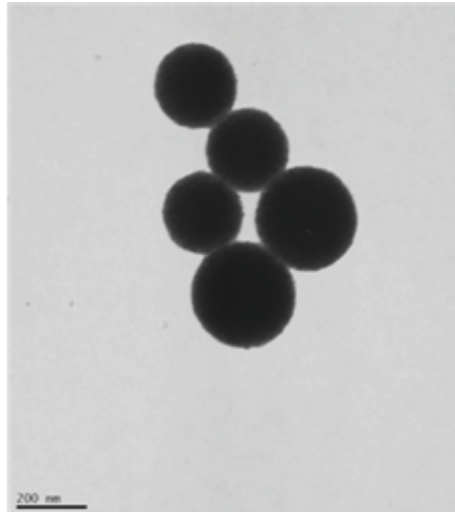


Figure: 2 TEM micrograph of (o/w) magnetic emulsion.

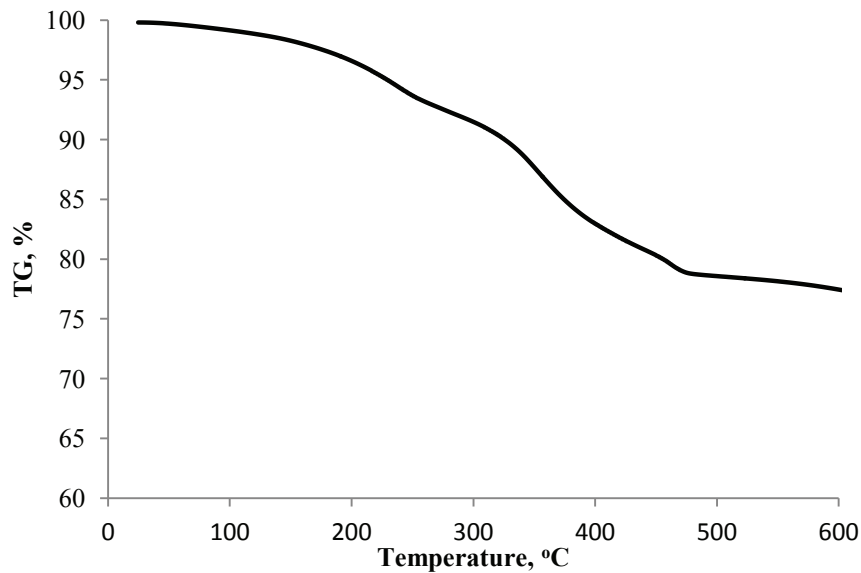


Figure: 3 TGA thermogram of (o/w) magnetic emulsion.

Further, it is worth to mention that the long term colloidal stability of the magnetic emulsion is of paramount importance *in vivo* biomedical applications. Accordingly, Zeta potential (ζ) measurements were carried out in order to study the electro-kinetic phenomenon based on the surface charge of the magnetic nanoparticles at different pH (3-11) environments. As shown in Figure (5), the magnetic colloidal particles exhibit negative charge irrespective of the investigated pH domain. That could be attributed to the strong negatively charged sulfate group of SDS which used during (o/w) magnetic emulsion formation [20].

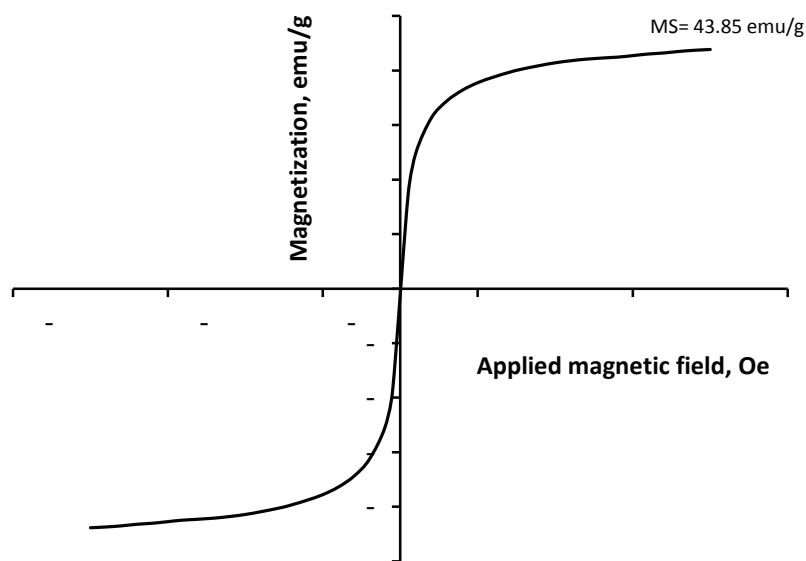


Figure: 4 Magnetization curve of dried (o/w) magnetic emulsion.

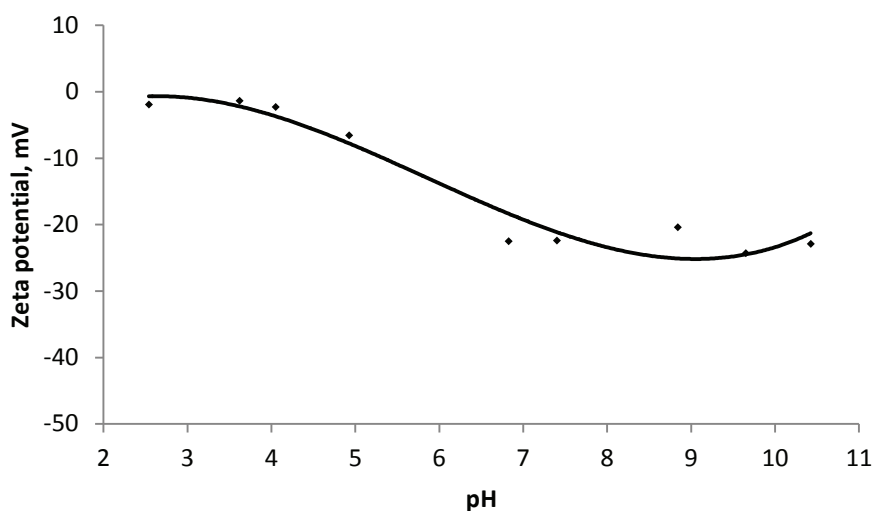


Figure: 5 Zeta potential (ζ) of the (o/w) magnetic emulsion as a function of pH in 1m M NaCl solution.

***In vivo* MRI**

MRI images were taken before and after I.V. injection of the highly diluted magnetic emulsion (500 μ L, 0.05wt %) to Sprague dawley rats. The contrast was investigated by comparing the post and pre-injection images or by subtracting pre-injection image from post-injection one considering the same imaging site. The MRI images obtained showed a significant contrast, which was found in the liver, spleen and kidneys of the Sprague dawley

rats. These preliminary results were satisfactory according to the aim of the work that was the feasibility of using a new type of o/w magnetic emulsion as a contrast agent in MRI.

T₂ enhancement of contrast

For the T₂ weighted scan, firstly, a low resolution scan was taken to set up the geometry for high resolution MR images which was done as RARE sequence, as previously described in methods section. Practically, the use of magnetic iron oxide nanoparticles in imaging cause the reduction in T₂ (relaxation time) signal absorbance of tissues, so the iron oxide contrast agents are preferred for T₂ weighted MRI. T₂ scans with RARE explained the T₂ enhancement of contrast, which was found in liver as it can be seen in Figure 6. The contrast has been verified by comparing scan before injection of the magnetic emulsion (Figure 6a) and scan after injection (Figure 6b). Furthermore, T₂ enhancement of the contrast was observed in other organs than liver like kidneys and spleen, as it can be observed in Figure 7. Where, in the same time delay, the contrast has been seen by comparing MR images before (Figure 7a) and after injection of the magnetic emulsions (Figure 7b). However, in this work, the detailed study had not done since the aim just for the feasibility of using the magnetic iron oxide emulsion *in vivo* MRI, so the results are based on qualitative analysis.

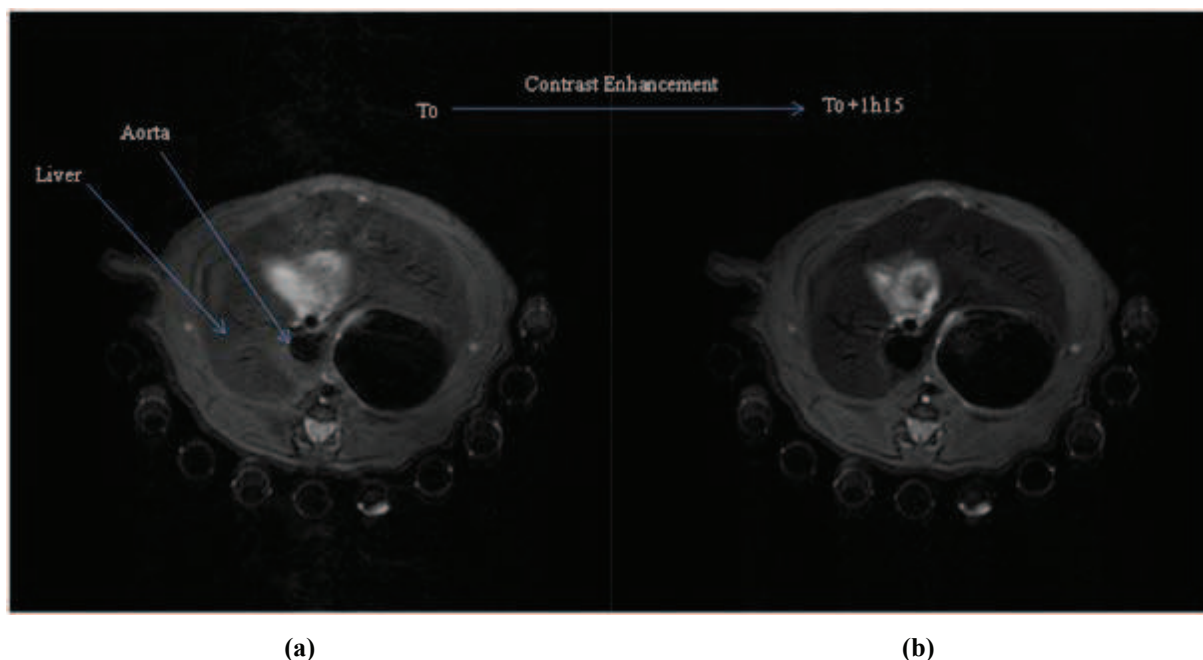


Figure: 6 T₂ enhancement of contrast in liver of Sprague dawley rats (a) MR image before and (b) after injection of magnetic iron oxide emulsion.

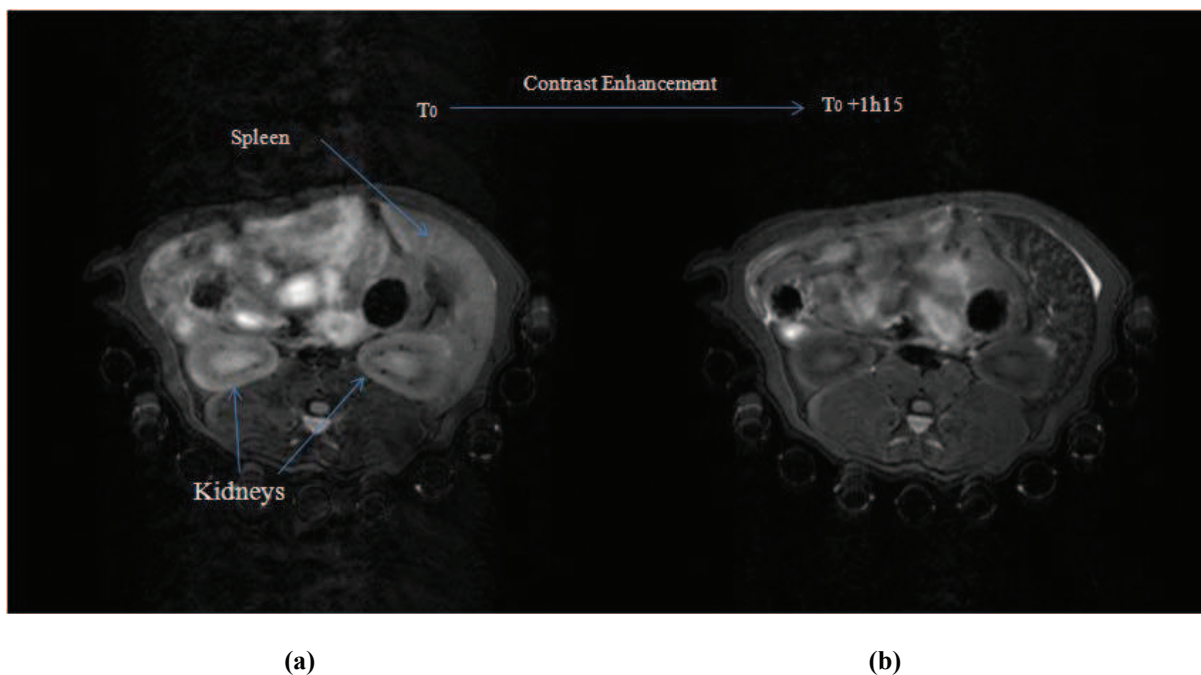


Figure: 7 T_2 enhancement of contrast in kidneys and spleen of Sprague dawley rats (a) MR image before and (b) after injection of magnetic iron oxide emulsion.

T_1 enhancement of contrast

The T_1 weighted gradient-echo images of spleen and kidneys of Sprague dawley rats, on the basis of FLASH sequence, were acquired with a TE of 3.36 ms, and shown in Figure 8. By comparing the two scans before (Figure 8a) and after injection of the magnetic emulsion (Figure 8b), it can be explored that the contrast has been seen. A further comparison was done by subtracting the pre-injection scan from the post-injection one to see the difference between them. The difference was observed but it was not as visible as for T_2 . This very low contrast (approaches to be zero) is apparent in the images (a) and (b), which seems to be identical without any detectable contrast. Consequently, this observation supports the preferential use of the magnetic iron oxide nanoparticles for the T_2 acquisition.

Here, it is worth to mention that, the acquisition of MR images was stopped after 75 minutes of magnetic emulsion injection because no more enhancements in contrast were observed in liver, spleen, kidney or bladder. In addition, after 75 minutes, the contrast started to disappear. This might be helpful for further investigation on pharmacokinetic profile of the magnetic emulsion. In addition, it could be concluded from the absence of signals that the magnetic emulsion may undergo complete degradation or excretion from the body as the administered amount (500 μ l) and concentration (0.05wt%) were very low. More interestingly, the second important thing is related to the safety of the animal. It was established that rats administered with magnetic emulsion were alive for long time after the completion of MRI

studies. As a result, it can be concluded that such type of magnetic emulsion is safe for animals, but further investigation is necessary to express this statement.

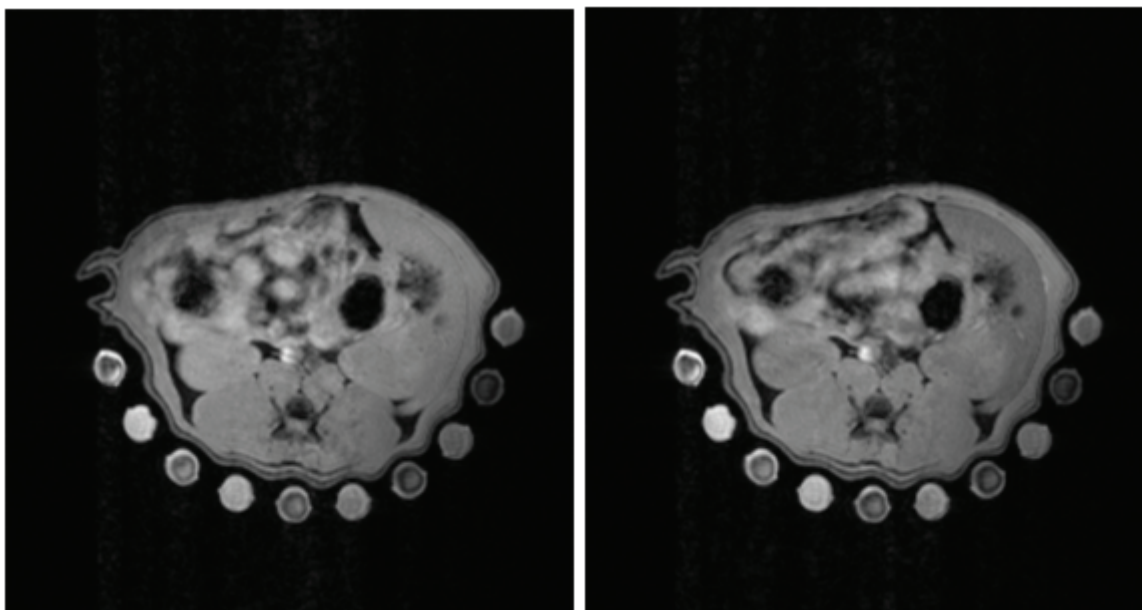


Figure: 8 T₁ enhancement of contrast of spleen and kidneys of Sprague dawely rats (a) MR image before and (b) after injection of magnetic emulsion.

Conclusion

Magnetic resonance imaging (MRI) is an important and beneficial imaging technique, especially for the early diagnosis of cancer. Magnetic iron oxide nanoparticles (IONPs) are one of most attractive contrast agents that have been successfully used for MRI. Various iron oxide products have been commercialized, but based on various reasons; the production has been stopped. Despite this, IONPs are still attractive for MRI due to their biodegradability, biocompatibility and superparamagnetic properties. In this study, superparamagnetic and high colloidal stable oil in water (o/w) magnetic emulsion (250 nm in size) was successfully used as a contrast agent in magnetic resonance imaging (MRI) for Sprague dawely rats. The magnetic emulsion used was diluted and administered intravenously in Sprague dawley rats in order to estimate the limit of detection in MRI as well as *in vivo* distribution within animal organs. The detection was successful and the contrast started after 40 minutes of injection while it reaches maximum after 75 minutes of injection and then started to disappear. These results provide some basic idea for pharmacokinetic profile of the iron oxide particles, but for the complete pharmacokinetic profile, a separate study is required. As the purpose of this work was to study the feasibility of iron oxide to be used for MRI and to estimate the detectable quantity of iron oxide for MRI, it was found that 500 μ l of magnetic emulsion (10

wt % solid content) in concentration of 0.05% is sufficient for *in vivo* MRI. The iron oxide nanoparticles in the colloidal form are also feasible knowing the exact concentration and solid content of iron oxide. These promising results push us to expand our basic aim for the theranostic applications of IONPs as contrast agents in MRI for the diagnosis of cancerous cells in future studies.

Acknowledgment

We are thankful to Mr. Radu Bolbos and J.B. Langlois for MRI acquisitions (Animage department, Cermep-imagerie du vivant, Lyon). We are also thankful to science and technology cooperation program between Pakistan & France via higher education commission (HEC) of Pakistan for supporting Ph.D. studies of Mr. Naveed Ahmed.

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PART IV

GENERAL DISCUSSION, CONCLUSION

AND FUTURE ASPECTS

Résumé

Cette dernière décennie, la nanotechnologie a été appliquée avec grand succès dans le domaine biomédical. Cette nouvelle science est prouvée comme une innovation utile dans le domaine de la technologie biomédicale. Les applications dans le diagnostic, l'administration de médicaments, la vectorisation de gènes, la diminution des effets secondaires liés aux médicaments ont ouvert de nouveaux horizons dans le domaine de la biologie, le diagnostic et la thérapie. Le concept théranostics est très convoité, car il offre la combinaison des options diagnostiques et thérapeutiques simultanément. Le procédé de la double émulsion /évaporation de solvant a été une méthode de choix, optimisée, examinée via une étude systématique et validée par IRM après encapsulation d'oxyde de fer comme agent de contraste. Ces résultats encourageants ont stimulés notre envie d'introduire un agent anticancéreux pour mieux se rapprocher de la réalité.

Dans cette partie, la discussion générale sur les problèmes ou les difficultés rencontrées lors de l'élaboration du processus est donnée. Les résultats et les observations sont également inclus dans cette partie. Il ressort de cette étude que la modification de la technique double émulsion et évaporation de solvant est un choix approprié pour la préparation d'une nouvelle génération de particules à double fonctionnalité diagnostic *in vivo* et thérapie localisée (application théranostiques). Les particules préparées via ce procédé optimisé et parfaitement maîtrisé ont également montré un bon pouvoir contrastant pour l'IRM.

En perspective, il sera indispensable de valider ces particules contenant un agent de contraste et un anticancéreux chez les rats afin de mettre en évidence si, le positionnement d'un aimant sur la tumeur permet la concentration locale de ces particules et par conséquent favoriser le traitement rapide de la tumeur. Cette approche doit permettre de réduire les effets secondaires et probablement une guérison rapide.

GENERAL DISCUSSION

Application of nanotechnology can be brought in account for the improved bioavailability of the drugs in the body. The primary stage is preparation of nanoparticles containing drugs using any suitable nanoparticles preparation method. Whereas to realize the aim of theranostics much attention is required for many other caring steps involved. A pharmaceutical technique is chosen for the preparation of nanoparticles depending upon the aim of the work. The technique selected was emulsion evaporation and modified according to the requirements. Instead of single emulsion, a double emulsion method and particularly w/o/w was coupled along with evaporation. This modification allowed us to prepare nanoparticles with high energy stirring apparatus called ultra-turrax. The choice of the polymer was made on the basis of biodegradability and biocompatibility and Dichloromethane is selected on the basis of low boiling temperature and immiscibility with water. The method was investigated with several stabilizers responsible for the stabilization of system. A number of agents tried with this modified method including triton-405, tween 80, poloxamer, PVA (poly vinyl alcohol) and PEG (poly ethylene glycol) and screened for the good results. PVA is proved to be good and a concentration of 0.5 % produced best results among the concentrations investigated. The aim was to obtain a nanoparticles system hence the factors/parameters affecting the size of particles were studied. It should be noted that along with this validation of method a model hydrophilic drug, derivative of Stilbene was encapsulated that is a fluorescent compound. A fixed quantity of Stilbene derivative was introduced in every experiment. Among these parameters, the primary investigation was done on the stirring speed as ultra-turrax was used for stirring purpose that is a high energy stirring apparatus. The stirring speed for both emulsification steps was screened. For first emulsion preparation oil in water the stirring speed was investigated from 9.500 rpm to 24.000 rpm keeping all other parameters constant including the stirring speed for second emulsion step (w/o/w). As explained in the results of preliminary study the nanoparticles formation varying first stirring speed was obtained and smallest particle size was achieved with 16.000 to 17.500 rpm range. It should be kept in mind that no stabilizing agent or surfactant was used in first emulsification step. Similarly the stirring speed screening for second emulsification step was done keeping all other parameters constant and varying the stirring speed from 9.500 to 24.000 rpm and the best emulsion was produced using 21.500 rpm. Stirring time is important parameters to define the hydrodynamic size of the resulting particles. As there are two emulsification steps, stirring time for both emulsions was investigated keeping all other

parameters constant in each case and interestingly 15 minutes found suitable time for both emulsification steps. Two superimposed curves were found at 15 minute time when the screening results for time of stirring were drawn against hydrodynamic particle size producing the particles in range of 250-350 nm. After validation of the stirring time and stirring speed, polymer concentration was the next important parameter to consider as after evaporation step the polymer is responsible for matrix formation due to its precipitation in aqueous phase. A range of PCL (polymer) using weight /volume ratio to the oily phase was investigated and 25 % was found to be suitable. The smallest particle size has been achieved in this case (25 %). With the decision of polymer concentration in a given volume of oily and aqueous phase, nearly whole recipe was prepared for the modified double emulsion evaporation method including the study of factors affecting the final particle size.

The successful investigation of parameters/factors along with encapsulation of a model active ingredient allowed us to proceed further. The next step was the preparation of iron oxide nanoparticles that can be loaded to these nanoparticles to be used as contrast agent for MRI. A chemical method was adopted termed as co precipitation method in which two salts of iron were reacted in basic medium with stirring. At the end of the preparation of iron oxide nanoparticles the dispersion was done in different phases i-e aqueous and organic phases. The dispersion in aqueous and organic phases allowed encapsulating iron oxide using above mentioned method of double emulsion evaporation. The advantage obtained was the ability to encapsulate any type of iron oxide with choice of solvent. For the organic medium dispersion the IONPs were treated with oleic acid and the dispersed in octane after washing with acetone. The characterization was done using DLS, thermogravimetric analysis and magnetization studies. It was proved that the iron oxide particles were of superparamagnetic characteristic with high saturation. DLS revealed the particle size below 40 nm and after filtration the particle with 10-20 nm size were separated. Successful stability of iron oxide nanoparticles in aqueous medium was obtained and octane dispersed IONPs were dried and stored for the next step of encapsulation.

The preparation of iron oxide particles with small size range was a success. Hence the double emulsion evaporation method was used to encapsulate the IONPs as encapsulation of an active ingredient via this method was already established showing the encapsulation of Stilbene. For the encapsulation of IONPs in aqueous media, a quantity equal to first aqueous phase was introduced and all other parameters were used as such. A loading of IONPs till 13 % weight/volume solid contents was achieved using this modified double emulsion method.

Above this limit, the excess of iron oxide particles was found at the bottom of the beaker where emulsion was formed or in evaporation flask. But the best results were obtained with iron oxide particles having solid contents below 10 %. The final size of the particles was in range of 250-350 nm and hence in comparison with blank and studying various concentrations of IONPs, it revealed that quantity of iron oxide loaded has no significant effect on final size of particles. However in case of iron oxide in organic medium, different aspects were observed. IONPs were dispersed in octane so it was investigated to encapsulate these nanoparticles in octane using same double emulsion evaporation technique. Nonetheless, DCM was used as oily phase in this double emulsion technique. Introduction of IONPs dispersed in octane in DCM surely would disturb the ratio between polymer and oily phase. To avoid this oily phase concentration was kept constant and a ratio was tried to settle for DCM and octane. 16 % and 33 % of octane was investigated against respective DCM quantity to form 100 % oily phase. The results were not interesting as a new octane layer was obtained at the top of final preparation and most of iron oxide went to this new layer. This leads to the failure of choice of octane as solvent for IONPs. Hence IONPs dispersed in octane were dried and then the dispersion was done in DCM. Redispersion was achieved but to a certain limit and after that the good dispersion of octane dried iron oxide nanoparticles was impossible. However this limit permitted the encapsulation of iron oxide nanoparticles sufficient for the magnetic resonance imaging purpose. Various quantities of IONPs treated with oleic acid were introduced in DCM and encapsulated using the same method of encapsulation with same predefined parameters. The characterization was done and it was proved that the contents of iron oxide nanoparticles have no specific effect on the final particle size. The method was proved successful for the encapsulation of iron oxide nanoparticles. The characterization in TEM for IONPs loaded particles revealed that in case of aqueous ferrofluid, the iron oxide particles tend to come out of the internal aqueous phase and move towards external aqueous phase. This resulted in the observation of the particles on the surface as well as inside the polymeric matrix. This trend found because of solubility of aqueous ferrofluid in water or it may be due to some interaction between the particles and polymer. On the other hand in case of IONPs in organic media, the particles restricted well inside the polymer and did not found on the surface of the polymer. This result provoked us to use an agent for the surface modification or charge creation on the particle surface during preparation of iron oxide particles. To study this, preparation of iron oxide nanoparticles coated with dextran and other such materials is in process and further investigation on any type of interaction between

polymer and particles is under process. The other type of iron oxide particles used for this investigation includes commercially available iron oxide emulsion from ademtech and particles prepared with slight modification in drying process.

Particles without evaluation for final applications are useless so evaluation *in vitro* and *in vivo* for MRI was done. In case of *in vivo* evaluation first of all commercially available emulsion from ademtech were used for the estimation of minimum detectable quantity in MRI. An emulsion of 10 % solid contents was used and the analysis was done on the Sprague dawley rats as animal specimen. Various dilutions of this commercialized emulsion were injected intra venously. It was found that 500 μ l of magnetic emulsion that was diluted to 0.05 % is detectable in MRI. These results encouraged to proceed with our method of encapsulation as a little amount is required in MRI. *In vitro* investigations were done in comparison with water for T₂ enhancements of contrast. For the analysis both types of iron oxide nanoparticles (aqueous and organic) and the emulsions loaded with aqueous and organic IONPs were investigated. T₁ images were also taken and compared with commercially available gadolinium i-e Gadovist. Successful results were obtained guaranteeing the success of double emulsion evaporation method for loading of iron oxide nanoparticles.

These all above mentioned results allowed further proceeding for the encapsulation of an active ingredient that will be an anticancer drug using this modified double emulsion evaporation method. Pharmacodynamic and pharmacokinetics studies will evaluate further the method for the theranostic purpose. The change of route of administration is another step and loading drug and contrast agent together is another task to be done. Passing through all these required steps the nanoparticles will evolve as suitable administration product. However this could be a small hope in the treatment or combat against cancer but still it will take a long way ahead to arrive the patient level which required a lot of patience and care.

CONCLUSION AND FUTURE PERSPECTIVES

Nanotechnology has been successfully applied in biomedical field since last decade and is proved as a helpful innovation in the field of biomedical technology. The applications in diagnosis, drug delivery, gene delivery, reducing secondary effects of the medicines, *etc.* opened new horizons for the scientists in the field of biology, diagnosis and therapy. Biomedical nanotechnology also allowed taking multiple advantages from the single moiety and among such advantages; theranostic approach is extremely faithful allowing rapid diagnosis and treatment simultaneously. Considering cancer as one of the most fatal diseases, newer domain of biomedical nanotechnology is seriously applied in oncology. The secondary effects of therapeutic agents on normal human tissues/cells and the early diagnosis in cancer are the main problems to deal in oncology. Due to this reason, biomedical nanotechnology tried to deal primarily with early diagnosis and drug targeting. Theranostic approach proved praiseworthy in this regard giving diagnosis and therapy at same time. However in case of theranostic magnetic nanoparticles are very helpful. For diagnostic purposes, the emphasis is still on imaging mechanisms e.g. MRI, CT-scan, PET, *etc.* Magnetic materials could be used for diagnosis of cancerous cells utilizing imaging mechanisms mentioned earlier. Imaging techniques especially MRI required a contrast agent for visualization of the specific cells/tissues in body for differentiation from other cells/ tissues. Hence these magnetic nanoparticles are used as contrast agents in MRI for contrast/ differentiation from other normal tissues of the body. Various materials are being used for the contrast including Gadolinium, iron oxide, manganese, gold, *etc.* Addition of a therapeutic agent with these contrast agents gave the idea of theranostic and with help of nanotechnology this becomes a useful and easy to bring in practice. Thus, dealing cancer in a well managed way for the betterment of human being.

In this work the particles are prepared for theranostic application. It has been studied to prepare the nanoparticles containing iron oxide nanoparticles (IONPs) with an active ingredient. The iron oxide is chosen for easy availability and more economic approach with the good contrast in MRI. These iron oxide nanoparticles were prepared through co precipitation method: one of the mostly used chemical methods for the preparation of iron oxide nanoparticles. Iron oxide nanoparticles were prepared in such a way that can be dispersed in both aqueous and organic medium with a small modification of the procedure. The IONPs obtained were in size range of less than 50 nm with well stability properties in their respective dispersion medium.

The next step was the preparation of nanoparticles containing these IONPs with an active ingredient most specifically an anticancer agent to be used for therapeutic purposes. At present different pharmaceutical techniques are being used for preparation of nanoparticles including nanoprecipitation, different emulsification techniques, layer by layer and supercritical fluid technology, *etc.* To encapsulate an active (anticancer agent) with IONPs, a multiple emulsion evaporation method was chosen. More specifically a W/O/W emulsion evaporation method was used for the preparation of nanoparticles target to be used as theranostic agents. The choice was made on the basis of the feasibility of encapsulation of both hydrophilic and hydrophobic active ingredient with same ease.

This newer modified double emulsion evaporation method was validated using a preliminary study. In this preliminary study, various parameters affecting the particle size of the nanoparticle/nanoemulsions has been investigated with the encapsulation of a model hydrophilic active agent (Stilbene derivative). The encapsulation of Stilbene was successful and within range of 250-350 nm particle size. Further this method was applied for the encapsulation of iron oxide nanoparticles dispersed in both aqueous and organic medium. The final IONP loaded nanoparticles were well characterized and especially for their magnetic properties. The IONPs were analyzed *in vivo* for the detection of minimum quantity to be required for MRI *in vivo*. For this short study rats of the species Sprague dawley were used and promising results were obtained. Later the IONPs containing emulsified nanoparticles were investigated *in vitro* MRI. Final step was the encapsulation of an active ingredient using modified double emulsion evaporation method. The choice of an anticancer agent was made and then encapsulation using aforementioned method was performed with well characterization of the particles.

It has been found that the modified double emulsion evaporation method is an interesting approach for the encapsulation of an active ingredient. In case of theranostic approach, it allows the nanoparticles preparation containing a contrast agent and an active ingredient. Having interior aqueous and organic phases, the encapsulation of both types (hydrophobic and hydrophilic) of drugs is possible. As Iron oxide was used as contrast agent for MRI and prepared in both aqueous and organic phases, this method gave us choice for the encapsulation of both types of iron oxide and active ingredients without any difficulty. Preliminary investigation explained about the various factors affecting the hydrodynamic size of the nanoparticles. Normally double emulsion is prepared using two types of surfactants i-e hydrophilic and hydrophobic but in this case only single stabilizing agent is used in second

emulsification step of oil in water emulsification. Second emulsification is instantaneous after the first emulsion preparation; hence the first emulsification step is done without using any stabilizing agent. Stirring speed and time were the most influencing factors and better control of these two allowed us to prepare the particles with size range of 250-350nm. Polymer and Stabilizing agent concentration also played an important role in the size determination of the final particles. In this preliminary study a model fluorescent hydrophilic active (Stilbene derivative) was encapsulated successfully which encouraged us about the success of this approach.

The preparation of iron oxide nanoparticles was done using modified co precipitation method. For iron oxide nanoparticles prepared and dispersed in aqueous medium, the iron oxide particles were treated with nitric acid at final stages of the preparation while in case of organically dispersed iron oxide particles, final treatment with oleic acid was done. The iron oxide nanoparticles dispersed in respective media gave a particle size of less than 50 nm which is too promising for the encapsulation and the final use of IONPs in MRI. These IONPs were encapsulated with double emulsion evaporation method using aqueous ferrofluids in first water phase and organic ferrofluids in organic phase respectively. The characterization results were encouraging having final hydrodynamic size in 250-350 nm range with good magnetic properties and about 60 % encapsulation efficiency. These investigations encouraged us a lot for the encapsulation of IONPs using this modified double emulsion evaporation technique.

The successful results for iron oxide encapsulation encouraged to encapsulate the active ingredients. A hydrophilic anticancer drug, 5-flourouracil (5-fu) was chosen and encapsulated separately and with organic ferrofluids. Similarly a hydrophobic anticancer (Paclitaxel) has been chosen and encapsulated using same method primarily and latterly co encapsulated with aqueous ferrofluids. The successful encapsulation of anticancer drugs with iron oxide will be realization of our aim for preparation of theranostic agent although the *in vivo* and *in vitro* investigations will be the final deciding factor.

For *in vitro* investigations, iron oxide prepared by co-precipitation method, blank emulsions, emulsions containing aqueous ferrofluids and the emulsions containing organic ferrofluids were analyzed and compared with commercially available gadolinium (Gadovist) used as contrast agent for MRI. The distill water was used as control and a sufficient contrast was obtained by iron oxide nanoparticles.

For preliminary study *in vivo*, as more care is required the iron oxide nanoparticles alone were analyzed using Sprague dawley rats. For this purpose the commercially available

emulsion prepared with a similar method were investigated for the detection of minimum quantity of iron oxide in MRI. It has been found that the 500 μ l (0.05%) of magnetic emulsion having 10% solid contents was detectable. These results were according to the expectations and fitted well with our method as aforementioned double emulsion evaporation method has some limitations of quantity to be loaded.

The *in vitro* investigations on iron oxide containing nanoparticles and the particles with dual moieties i-e IONPs and anticancer agent are in progress. While the investigations *in vivo* for the applications of theranostic agent are also under study. We are hopeful that with the completion of these studies we will be able to prepare a theranostic agent containing anticancer and IONPs. Anticancers will be helpful in therapy of cancer while IONPs will serve for the diagnosis of cancerous tissues/cells.

In future, a complete study will be carried out on the pharmacokinetics and pharmacodynamic aspects of iron oxide and encapsulated anti cancer drug with emphasis on the toxicity profile. This study will be helpful in evaluation of the technique in practical way. The next step will be the application of this theranostic approach on the other anticancer drugs using other contrast agents by involving different suitable imaging modalities accordingly. Further pharmaceutical techniques will be applied for other routes of administration than intra tumoral or intravenous injection depending upon the type, stage and location of cancer. All these investigations, development and progress in bio nanotechnology will allow human being to be well equipped to combat cancer. The success of all the steps and process will lead to the betterment of humanity and ability to get rid of this fatal disease.

THE END

List of publications included in manuscript

1. Naveed Ahmed, Ahmed Bitar, Hatem Fessi and Abdelhamid Elaissari. Magnetic nanoparticles applications for drug targeting/release and hyperthermia with special focus on cancer. **In preparation, to be submitted as book chapter** in ‘Control release systems: Advances in nanobottles and active nanoparticles’ will be published by Pan Stanford Publishing, Singapore.
2. Naveed Ahmed, Hatem Fessi and Abdelhamid Elaissari. Theranostic Applications of Nanoparticles in Cancer. **Published** in Drug Discovery Today 2012.
3. Naveed Ahmed, C.E.Mora-Heurtas, Chiraz Jaafar-Maalej, Hatem Fessi and Abdelhamid Elaissari. Polymeric drug delivery systems for encapsulating hydrophobic drugs. **Accepted as book chapter** in ‘Drug delivery strategies for poorly water -soluble drugs’ will be published by John Wiley & Sons, Ltd.
4. Mohamed Ayoub, Naveed Ahmed, Nader Kalaji, Catherine Charcosset, Ayoub Magdy, Hatem Fessi, and Abdelhamid Elaissari. Study of the effect of formulation parameters/ variables to control the nanoencapsulation of hydrophilic drug via double emulsion technique. **Published** in J. Biomed. Nanotechnol. 2011, vol. 7, No.2
5. Naveed Ahmed, Millán Michelin-Jamois, Hatem Fessi and Abdelhamid Elaissari. Modified double emulsion process as a new route to prepare submicron biodegradable magnetic/polycaprolactone particles for in vivo theranostics. **Published** in Soft Matter, 2012, 8, 2554
6. N. Ahmed, N. M. Ahmad, H. Fessi and A. Elaissari. *In Vitro* MRI of hybrid (iron oxide/poly caprolactone) magnetic nanoparticles prepared via modified double emulsion evaporation mechanism. **In preparation for submission.**
7. Naveed Ahmed, Chiraz Jaafar-Maalej, Mohamed M. Eissa, Hatem Fessi and Abdelhamid Elaissari. Magnetic nanoemulsion for detection of minimum iron oxide quantity for *in vivo* magnetic resonance imaging. **Submitted** in J. Biomed. Nanotechnol.

Titre: *Elaboration de nanoparticules pour l'application theranostic: In vivo imagerie et délivrance de médicament.*

Résumé

L'objectif de ce travail a été la préparation de nanoparticules pour l'administration intratumorale. La double fonctionnalisation de ces nanoparticules est rapidement approuvée comme extrêmement intéressante, car elles peuvent être utilisées pour le diagnostic *in vivo* et la thérapie (théranostics). Pour réaliser ce type de nanoparticules, l'oxyde de fer a été choisi comme agent de contraste pour une utilisation en imagerie par résonance magnétique (IRM) pour le diagnostic de cellules cancéreuses. Les nanoparticules d'oxyde de fer ont été préparées dans des milieux aqueux et organiques. La méthode d'émulsification multiple suivie de l'évaporation de solvant a été utilisée pour l'encapsulation concomitante d'un principe actif et des nanoparticules de l'oxyde de fer. Tous les paramètres affectant la taille des nanoparticules pendant le procédé ont été étudiés en utilisant une molécule active hydrophile modèle (une dérivée de la Stilbene) et le protocole standard a ensuite été évalué. Dans une seconde étape, les particules d'oxyde de fer ont été encapsulées par la même méthode d'émulsion évaporation et entièrement caractérisées en termes de morphologie, taille, magnétisation, et composition chimique. La visualisation *in vitro* des particules modèles a été réalisée par IRM et comparée à un produit commercial à base de Gadolinium (Gd). D'autre part, la quantité nécessaire d'oxyde de fer permettant une bonne visualisation par IRM a été déterminée par une étude *in vivo* menée sur des souris. Enfin la double encapsulation d'oxyde de fer avec une molécule anticancéreuse a été effectuée par la méthode développée, la formulation obtenue a été entièrement caractérisée.

Title: *Elaboration of nanoparticles for theranostic applications: In vivo imaging and drug delivery*

Summary

The objective of this work was the preparation of nanoparticles for intratumoral administration. Important characteristic was dual functionality of these nanoparticles that they can be used for diagnosis and therapy so the resulting nanoparticles will serve as theranostic agents. For this purpose iron oxide was chosen as a contrast agent to be used in Magnetic resonance imaging (MRI) for diagnosis of cancerous cells. Iron oxide nanoparticles (IONPs) were prepared in aqueous and organic medium. A multiple emulsion evaporation method was designed for the encapsulation of active ingredient (hydrophilic drug i.e Stilbene) and the IONPs. All the parameters affecting the colloidal properties final hybrid particles were studied and characterization was done for final particles. Then prepared particles were evaluated for *in vitro* MRI and also compared with commercially available products such as gadolinium (Gd). At same time, the minimum detectable quantity of iron oxide *in vivo* was determined using a commercialized iron oxide emulsion on rat models. Finally an anticancer agent was encapsulated with IONPs using same multiple emulsion method and characterization was done.

MOTS-CLES: Cancer, Nanoparticules, Délivrance de médicament, Encapsulation, Anticancéreux, Agent de contraste, Oxyde de Fer, *In vitro*, IRM, Theranostic

KEY WORDS: Cancer, Nanoparticles, Drug delivery, Encapsulation, Anticancer, Contrast agent, Iron oxide, *In vitro*, MRI, Theranostic

Laboratoire d'Automatique et de Génie des Procédés (LAGEP) UMR 5007 : Université Claude Bernard Lyon 1
bât 308G ESCPE-Lyon, 2ème étage, 43 bd du 11 Novembre 1918, 69622 Villeurbanne Cedex FRANCE

