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Magnetic targeted drug delivery system in gene therapy

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ABSTRACT

MAGNETIC TARGETED DRUG DELIVERY SYSTEM IN GENE THERAPY

by
Weilong Lu

Drug delivery system is a method to transport the drug of the required amount accurately to the targeted diseased part. Recently, the concept of Magnetic Targeted Drug Delivery System (MTDDS), based upon magnetic particles under the action of an external magnetic field, exhibits considerable potential for a wide range of medical applications. Gene therapy is the insertion of genes into an individual's cells and tissues to treat diseases. As the recombinant virus vector has many limits and problems, more studies turn to the nonvirus vectors, which have transfection efficiency and low cytotoxicity. PLLA(poly(L lactic acid)) can be biodegradable and has high biocompatibility. PLLA is already used in functional biomedical materials, including functional gene vectors.

This thesis covers the synthesis and characterization of superparamagnetic iron oxide nanoparticles and summarizes the research process of polymeric coating as a function of gene vectors. Nuclear magnetic resonance technology is used to identify the structure of the polymer step by step. At last, the use of PLLA(poly(L lactic acid)) in gene therapy is combined with the MTDDS. The PEI-PLLA-SS-PLLA-PEI was successfully made and could be applied for fabrication of superparamagnetic iron oxide nanoparticles as polymeric coatings at nanometer scale.

MAGNETIC TARGETED DRUG DELIVERY SYSTEM IN GENE THERAPY

by
Weilong Lu

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APPROVAL PAGE

MAGNETIC TARGETED DRUG DELIVERY SYSTEM IN GENE THERAPY

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Nothing is impossible

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CHAPTER 1

INTRODUCTION

Targeted Drug Delivery System and Gene Therapy

Nanotechnology for diagnosis, treatment and monitoring diseases is a fast growing field in biomedical research, in which drug delivery system is the most promising area [1]. Drug delivery system is a method to transport the drug of the required amount accurately to the targeted diseased part, so that the drug efficiency is enhanced and the side effect is reduced. Recently, the concept of Magnetic Targeted Drug Delivery System (MTDDS), based on magnetic particles under the action of an external magnetic field, has shown considerable promise for a wide range of medical applications including cancer therapy, gene therapy, blood detoxification, and radiation treatment.

Compared with the traditional methods, MTDDS has the advantages of quick-impact, high efficiency, reduction in toxicity as well as minimizing some adverse side effects in the non-target region when the drug is concentrated in the specific diseased part [2]. MTDDS technology has significantly evolved since the early 1970s; two most important aspects are the use of superparamagnetic iron oxide nanoparticles (SPIONs) and the utilization of external magnetic fields. Firstly, biocompatible superparamagnetic iron oxide nanoparticles (SPIONs) with proper characteristics, including colloidal stability, shape, size, size distribution, surface charge and toxicity, are the best nanoparticles among all types for various biomedical applications. Secondly, in this technology, one of the most significant issues is the methodology to guide and locate the transport of SPIONs. There are two limitations. One is that the high blood flow velocity in the human arteries has

negative influence on the accumulation of drug-filled nanoparticles. Another main problem for translating fundamental research into clinical practice is the depth the magnetic fields can reach at a given target site [3]. Therefore, the optimization of the external magnetic field and the improvement of the magnetic properties of nanoparticles are the key to satisfy the requirements of MTDDS for the control of transport of the SPIONs in the human body. Most of the experiments that have been performed in the literature are related to the improvement of the coating on the magnetic carriers and the method of synthesis in targeting. Figure 1.1 describes the processes of preparation of magnetic particles for drug delivery.

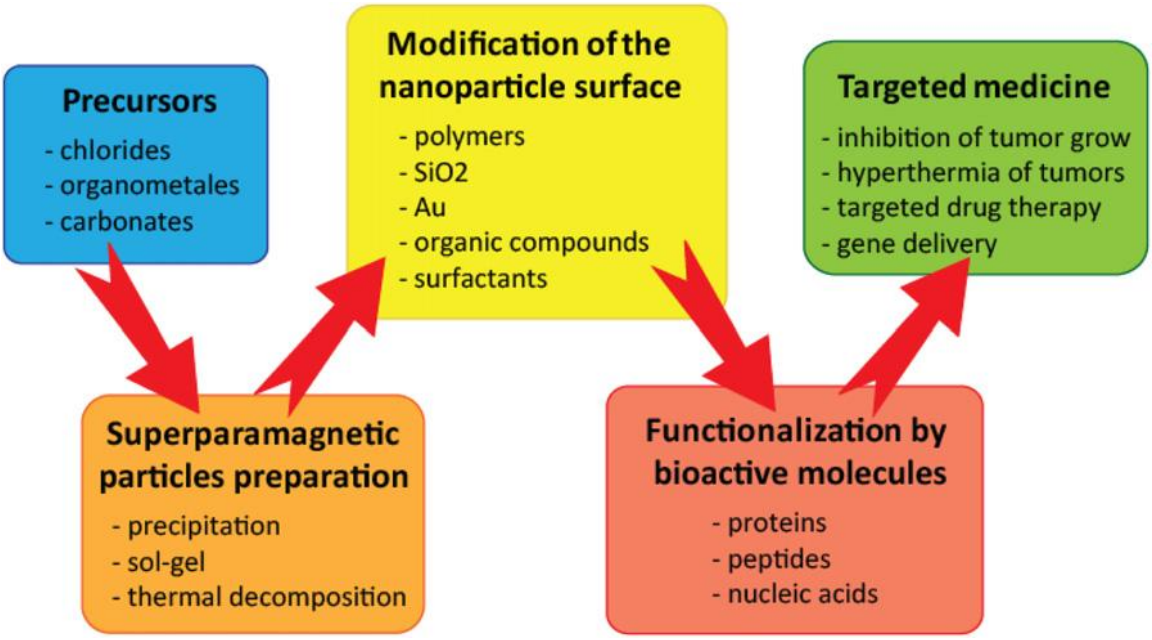

Figure 1.1 Processes of magnetic particles preparation for drug delivery. Source: [4]

Coating of SPIONs with polymers used for drug delivery is the most commonly used way, because the surface of polymers provides better physical and chemical

properties [4]. Moreover, the polymer shell of SPIONs has the advantage of good dispersion, high stability against oxidation and low toxicity. Polymer coatings are widely used in gene therapy for targeted drug delivery.

Gene therapy is the insertion of genes into individual cells and tissues to treat diseases, such as hereditary diseases where deleterious mutant alleles are replaced with those that are functional. For instance, using the specific gene carriers to transport treatment materials such as genes and nucleic acid drugs into the targeted cells in order to produce bioactivators to cure the disease due to the loss of genes [5] is a good example. The supporter of the gene is DNA which is hard to get access into the cells and is easy to be degraded in the blood and cells. Thus, a specific carrier must be used to transport the DNA to targeted area of the human body. Normally, the carriers of the DNA can be classified into virus vector and non-virus vector. As the recombinant virus vector has limitations and problems, studies are being conducted on the nonvirus vector, which have transfection efficiency and low cytotoxicity. PLLA(poly(L lactic acid)) can be biodegradable and has high biocompatibility. PLLA is already being utilized in functional biomaterials, including functional gene vectors.

This thesis covers the synthesis and characteristics of SPIONs and summarizes the research that is focused on the process of coating polymers to functionalize gene vectors. NMR (Nuclear Magnetic Resonance) spectroscopy has been used to identify the structure of the polymer step by step.

CHAPTER 2

METHOD AND CHARCTERISTICS

2.1 Synthesis of SPIONs

In the field of implant-assisted magnetic drug targeting, magnetic nanoparticles (NPs) such as octahedral magnetite NPs are always synthesized, silanized, and attached covalently to tPA (tissue plasmonigen activator). It is known that the NPs can be targeted accurately to a ferromagnetic coil with the coil placed in a magnetic field. These are based on in vitro flow-through studies [6].

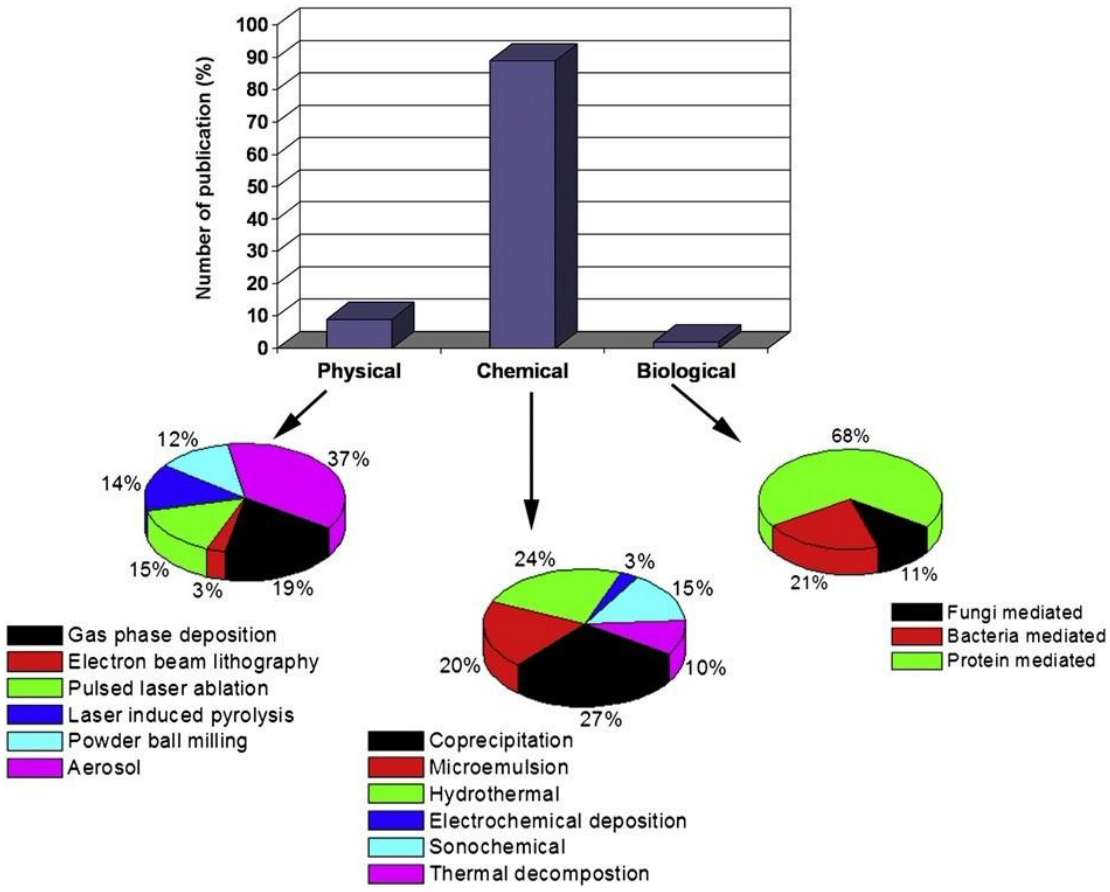

Figure 2.1 A comparison of published work (to date) on the synthesis of SPIONs by three different routes. Source: [7]

For the category of SPIONs, magnetite, maghemite and hematite are mainly used. Other reported superparamagnetic oxides are ferrites, which are mixed oxides of iron and other transition metal ions (for example, Cu, Co, Mn, and Ni).

There are three most significant published routes for the synthesis of SPIONs as presented in Figure 2.1. As for the past chemical routes, one report suggests using ferrous salt in the presence of potassium hydroxide to make spherical magnetite NPs by a co-precipitation method. Recently, sol-gel methods, in which precipitation from the solution as a basic way of crystallization, are being used [7]. In this case, under the diffusion from the solution to the surfaces, the nuclei can grow uniformly. However, due to the Oswald ripening, large uniform crystals will form through crystal growth by the dissolution of small crystallites. Besides, the aggregation of small crystallites through coalescence may also lead to large uniform particles.

The method of using the addition of base to an aqueous solution of ferrous and ferric ions in a 1:2 stoichiometry to make a black precipitate of spherical magnetite NPs of uniform sizes under an oxygen free environment is the most common one [8]. Compared with this method, another method which produces larger nanoparticles is reported recently by controlling the ratio of ferrous and hydroxide ions.

In order to produce sub nanometer size SPIONs, the most important factor is to control the crystal growth step during co-precipitation. The micro-emulsion method is an alternative and more controlled method compared with another method that uses surfactants.

A recent breakthrough in the aspect of SPION synthesis is the sonochemical routes, in which the formation and growth of nuclei and the implosive collapse of bubbles are

created at very high temperatures [9]. Another method called electrochemical deposition under oxidized condition has also been used for the synthesis of SPIONs. Both the methods cannot produce large scale synthesis. In order to solve this problem, using the hydrothermal method by microwaves can scale up the size of SPIONs. Besides, from earlier studies, bacteria have been used in the biomimetic synthesis of SPIONs.

2.2 Shape and Size

The biodistribution of non-spherical and rod shaped nanoparticles must be studied when doing research on the effects of nanoparticles on biodistribution. From some old reports, it is shown that anisotropically shaped nanoparticles can avoid biolimitation better than spherical nanoparticles [10]. The ratio of in vivo blood circulation time of anisotropic nanostructures to spherical nanoparticles has been proved to relate to the increase in the length-to-width aspect ratio of the nanostructure. Also, the high aspect ratio shaped magnetic nanoparticles are shown to increase the blood circulation times over the spherical counterparts in vivo. Detailed research is needed to understand what aspect ratios yield most dramatic influence in the study of nanoparticles pharmacokinetics.

The size and size distribution play very important roles in the application of SPIONs. Mahmoudi et al. [11] have investigated the effects of the applied magnetic field on the SPIONs of various sizes using a Multiphysics finite element model in a simulated blood vessel. In this case, a 2D model triangular mesh consisting of a blood vessel, 1 cm in width, tissue, 1 cm in width, and an external magnet are used. From Figure 2.2A, the fluid flows from the left side to the right side. In order to make different sizes and shapes of SPIONs, various synthesis parameters were used. From Figure 2.2, the FEM model can be

used to predict both the induced magnetic flux density and fluid velocity fields by solving coupled Maxwell and Navier-Stokes equations. It is also shown that the velocity field fluctuations (flow turbulence) and amplitude (peak velocity) are affected by the strength of the applied magnetic field and magnetic properties of the SPIONs. Interaction exists between these parameters in determining the shape and amplitude of the velocity field. Less fluctuation and higher amplitude of the velocity field are suggested by the author, which is preferable to facilitate the drug delivery, especially in the direction normal to the tissue.

It has been proved that the magnetic dipole-dipole interactions in SPIONs are efficiently decreased by the fact that they scale as r^6, where, r is the particle radius. One advantage was reported that the saturation magnetization and size of SPIONs are correlated, as the surface curvature changes with size linearly, by Varanda et al. [12]. Another important advantage is their higher effective surface (easier attachment of ligands), increased tissular diffusion and lower sedimentation rates (i.e., high stability in suspension) when the used nanoparticles are smaller than 100nm. In order to escape from the reticuloendothelial system, the size of the particles must be smaller than 100 nm. They should remain in the circulation system when the injection is done and can pass through the capillary systems of organs and tissues without vessel embolism. In addition, the stable permeability and retention effect are affected by the size of the particle. For example, particles larger than 10 nm can penetrate the endothelium at pathological conditions such as tumour infiltration but cannot penetrate in physiological conditions.

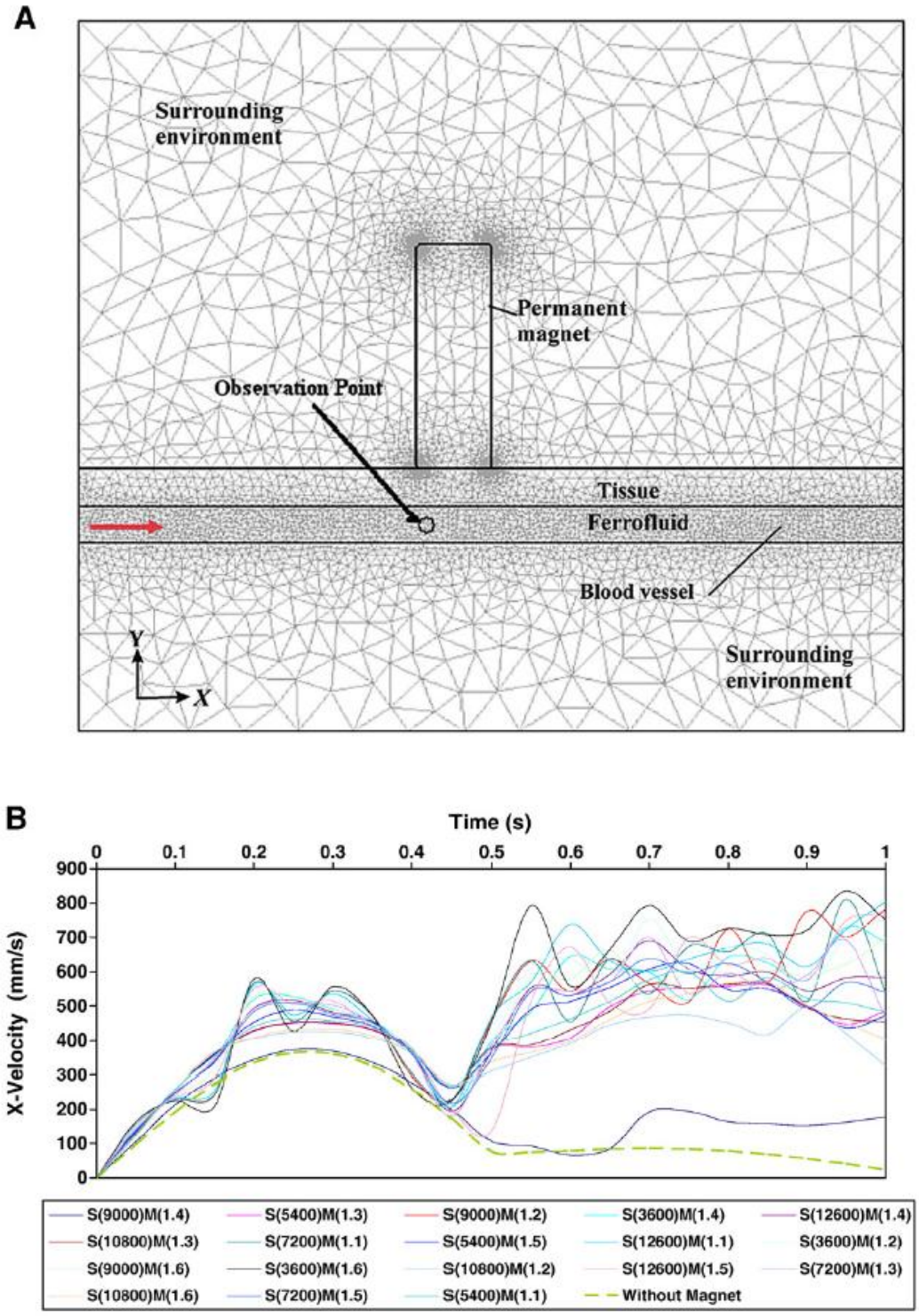

Figure 2.2 (A) 2-D geometry and finite element mesh model of a blood vessel under a permanent (static) magnetic field for drug delivery. The (B) x is velocity of the ferrofluid containing SPIONs with various sizes. Results are presented for the observation point shown in (A). Source: [11]

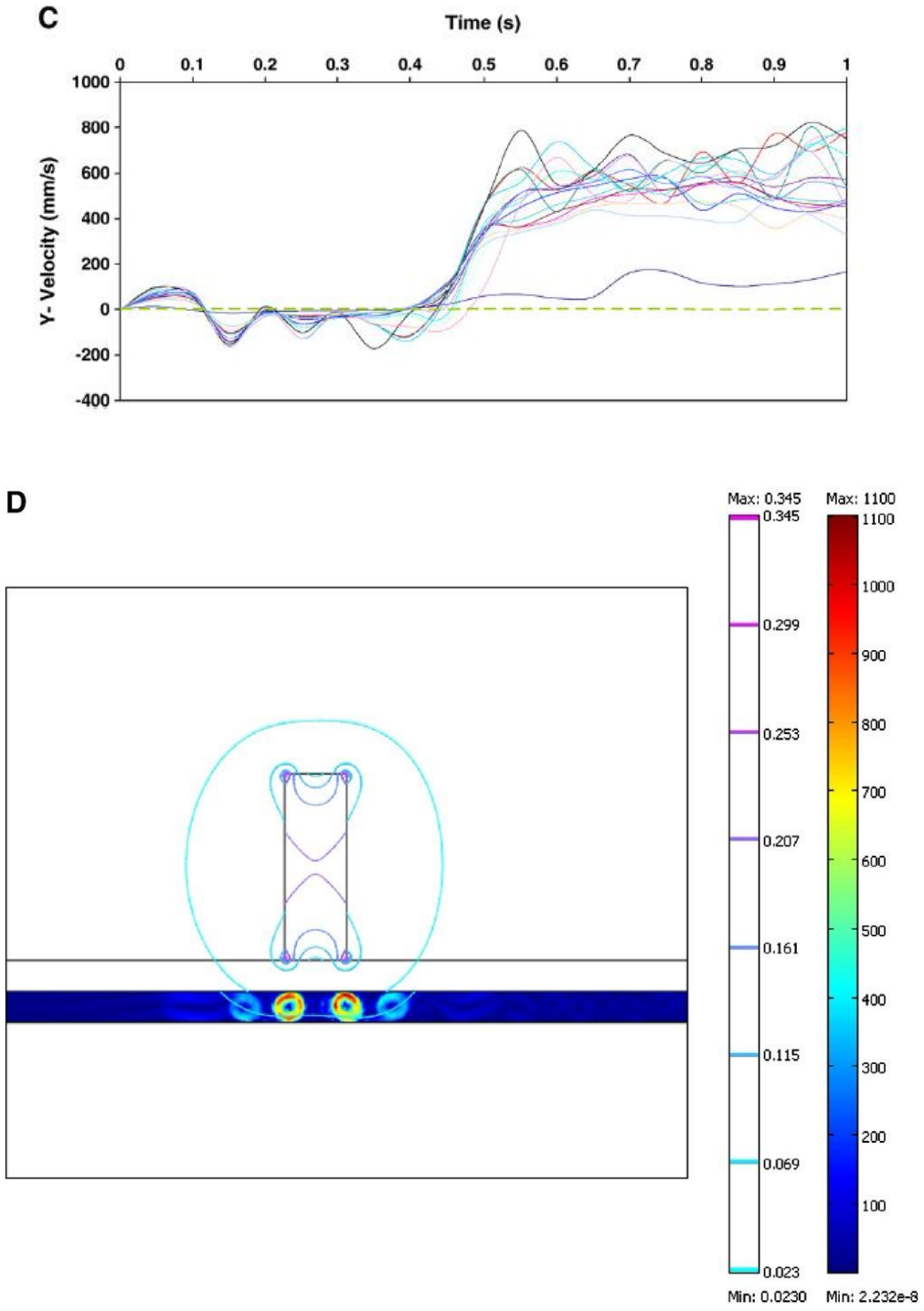

Figure 2.2 The (B) x and (C) y are velocities of the ferrofluid containing SPIONs with various sizes. Results are presented for the observation point shown in (A). Samples are herein referred to as S(x)M(x), where, S is the stirring rate and M is the NaOH molarity. (D) contour lines of magnetic flux density and ferrofluid velocity surface contours in the blood vessel model (note: results are shown for t = 1 s for S(12,600)M(1,1); at the observation point marked in (A), the value of magnetic flux density is 27.7 mT. Source: [11]

The sizes of SPIONs are measured either in dry state or in suspension by many different ways. However, in the application, the size in dry state is more important because of the solvation shell around the SPIONs. The aggregation behavior is affected by the drying step. Dynamic light scattering (DLS), transmission electron microscopy (TEM), Scherrer analysis of X-ray diffractograms (XRD) and extended X-ray absorption fine structure (EXAFS) are used to investigate the size of the nanoparticles. It is the DLS technology which determines the size of the particles in suspension while other methods determine the crystallite size in the dry state. The DLS technology is based on volume, intensity and number distribution and Scherrer analysis of XRD is based on the broadening of the diffraction peak. The most powerful technique is TEM which gives information on both the core-shell structure and the size distribution by the difference in electron density of core and shell materials, so that it can determine the crystallite and sizes of nanoparticles and their morphology.

After the drug-loaded nanoparticles are injected into the human bloodstream, the three most important parameters are the size, size distribution and surface charge. The size of the nanoparticles should be above 200 nm or below 10 nm since other sizes will cause the particles' uptake by the RES. As for the behavior of injected nanoparticles, the biodistribution and the surface charge of SPIONs determine the colloidal stability due to the nature and behavior of the surface groups in solution at a certain pH in the presence of an electrolyte.

2.3 Toxicity

Many well known examples of the use of SPIONs in biomedical applications have been reported including drug delivery, cellular labeling/cell separation, tissue repair, magnetic resonance imaging, magnetic hyperthermia and magnetofection. Toxicity of the SPIONs and the individual parts must be investigated to avoid harm to the patient when the MTDDS is developed. There are two things that must be taken into consideration when evaluating nanoparticle toxicity. One is how the assembled nanoparticle system will interact with the body in its functional lifetime; another is how the independent components will affect the human body during biodegradation and liver processing [13]. Though nanotoxicology is a promising field of research, more experiments are needed to investigate the human body's response to the SPIONs.

2.3.1 Toxicity of SPIONs

Mahmoudi et al. [11] have explored the cytotoxicity of polymer coated SPIONs with different shapes and morphologies including nanospheres, nanorods, nanoworms, magnetite colloidal nanocrystal clusters and nanobeads in mouse fiberblast cells and human leukemia cells. Few or no toxicity is found in his research. In addition, when the toxicity is studied in human lung cancer cell lines, neither DNA damage nor intracellular toxicity effects were observed. However, small oxidative DNA lesions were detected. In another study by Mahmoudi et al. [14], gas vesicles in SPION-treated cells were observed with increased granularity of the cells (Figure 2.3).

As a result, it showed that the cytotoxicity may lead to the autophagy. Since large amount of protein absorption exists on the surface of SPIONs, the in vitro cytotoxicity studies can encounter a great deal of error. Modifications have been made in the method

that uses cell medium to introduce the SPIONs and keeping the solution in contact for a period of 24h in order to create a stable protein corona on the surface of the SPIONs [15]. As a consequence, reliable and exact cytotoxicity results were obtained.

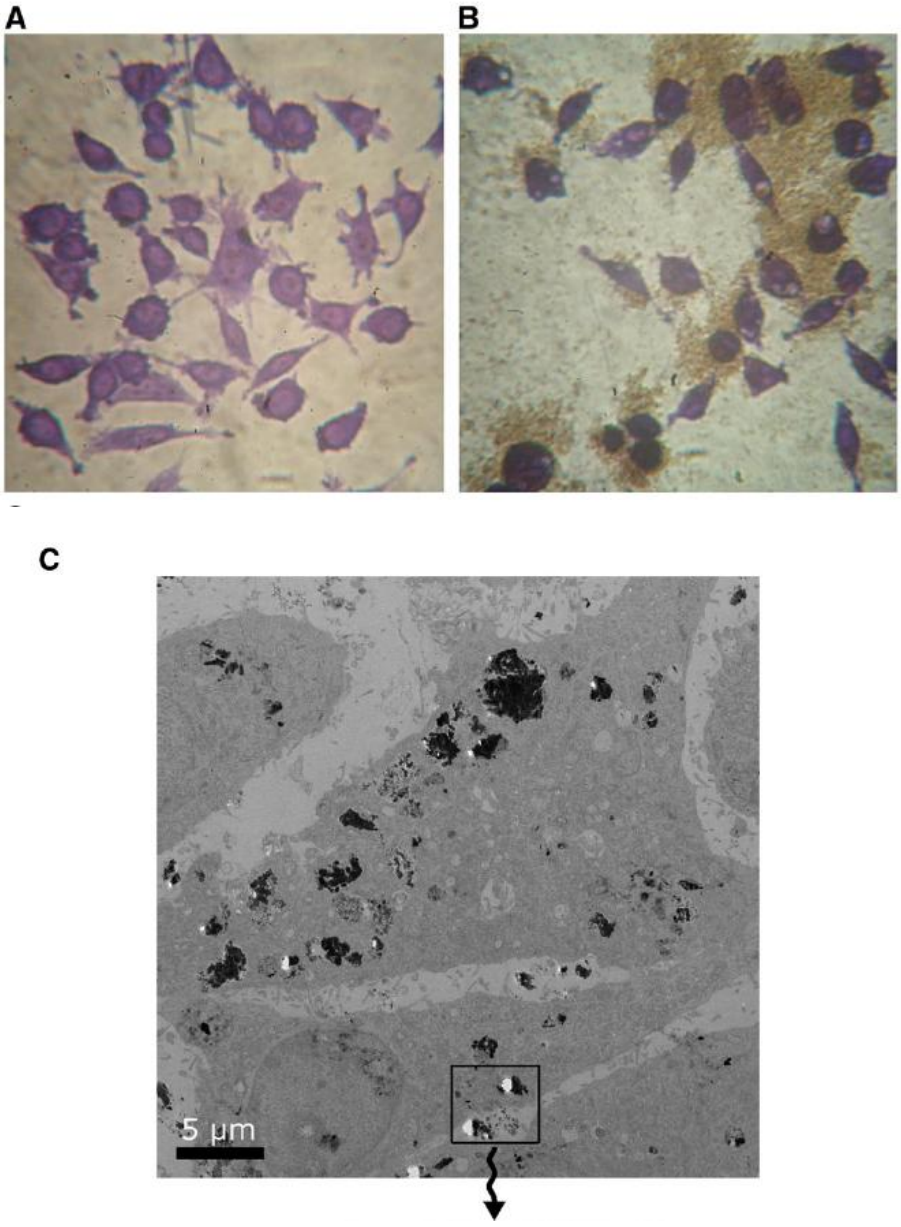

Figure 2.3 Optical microscopy (800X) of dyed L929 cells for (A) control, (B) cells containing 800 mM (millimolar) uncoated SPIONs after a 72h interaction with cells showing the existence of gas vehicles. (C) TEM images of SPIONs-treated cells showing gas vehicles.Source: [14]

After the medium was replaced with a fresh one, the SPIONs obtained were used for toxicity assays. Since the nanoparticles can lead to significant changes in the cell medium, namely denaturation of proteins, the conventional in vitro examination methods contain large errors compared to the modified method. In this case, the toxicity of SPIONs can be decreased by this modified method.

2.3.2 Protein-NP Interaction

Having been developed intensively for a long time, the study of the interactions between nanomaterials and proteins becomes very important in the medical application of nanoparticles. Proteins can be adsorbed or associated in a biological fluid which will have significant influence on the biological, biochemical and cellular behavior. It is well known that the nanoparticle-protein interaction plays a very important role in defining the toxicity of nanoparticles [16]. In order to foresee biological injuries caused by changes such as fibrillation, the unavoidable changes in the protein configurations should be probed. It is the SPION-protein interaction that causes this change. What is more, a new immune response will take place followed by the denaturation of the protein after interaction with the SPIONs due to the exposure of new antigenic sites. In order to study the interactions, there are three normal ways including size-exclusion chromatography (SEC), surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). ITC and SEC studies are more related with the affinity and stoichiometry of protein bonded to particles, SPR gives the rates of protein association and dissociation.

For the interaction between the large numbers of potential proteins, there exists one problem which is to find a way that has the capability to measure such interactions. One

method has been introduced to overcome this problem using an interaction analysis platform based on a highly parallel and sensitive microfluidic affinity assay [17].

CHAPTER 3

POLYMER COATING

Although the unmodified SPIONs are stable in high and low pH solutions after synthesis, they need to be coated before using them in vivo study. Polymers and small organic molecules are most commonly used materials for coating of MNPs for drug delivery, as demonstrated in the several following functions: (1) enhance the stability of nanoparticles against oxidation, (2) chemical handles for the conjugation of drug molecules, targeting ligands, and reporter moieties and (3) limit non-specific cell interactions. Additionally, it was found surface functionalization plays the key role in nanoparticle toxicity. In order to enhance SPION pharmacokinetics, endosomal release, and tailored drug loading and release behaviors, polymeric coatings have been widely engineered including PEG, dextran, chitosan, PEI, and phospholipid.

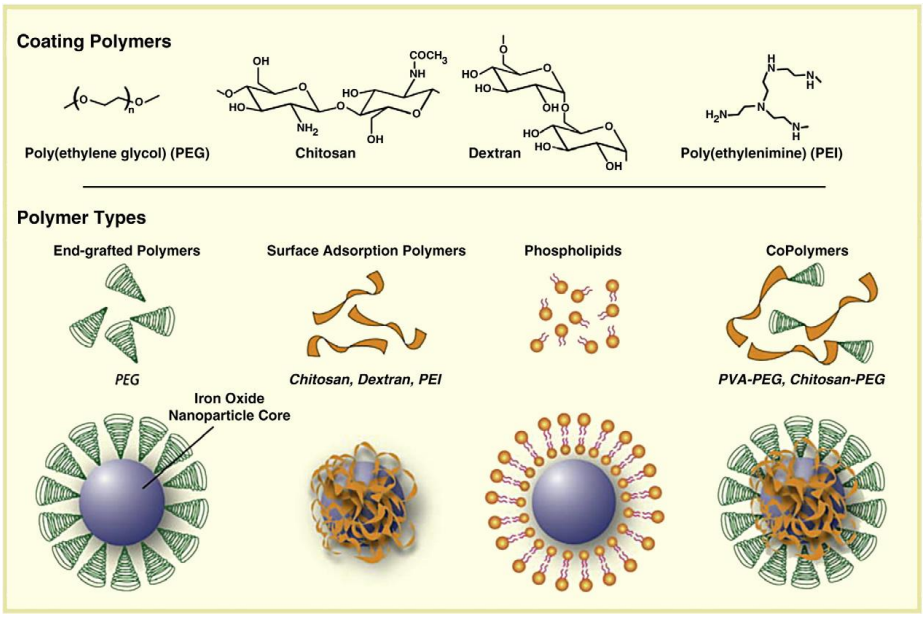

Figure 3.1 Illustration depicting the assembly of polymers onto the surface of magnetic nanoparticle cores. Source: [18]

There are many approaches which can be used to build SPION coating such as in situ coating, post-synthesis adsorption and post-synthesis end grafting [18] (Figure 3.1). In situ and post synthesis modification with copolymers and polysaccharides lead to coatings that uniformly encapsulate cores.

Reshmi et al. made a preparation of composite colloidal core/shell nanoparticles, which are made of magnetic core (carbonyl iron) and biodegradable polymeric shell (cellulose acetate hydrogen phthalate) [19]. During the biodegradation of the polymeric shell, it could transport and release the drug.

Magnetic nanoparticles tend to aggregate because of the strong magnetic dipole-dipole attractions between particles. Dextran and chitosan are usually coated on the surface of the MNPs in order to avoid the aggregation [20, 21].

Dextran is a branched polysaccharide consisting of glucose subunits which can be used to prepare with sizes ranging from 10 to 150kDa (1 kDa = 1000 molecules). It has the properties of biocompatibility and polar interactions (chelation and hydrogen bonding) that make dextran a high affinity for the wide use in iron oxide SPION coatings. In 1982, Molday and Mackenzie first described the dextran coatings prepared by in situ techniques [22]. Since then, many different forms of dextran polymers have been used such as carboxydextran and carboxymethyl dextran. Conventional dextran coatings are based on hydrogen bonding, which makes the polymer susceptible to detachment. However, after using epichlohydrin and ammonia, forming a CLIO (cross-linked iron oxide (CLIO)-NH_2), the dextran polymers have been crosslinked due to the SPION attachment [23]. In addition, their use in clinical setting becomes unlikely because of the use of epichlohydrin and the inability to degrade and clear from the body.

Recently, chitosan is becoming more and more popular in drug delivery due to its nontoxic, biocompatible and bioabsorbable properties. It is a cationic, hydrophlilic polymer that has large abundance in nature, and ease of functionalization. In the past, chitosan and its derivatives have been used to make polymeric nanoparticles through electrostatic complexation with nucleic acids and different pharmaceutical processes [24]. Different from dextran coatings, the chitosan coatings are produced by physically adsorbing chitosan onto oleic acid-coated nanoparticles making spherically shaped SPIONs (15 nm diameter) [25]. Chitosan can be used as genetic materials for the preparation of gene delivery carrier, even when used as a SPION coating because of its cationic nature. The SPIONs serve to enhance the gene transfection. For example, Kumar et al. reported chitosan coating on Fe_20_3 MNPs [26]. This technology would constitute a useful tool for gene/drug delivery in vivo due to the safe transport and concentration in targeted area in the body. In addition, when the drugs are modified with antibodies, protein, or ligand, the in vitro drug delivery can be improved. For the in vivo study, before coating with chitosan, Fe_20_3 MNPs were synthesized in aqueous medium without surfactants; then they were conjugated with plasmid DNA expressing enhanced green fluorescent protein (EGFP). The aeration oxidized the colloidal magnetite suspensions and the Fe_20_3 MNPs formed. Besides its bioadsorbtive properties, chitosan can be used for SPION functionalization with targeting, imaging, and therapeutic agents due to the amino and hydroxyl radicals.

Another water soluble cationic polymer coating material is polyethyleneimine (PEI). PEI can be both linear and branched forms and has been used for gene delivery for a

long time due to its ability to synthesize with DNA, accelerate endosomal release, and lead to intracellular trafficking of the cargo into the nucleus [27].

Abdalla et al. performed some research on the drug loading efficiency of polylactide (PLLA) and poly (1-lactide acid- co-gylocolide) (PLGA) polymeric systems of many different molecular weights [28]. It is shown that the molecular weight of the polymer has significant influence on the capacity of the drug loading on the polymer surface.

Copolymers have been developed because of their distinct functionalities from their components. The advantages of these copolymers can be applied to SPION coatings.

CHAPTER 4

GENE VECTORS

Gene therapy is the insertion of genes into individual cells and tissues to treat diseases such as hereditary diseases where deleterious mutant alleles are replaced with functional ones. For instance, using the specific gene carriers to transport treatment materials such as genes and nucleic acid drugs into the targeted cells in order to produce bioactivators to cue the disease due to the loss of genes [5] is a good example. The supporter of the gene is DNA which is hard to get access into the cells and is easy to be degraded in the blood and cells. Thus, a specific carrier must be used to transport the DNA to targeted area of the human body. Normally, the carriers of the DNA can be classified into virus vector and non-virus vector.

4.1 Gene Vectors

4.1.1 Virus Gene Vectors

Virus have been evolved for billions of years and they have their own strategy to intrude into the cells. With the application of this strategy, people use virus as vector for gene therapy, remove the pathogenetic part of the gene and plant healing gene into the virus vector so that it can access into the cells and express related proteins. Among all the virus vectors, adenovirus and retrovirus are used frequently. But virus vector also has safety problems. As most of the virus vectors are with relatively high immunogenicity, human immune system will identify the virus and generate large amounts of antibodies which makes the virus and the DNA along with it both be phagocytosed and degraded at the same

time [29]. The selected cloning is defective, so the cloning protein will trigger the human immune system and cause damage to target cells, which deceases the transfected cells and then shortens the target cells' duration to a certain extent [30,31].

4.1.2 Non-virus Gene Vectors

As non-viral gene vectors have shown multiple potential advantages in the aspects of experiment and treatment, more and more researchers choose non-viral gene vectors for genetic therapy research. Thus more and more non-viral gene vectors appear, such as liposome or liposome complex vectors, cationic polyphosphate vectors, naked DNA, nanometer materials, human artificial chromosome and so on [32].

(1) Liposome or liposome complex vectors

Liposome is enclosed particles with bilayer structure. Cationic liposome has a relatively accurate molecular structure and its basic structure is hydrophobic chain attached to cationic head group. Cationic liposome compound can become Cationic liposome/DNA compound (Lipoplex) through static electricity self-assembly. Lipoplex gets into cells through endocytosis, thus releases the gene which it loads to complete transfection. Cationic liposome compound will not generate immune response and has advantages such as easily prepared liposome and stabilized storage. It is widely used in genetic therapy because of all these qualities. Also, it is recently considered as one of the supplies for supermolecule drug delivery system research. Now some cationic liposome compounds with excellent performance such as DOPE, DOTMA, Lipo2000 have been commercialized.

But, liposome complex is formed according to spontaneous dynamics, which makes it hard to control. So the cationic liposome that is obtained is usually a compound of

multiple structures. The physiochemical stability of cationic liposome compound in body fluid is not good enough and the compound is easy to be cleared and exit body cycles. Most of the cationic liposome/genetic compound is hard to be released under control, the cytotoxicity is relatively high and it is hard to make it on a large scale [33].

(2) Cationic polyphosphate carrier

Through the electrostatic combined action, cationic polyphosphate carrier, together with gene, can form a composite particle with appropriate grain size and the ability of transfection. This particle enters cell by endocytosis and moves further into nucleus to integrate with the gene. Then, in order to accomplish treatment by expression of target gene, PEI is a very effective gene transfer vector. Amphipathic triblock gene vector is the hottest research area of gene vectors.

(3) Naked DNA

Attach target gene to the plasmid to be expressed and inject this reorganized naked DNA into the cells directly to express without relying on mediation of other materials.

(4) New nanometer materials gene vectors

Nanometer material gene vectors can combine with the plasmid to be expressed very well and accomplish local gene transfection and expression within the body and play its relevant effect.

(5) Human artificial chromosome.

Since the traditional virus carrier has immunogenicity and will cause genetic mutation after inserting host genomes, it is difficult to achieve safety and efficiency transfection results. Thus people consider building bionic artificial gene for the gene transfection, which will become a reasonable gene vector in the future.

4.2 Linear PLLA Amphiphilic Cationic Gene Vector

The high polymer material, poly-lactic acid (PLA) which is made by lactic acid polycondensation not only has excellent mechanical strength and chemical stability, but also has good biocompatibility, absorbability and environmental degradability [34]. Therefore, PLA is a very promising medical physiological material and, generally, environmentally friendly high polymer material. It is also one of the key areas for international biodegraded materials research. The linear polylactic acid cationic genetic vector that will be introduced is synthetized by L-Lactic Acid and D-D-Lactide. Linear PLLA amphipathic triblock gene vector has become a new candidate in macromolecule genetic vectors with its excellent functions and biodegradability.

CHAPTER 5

EXPERIMENTAL RESULTS AND DISCUSSION

Nuclear magnetic resonance (NMR) is a physical phenomenon in which magnetic nuclei in a magnetic field absorb and re-emit electromagnetic radiation . This energy is at a specific resonance frequency which depends on the strength of the magnetic field and the magnetic properties of the isotope of the atoms; in practical applications, the frequency is similar to VHF (Very high frequency) and UHF (Ultra high frequency) television broadcasts (60--1000 MHz). NMR enables the observation of specific quantum mechanical magnetic properties of the atomic nucleus. Many scientific techniques exploit NMR phenomena to study molecular physics, crystals, and non-crystalline materials through NMR spectroscopy. NMR is also routinely used in advanced medical imaging techniques, such as in magnetic resonance imaging (MRI) [35].

In this chapter, the experimental methods used in this research will be introduced. All the samples were made by Ziqian Fan, a Master's degree candidate in the lab. Fan used HEDS (Hydroxyethyl Disulfide) to initiate the ring-opening polymerization to form PLLA-SS-PLLA. Then, cationic groups were attached to the PLLA-SS-PLLA to form embedded zone molecules PEI-PLLA-SS-PLLA-PEI.

In this work, NMR spectroscopy was utilized to investigate the structure of the polymers in every polymerization step. This chapter focuses on the results of NMR spectroscopy.

5.1 Purification of Reagent

The reagents were purified to avoid the influence of impurities on the experimental results.

In Figure 5.1, the NMR spectra of lactide, after purification, are shown from region 0 to 7 PPM. Two peaks at 1.68 and four peaks at 5.05 are observed. It can be seen clearly that the two peaks represent the a-H on the methyl of lactide at 1.68ppm. Also, it can be seen that the four peaks at 5.05 PPM represent the b-H on the ring of lactide.

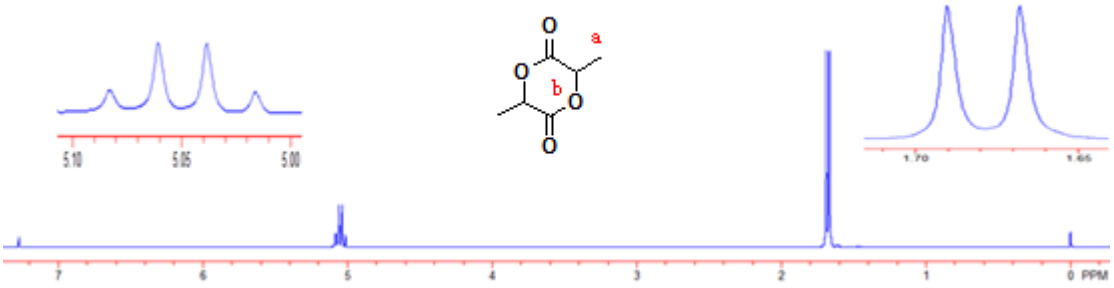

Figure 5.1 NMR spectra of lactide after purification .

Reduced pressure distillation was used to purify the HEDS. The purified HEDS are divided into three parts. In Figure 5.2, the NMR spectra of purified HEDS are shown. It can be seen that the first cut fraction and the second cut fraction are the same HEDS.

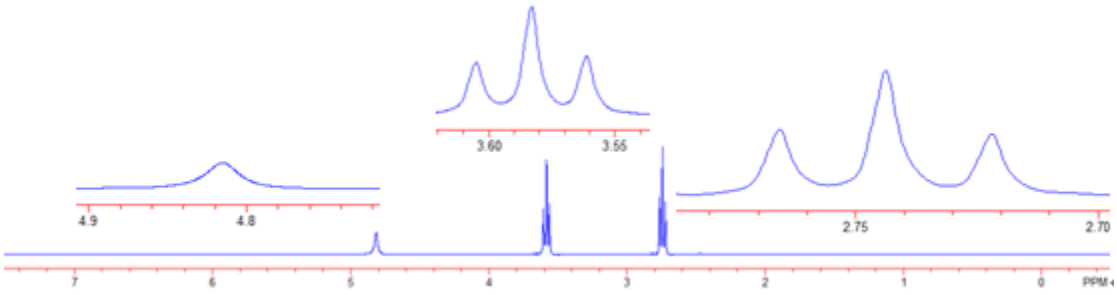

Figure 5.2 NMR spectra of HEDS after purification.

5.2 Characterization of PLLA-SS-PLLA

In 2001, Hedrick [36] has reported synthetizing polylactic acid by using DMAP (Dimethylaminopurine) to catalyze ring-opening polymerization of lactides. This catalyst system requires mild condition. It can react successfully under room temperature. It will not have racemization and chain transfer reactions during the polymerization process. It can form structured linear polymer. Hou [37] and others have synthetized PLLA-SS-PLLA, which has a more narrow molecular weight distribution and the reaction can be easily controlled.

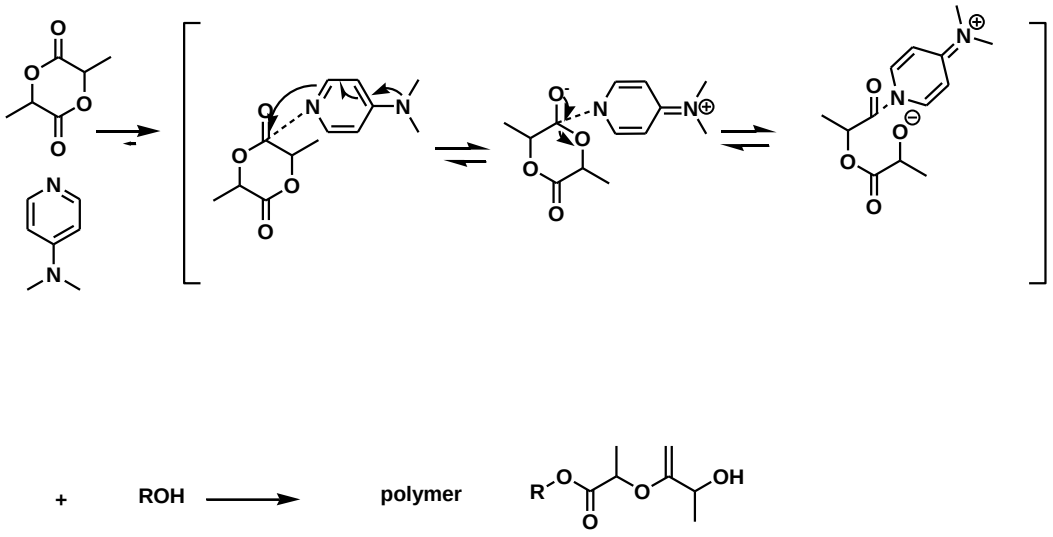

Figure 5.3 The principle of ring-opening polymerization of lactide catalyzed by DMAP.

Another advantage of using DMAP as the catalyst is no metals will be left thus avoiding their influence on the performance of polylactic acid.

For the macromolecules with lipophilic cations, its ability to integrate with DNA will be changed due to change in the ratio of hydrophobic chains and hydrophilic chains. The DNA will be released after the bond break in the disulfide.

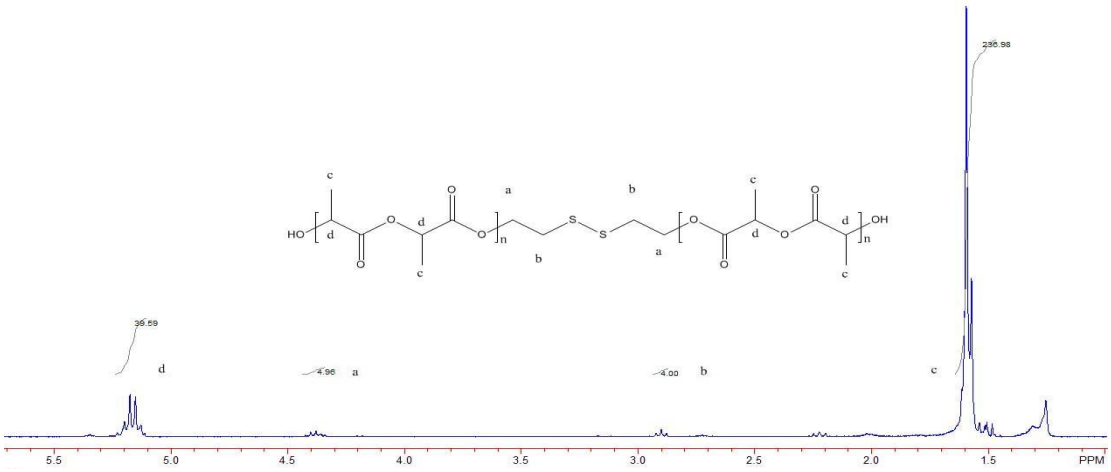

Figure 5.4 NMR spectra of PLLA-SS-PLLA.

The H in the chemical formula can be observed in Figure 5.4. Since the chemical environment of the b-H is simple, the peak that represents b-H is clearer. The area of other peaks can be integrated by setting the area of the b-H peak as a standard. Then the degree of polymerization can be calculated through the number of H in the main chain. For instance, the area of b-H is 4, the integral quantity of b-H in Figure 5.4 is 39.68. Thus, the polymer is PLLA10-SS-PLLA10.

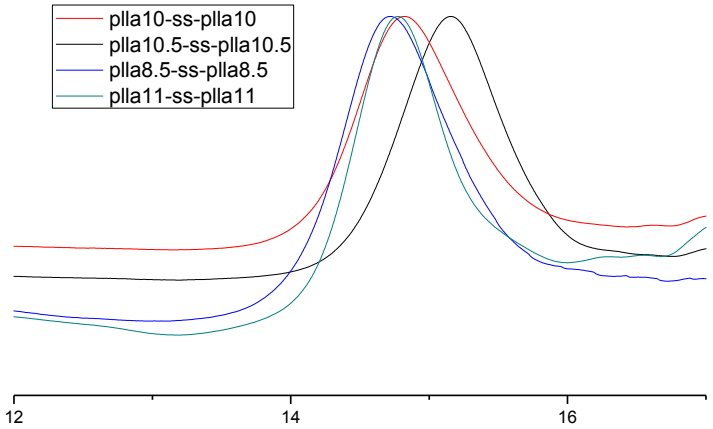

Figure 5.5 GPC (Gel permeation chromatography) spectrogram of PLLA-SS-PLLA.

In Figure 5.5, it is shown that the polymers with the same molecular weight are detected at the same time (via the peaks). The peak for each polymer can be observed in the same area, which means that this reaction method is easy to control in a simple experimental condition.

Table 5.1 Important Displacement, Crack and Integration of Figure 5.4

Number	1	2	3
Displacement of H	2.9	4.4	5.2
Crack points of H	3	3	4
Integral quantity	4(Standard)	4.96	39.68
Notes	HEDS(SS)	HEDS(O)	PLLA

Since polymers remain in the shape of a sphere when dissolved in solvent, the methyls of PLLA that are not located in the main chain may interact with each other. As a consequence, the integral quantity of methyl-H at 1.6 deviated significantly from the theoretical value.

Table 5.2 Molecular Weight of PLLA-SS-PLLA From NMR

Name	Molecular Weight	
	Mn	Mw
PLLA10.5-SS-PLLA10.5	3178	5154
PLLA10-SS-PLLA10	3034	5111
PLLA8.5-SS-PLLA8.5	2602	3943
PLLA11-SS-PLLA11	3322	5360

5.3 Characterization of Activated PLLA-SS-PLLA

Before the reaction with the cationic groups, the hydroxyl groups of polylactic acid must be activated.

In Figure 5.6, the integration quantities of peaks at 7.4 and 8.3 are calculated by setting the b-H (four H) next to SS as a standard. The activation degree of the products is 1.81/4*100%=45.3%, which means 45.3% of terminal hydroxyl groups were connected with the activation ester.

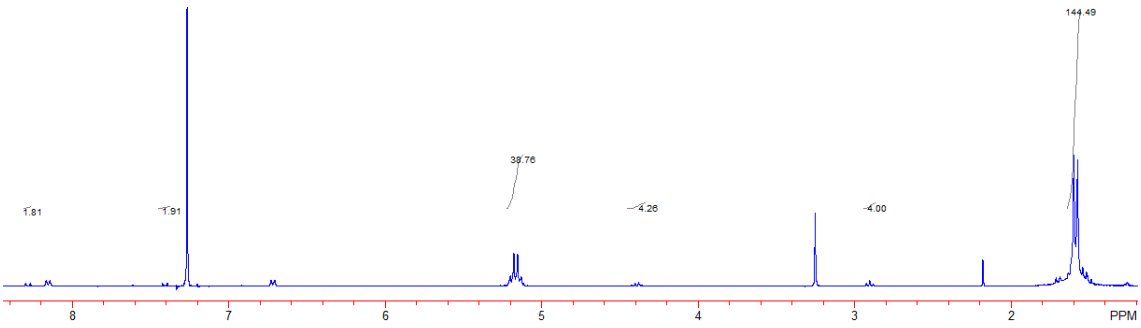

Figure 5.6 NMR spectra of activated PLLA-SS-PLLA.

Table 5.3 Important Displacement, Crack and Integration of Figure 5.6

Number	1	2	3	4	5
Displacement of H	2.9	4.4	5.2	7.4	8.3
Crack points of H	3	3	4	2	2
Integral quantity	4(Standard)	4.26	38.76	1.91	1.81
Notes	HEDS(SS)	HEDS (O)	PLLA(Main chain)	Benzene ring(NO_2)	O(Benzene ring)

The integration quantities of peaks at 7.4 and 8.3 were less than 2; it means not all the terminal hydroxyl groups were activated. In other words, parts of the terminal hydroxyl groups of the PLLA were not activated or fell off after activation.

5.4 Characterization of PEI-PLLA-SS-PLLA-PEI

Arborescent cationic molecules have been extensively studied due to their enormous ability to combine with genes [38-44]. Among these, the PEI2K can not only combine with gene very well, but also has excellent transfection effect. But, its relatively high cytotoxicity limits it to be applied as gene vectors [45]. Many studies in the literature [46-48] have reported that adding hydrophobic parts to cationic gene vectors can enhance its ability to combine with the DNA. Li Yang [49,50] has specially studied the proportion of hydrophilic and hydrophobic in PLLA arborescent cationic marcromolecules that can affect its ability to combine with the gene. When the hydrophobic chain part is bigger, it will help the cationic marcromolecules to combine with the gene. Therefore, Li Yang tried to use micromolecule quantized PEI423 as the end of the arborescent cationic group.

PEI contains amino acid. Stable conjugated molecules of p-nitrophenol can be produced through the ammonolysis reaction between the terminal carbonic esters of activated PLLA-SS-PLLA and PEI.

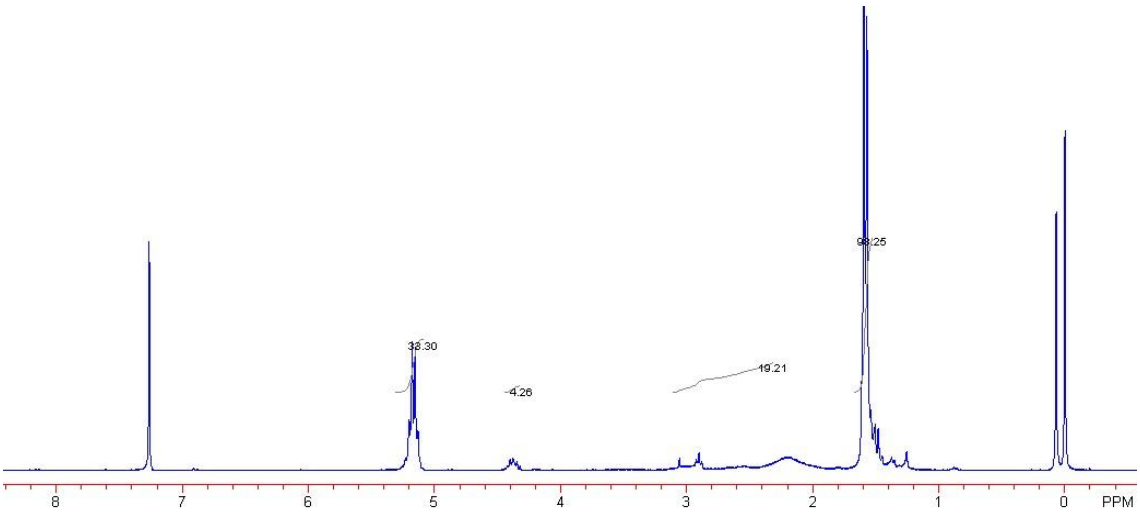

Figure 5.7 NMR spectra of PEI-PLLA-SS-PLLA-PEI.

Table 5.4 Important Displacement, Crack and Integration of Figure 5.7

Number	1	2	3
Displacement of H	2.3-3.1	4.4	5.2
Crack points of H	3(diffuse)	3	4
Integral quantity	19.21	4.26(Standard)	33.3
Notes	H(PEI, SS)	HEDS	PLLA(Main chain)

Because PEI423 is a hyperbranched PEI molecule, its structural uncertainties are relatively high. Thus it is hard to speculate its peak location and split situation. We can only speculate its magnetic cores when it exists alone in deuterated chloroform and speculate the peak of hyperbranched PEI by referring to articles with small molecular NMR spectra.

Some studies [51] have reported that magnetic nuclear displacement of hyperbranched PEI is somewhere between 2.3-3.1 PPM. According to the NMR spectra of hyperbranched PEI423 in Figure 5.8, the peaks between 2.3-3.1 contain H PEI and H on the disulfide bond. Because there is no change at the SS bond of the reactants that we use, and by comparing the peak of the part connected to acyloxy of the reactant and the product

HEDS, we can find that the location and shape of the peak merely change. So using the 4.26 activated H at the 4.4 of PLLA-SS-PLLA as the standard to do numerical integration of other peaks, there are about 19 H between 2.3-3.1, among which 4 H belong to the center of polymer PLLA-SS-PLLA near disulfide bond. It indicates that the product contains PLLA partially connected to the PEI and a cross-linked macromolecule with the PEI connected to two or more PLLA-SS-PLLA.

Molecules, partially connected with cross-linked macromolecule or more PLLA-SS-PLLA, will not affect the cationic groups. With the combination of PEI and DNA reduction and the response of the release, this water group has smaller proportion of hydrophilic groups and hydrophobic groups, and it is very good for combining DNA with cationic macromolecules. When adding a reducing material to the molecules joined with the DNA, the disulfide bond at the center of the hydrophobic chain will break into sulfydryl. After the disulfide bond break, the proportion of hydrophilic groups and hydrophobic groups will increase rapidly, which will cause the pyrolysis of composite microsphere core and then the release by combining with DNA at the spherical shell. The ability to combine with DNA can assure that these macromolecules have the potential properties as a gene vector.

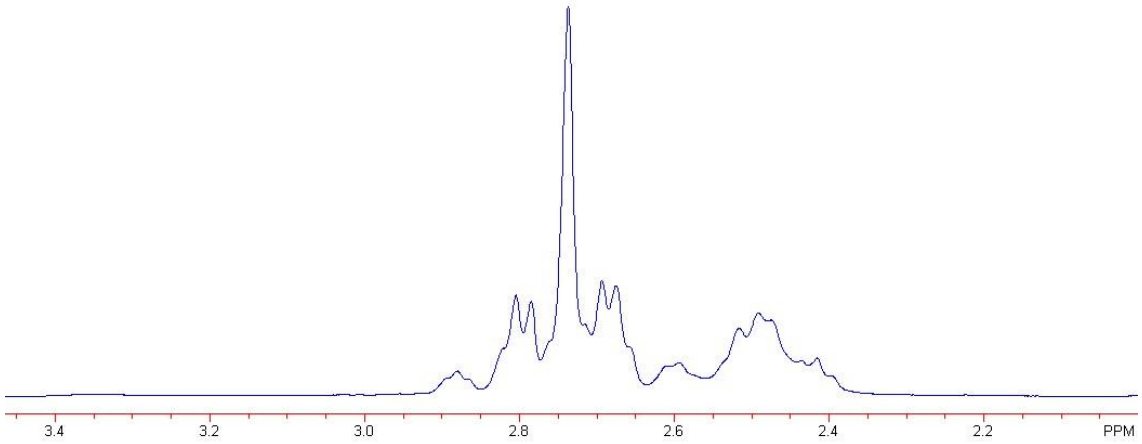

Figure 5.8 NMR spectra of PEI423 in $CDCl_3$.

In Figure 5.8, it is shown that the peaks of PEI423 distributed in the range of 2.4 to 3.0 are diffused.

CHAPTER 6

CONCLUSIONS

The cationic nature of PEI-PLLA-SS-PLLA-PEI allows complexation with genetic materials making it suitable for use as a gene delivery carrier, even when used as a polymeric coating on superparamagnetic iron oxide nanoparticles. In addition, these superparmanetic iron oxide nanoparticles are used to enhance gene transfection. The NMR spectra showed that the PEI-PLLA-SS-PLLA-PEI was successfully made. It can be applied for fabrication of SPION as polymeric coating at nanometer scale.

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