CONTROLLED RELEASE OF ANTICANCER DRUGS, PROTEINS AND LIPOSOMES BY POLYMERIC MICROSPHERES

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This thesis is dedicated to my father

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SUMMARY

A simple but great idea in rational drug design is that loading drugs to a vehicle made of biocompatible materials may provide better therapeutic effects of the drugs, because the vehicle, very much like that for macroscopic cargo, is potentially able to protect the drug, control the drug release rate, and target the drug to desired sites. This idea has led to the area of controlled drug delivery, which has achieved remarkable success in both laboratory research and clinical applications in the last decades, and is now drawing more and more attention of the pharmaceutical industry.

Polymeric microspheres are such a vehicle for controlled drug delivery. The materials used in their preparation play critical roles for their applications. In the present thesis, novel polymers such as poly(lactic acid)-poly(ethylene glycol)-poly(lactic acid) (PLA-PEG-PLA), organic solvents such as ethyl acetate and acetone, and additives such as d-alpha tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS), which have different hydrophobicity from their conventional counterparts, were applied in the fabrication of microspheres encapsulating either water insoluble agents, with an anticancer drug paclitaxel as the prototype, or water soluble agents, with a protein human serum albumin (HSA) as the prototype. Their effects on the properties of the resulted products were investigated. It has been found that these materials could be useful for controlling or improving the properties of the polymeric microspheres. For instance, PLA-PEG-PLA facilitated the release rate of paclitaxel to meet the requirement of cancer chemotherapy. It could also increase the encapsulation efficiency of proteins. Ethyl acetate and acetone are

less toxic than the conventional solvent dichloride methane (DCM), and they showed good effect on adjusting the particle size of HSA-loaded microspheres. In addition, Vitamin E TPGS might improve the quality of the microspheres, and could be useful in controlling protein release.

Based on the understanding obtained in the study of polymeric microspheres encapsulating paclitaxel and HSA, a novel controlled drug delivery system liposomes-in-microsphere (LIM), in which drug-loaded lipid vesicles (liposomes) are encapsulated into polymeric microspheres, was created and its potential applications were probed. The microencapsulation process and the liposome structure were modified to maintain the integrity of the liposomes. The release of liposomes from the polymer matrix could be controlled by the properties of the liposomes and the microspheres. The LIM system could combine the advantages and avoid the disadvantages of the polymer-based and lipid-based systems, and open a room for new applications.

ABBREVIATIONS AND NOMENCLATURE

ABS	absorption
AFM	atomic force microscope
CLSM	confocal laser scanning microscope
DCM	dichloride methane
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
Double emulsion	water-in-oil-in-water double emulsion solvent extraction/evaporation
DPPG	1,2-Dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]
DSC	differential scanning calorimetry
DSPE-PEG 2000	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
DTA	differential thermal analysis
EDX	energy dispersive X-ray spectroscopy
FASEB	Federation of American Societies for Experimental Biology
FDA	Food and Drug Administration
HSA	human serum albumin
LIM	liposomes-in-microsphere
LUV	large unilamellar vesicle
MIT	Massachusetts Institute of Technology
MLV	multilamellar vesicle
Mw	molecular weight

NA	numerical aperture
PBS	phosphate buffered saline
PEG	poly(ethylene glycol)
PEO	poly(ethylene oxide)
PGA	poly(glycolic acid)
PLA	poly(lactic acid)
PLA-PEG-PLA	poly(lactic acid)-poly(ethylene glycol)-poly(lactic acid)
PLGA	poly(lactide-co-glycolide)
PVA	poly(vinyl alcohol)
RES	reticuloendothelial system
SEM	scanning electron microscope
Single emulsion	oil-in-water single emulsion solvent extraction/evaporation
STM	scanning tunneling microscope
SUV	small unilamellar vesicle
TEM	transmission electron microscope
TGA	Thermogravimetry analysis
TMA	Thermomechanometry analysis
Vitamin E TPGS	d-alpha tocopheryl polyethylene glycol 1000 succinate
XPS	X-ray photoelectron spectroscopy
o/w	oil-in-water
w/o	water-in-oil
w/o/w	water-in-oil-in-water
BE	binding energy
C_i	concentration in the water phase of each extraction

EE	encapsulation efficiency
KE	kinetic energy
I	Intensity of the incident light
V_i	volume of water phase of each extraction
W_{HSA}	weight of HSA used in the microsphere preparation process
W_{MS}	weight of microspheres used for EE analysis
$W_{additive}$	weight of additive used in the microsphere preparation process
$W_{emulsifier}$	weight of emulsifier used in the preparation of w/o emulsion
$W_{polymer}$	weight of polymer used in the microsphere preparation process
b	path length
c	sample concentration
dI	change of intensity of light
hv	energy of the photon
k	proportionality constant
γ_s	spectrometer work function

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CHAPTER 1 LITERATURE REVIEW

1.1 Controlled drug delivery

Introduction of high-throughput screening, combinational libraries and automated synthesis methods has immensely expanded the capacity of developing new drug compounds [Guarino, 2000]. However, it has been widely recognized that a large portion of the potential value of drugs has been lost due to inadequate delivery strategies [Saltzman, 2001]. Drug release from conventional drug delivery systems is largely dependent upon the biological environment, thereby making the release behavior difficult to be predicted and controlled. A drug concentration lower than the minimum level of the desired concentration range is not able to achieve sufficient drug efficacy, and a too high drug concentration may lead to serious side effects. In recent years, many controlled drug delivery systems have been developed. By encapsulating drugs in or attaching them to polymers or lipids, the drug release can be mostly controlled by the properties of the delivery systems. The advantages of controlled drug delivery systems can be summarized as below [Park, 1997].

(1) Continuous maintenance of drug levels in a therapeutically desirable range due to the sustained and controllable release manner.

(2) Decreased side effects due to targeted drug release to a particular cell type/tissue/organ.

(3) Protection of activity of drugs, especially proteins and genes which have delicate and function-related molecular structure.

(4) Enhanced bio-absorption of drugs, especially proteins/peptides, which are large hydrophilic molecules and can hardly penetrate the biological barriers formed by hydrophobic cell membranes.

(5) Improved patient compliance, thanks to decreased number of drug dosages and possibly less invasive dosing.

(6) Reduced drug amount required.

(7) Multiple drugs may be delivered simultaneously. This is advantageous not only for patient compliance, but for synergetic effect of some drugs.

Presently, annual sales of the controlled drug delivery systems in the United States are approaching $20 billion and are rising rapidly [Langer, 2001]. In comparison to the average 250-300 million dollars and 12-15 years needed for the development of a new drug, a drug can get a new life by developing a controlled delivery system with much less money and time consumed (20% of money and half of time) [Panchagnula, 1998].

1.2 Polymeric microspheres

Since 1971, when Boswell filed a patent on the use of poly(lactic acid) in drug delivery systems [Boswell, 1971], numerous polymers have been synthesized and a number of polymer-based systems, such as polymer particles [Pekarek et al., 1994], films [Klausner et al., 2002], pellets [Becker et al., 1990], etc., have been developed for controlled drug delivery. Among them, a most frequently used system is polymeric microspheres, referred in the present thesis as spherical particles that are made of polymers, have micron size, can encapsulate bioactive agents and release them in a controlled manner. The controlled

release behavior of polymeric microspheres is dependent upon both the properties of the polymers and the microencapsulation process.

1.2.1 Polymers

Polymers are large molecules formed by the union of many identical units (monomers). In the first half of the last century, research of materials synthesized from α-hydroxy acids was abandoned because the resulting polymers were too unstable for long-term industrial uses. However, this very instability, leading to biodegradation, has proven to be immensely useful for medical applications due to the elimination of the need to surgically remove the medical devices made of these materials. In particular, for the application of drug delivery, biodegradation of polymer matrix also offers a drug release mechanism, which is especially important for drugs of low diffusivity.

In addition to biodegradability, as the material for a drug delivery device, a polymer should meet the following requirements [Dumitriu, 2002]:

(1) Satisfactory biocompatibility and bioabsorption

(2) Easy to control drug release

(3) Satisfactory mechanical properties

(4) Easy for processing

(5) Acceptable shelf life

(6) Easy to be sterilized

1.2.1.1 PGA, PLA and PLGA

Currently most commonly used biodegradable polymers for drug delivery are poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and poly(lactide-co-glycolide) (PLGA), mostly due to the long history of their use as medical sutures.

PGA is the simplest linear aliphatic polyester. It is normally synthesized from glycolide, the dimer of glycolic acid. PGA was used to develop the first totally synthetic absorbable suture. It is highly crystalline (45-55%) with a high melting point (220-225ºC) and a glass transition temperature of 35-40ºC. Because of its high degree of crystallization, it is not soluble in most organic solvents. Fibers from PGA exhibit high strength and modulus and are actually too stiff to be used as sutures except in the form of braided material. Glycolide has been copolymerized with lactide, the dimmer of lactic acid, to reduce the stiffness of the resulting fibers.

PLA is normally synthesized from lactide. Lactide exists as two optical isomers, d and l. L-lactide is the naturally occurring isomer, and dl-lactide is the synthetic blend of d-lactide and l-lactide. Poly(l-lactide) is about 37% crystalline with a melting point of 175-178ºC and a glass transition temperature of 60-65ºC. The degradation of poly(l-lactide) is very slow, requiring more than 2 years to be completely absorbed. It exhibits high tensile strength and low elongation and consequently has a high modulus that makes it more suitable for load-bearing applications such as sutures. Poly(dl-lactide) is an amorphous polymer exhibiting a random distribution of both isomeric forms of lactic acid, and accordingly is unable to arrange into an organized crystalline structure. This material has

lower tensile strength, higher elongation, and a much more rapid degradation time, making it more attractive as a drug delivery system.

Using the properties of PGA and PLA as a starting point, it is possible to copolymerize the two monomers to extend the range of the homopolymer properties. Fig. 1.1 shows the synthesis of PLGA. PLGA has been developed for both sutures and drug delivery applications. It is important to note that there is not a linear relationship between the copolymer composition and the mechanical and degradation properties of the materials. For example, a copolymer of 50% glycolide and 50% dl-lactide degrades faster than either homopolymer [Stallforthe and Revell, 2000].

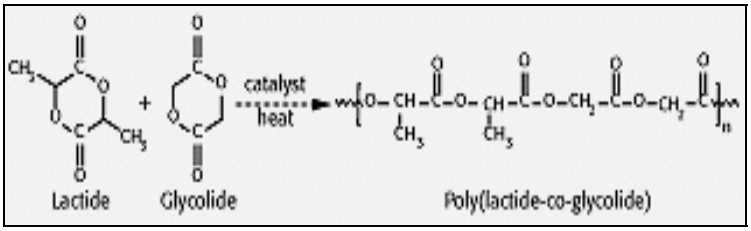


Figure 1.1 Synthesis of PLGA

1.2.1.2 PLA and PEG copolymers

Although the PLA and PLGA are now commonly used, they are far from perfect. A hot research topic has been to designing and synthesizing new polymers for the application of drug delivery. One very promising strategy is to copolymerize PLA and poly(ethylene glycol) (PEG). PEG has been known as an excellent biomaterial due to its biocompatibility, hydrophilicity and flexibility. It is also referred to as poly(ethylene oxide) (PEO) at high molecular weight. Copolymerization of hydrophobic PLA and hydrophilic

PEG can provide a balance between the two opposite parts. Furthermore, different supramolecular structures can be achieved by different monomer combinations and preparation processes to meet various medical requirements.

Surface modification of polymeric nanoparticles has been achieved with PLA-PEG copolymers [Feast et al., 1993]. The PEG chains minimize non-specific fouling of the device surface with bio-components such as proteins. The uptake of nanoparticles by the reticuloendothelial system (RES) can be reduced. Diblock PLA-PEG copolymer can also form micelles in aqueous environment with PEG on the surface. Compared to surfactant micelles, these polymeric micelles are more stable, have a lowered critical micellar concentration, and have a slower rate of dissociation, allowing retention of loaded drugs for a longer period of time and, eventually, achieving higher accumulation of a drug at the target site. Furthermore, they have a size range of several tens of nanometers with a considerably narrow distribution, which is crucial in determining their body disposition [Katooka et al., 2001].

A family of star-block copolymers from multi-arm PEO and l-lactide or l-lactide/glycolide has been reported. In vitro degradation test results on these polymers show that the biodegradation consists of an initial slow-rate period in the first 2-3 weeks, which makes them an excellent drug carrier, and an exhaustive degradation period, which provides the way for renal excretion. The star shape of the polymers also leads to small hydrodynamic radius and low solution viscosity, which make them excellent for complete excretion [Choi et al., 1998, Li and Kissel, 1998].

Thermosensitive hydrogels have been prepared from either PLA-PEO diblock or PEO-PLA-PEO triblock polymers. The hydrogel can be loaded with bioactive molecules in an aqueous phase at an elevated temperature (around 45ºC), where a sol is formed. The polymer is injectable in this form. On subcutaneous injection and subsequent rapid cooling to body temperature at 37ºC, the polymer forms a gel that can act as a sustained-release matrix for drugs. The gel-sol transition temperature can be well controlled by the molecular weight of PLA segment. Both high molecular weight proteins and low molecular weight hydrophobic drugs can be loaded and released. The release rate is controllable by the initial drug loading and the polymer concentration [Jeong et al., 1997].

Biotin has been conjugated to the PLA-PEG copolymer to form a new polymer PLA-PEG-biotin. In this new polymer, the PLA component provides structural integrity to the fabricated devices. The PEG block acts as a hydrophilic coating to avoid the uptake of RES. The third part of the polymer, i.e. biotin moiety, allows facile surface engineering using aqueous solution of avidin. Avidin posses a tetrameric structure with four binding sites for biotin. It binds to the biotin using one of these sites, and the other free binding sites are available for the attachment of biotinylated ligand motifs, which can then be used for targeting to tumor cells [Black et al., 1999, Cannizzaro et al., 1998].

1.2.2 Microencapsulation by polymeric microspheres

A number of techniques have been developed to prepare polymeric microspheres to encapsulate bioactive agents. Following is a summary of these methods [O'Donnell and McGinity, 1997, Rajeev, 2000, Wise, 1995].

1.2.2.1 Solvent extraction/evaporation method

Solvent extraction/evaporation is the most popularly used method to prepare polymeric microspheres. It involves forming an emulsion, in which an organic solvent dissolving a polymer and containing a bioactive agent is the inner phase while water is the outer phase, followed by removal of the organic solvent by either extraction to the outer water phase or evaporation to the air, leaving behind solid tiny polymer particles with the bioactive agent encapsulated. Initially it was applied mainly for microencapsulation of water-insoluble drugs. In this case, the emulsion formed is an oil-in-water (o/w) emulsion, in which the drug, polymer and organic solvent form the oil phase. Later, this method was successfully adapted by Vrancken & Clays and Dejaeger & Tavernier to microencapsulate water-soluble drugs, by forming a water-in-oil-in-water (w/o/w) emulsion, in which the drug is dissolved in the inner water phase and the polymer is dissolved in the oil phase [Dejaeger and Tavernier, 1971, Vrancken and Clays, 1970]. The method for encapsulation of water-insoluble drugs is often termed as the oil-in-water single emulsion solvent extraction/evaporation method, or simply single emulsion method, while the method for water-soluble drugs as the w/o/w double emulsion solvent extraction/evaporation method, or simply double emulsion method.

1.2.2.2 Other methods

In phase separation method (coacervation method), the drug is either dissolved (for water-insoluble drug) or dispersed (for water-soluble drug) in an organic solvent (solvent I) in

which the polymer has been dissolved. Another organic solvent (solvent II), which is miscible with the solvent I but doesn't dissolve the polymer or the drug, is then added under stirring to gradually extract the solvent I, leaving behind soft polymer droplets. Afterwards a large quantity of the solvent III, which is relatively volatile, is added to remove the solvent II and harden the droplets, which become microspheres eventually. Compared with solvent extraction/evaporation method, phase separation has the advantage of high encapsulation efficiency of water-soluble drugs because no aqueous phase is involved to cause drug loss. On the other hand, this method suffers from the disadvantages of difficulty in the control of the process and large amount of organic solvents used, which is harmful to the environment.

Spray drying method is rapid, convenient and easy to scale-up. A mixture of the drug, polymer and solvent is fed by a pump into a spray dryer, in which the mixture is atomized by compressed air and goes through a nozzle. Instantly the solvent is evaporated and the microsphere product can then be obtained from a collector located at the outlet of the equipment. However, a major drawback of this method is the adhesion of the microspheres, which are not hardened sufficiently yet, to the inner wall of the apparatus and with each other.

The particle size of microspheres formed by all the above processes is mostly controlled by the mechanical forces used. Therefore, ideally one can obtain nano-size particles, or nanospheres, by simply increasing the mechanical strength. However, there are often lower limits to the sizes obtainable by these methods. Some methods are available especially for producing nanospheres. Frequently used are (1) the emulsion polymerization

technique, in which the monomer is polymerized in an emulsion, (2) the nanoprecipitation technique, in which the solvent of a low viscosity polymer solution rapidly diffuses to a miscible solvent, and (3) dialysis technique, in which polymer solution is dialyzed against water [Courvreur, 1979, Fonseca et al., 2002, Mathiowitz et al., 1997, La et al., 1996].

1.2.3 Controlled release by polymeric microspheres

The mechanisms to control the release rate by a controlled delivery vehicle include dissolution, diffusion, erosion or chemical reactions, swelling, osmosis and external forces such as ultrasound and magnetic force [Langer, 1980, Van Brunt, 1986]. In polymeric microspheres, major mechanisms normally include polymer degradation and drug diffusion. In addition to controlling the drug release rate, releasing the drug at a desired site, or drug targeting, can also be very important, particularly for cancer chemotherapy.

1.2.3.1 Control of release rate

Almost all commercial biodegradable polymers for drug delivery have hydrolysable backbones and mainly undergo hydrolytic degradation. The possibility of enzyme degradation has also been considered [Schakenraad, 1990]. Two types of degradation are possible, namely bulk degradation and surface erosion. In bulk degradation, the rate of water penetration into the polymer matrix is faster than that of polymer degradation, so that degradation takes place throughout the whole matrix. In contrast, in surface erosion, water cannot enter the polymer matrix readily and the matrix has to erode gradually from the surface to the core. Surface erosion is normally desirable for drug release because of

its ability to offer zero-ordered release kinetics, i.e. constant drug release. However, up to now all the USA Food and Drug Administration (FDA) approved biodegradable polymers undergo the bulk degradation mechanism.

Bulk degradation normally experiences three stages. In the beginning, the backbone of the polymer is hydrolyzed and its molecular weight decreases gradually. At this stage (stage I) the strength and integrity of the delivery device is intact. After some time, the device is hydrolyzed so greatly that the strength is lost (stage II) and the mass is subsequently dissolved (stage III). The dissolved mass can be further metabolized and eliminated from the human body eventually.

The degradation rate of polymeric microspheres is dictated by their hydrophilicity since the degradation mechanism is mostly hydrolysis. Therefore, faster degradation can be caused by more hydrophilic molecular structure and more amorphous state of the polymer, and smaller particle size and higher porosity of the polymeric microspheres [Edlund and Albertsson, 2002].

By assuming that the driving force of diffusion is the concentration gradient, one may describe the drug diffusion in polymer matrix by Fick's first law in which the diffusive flux is proportional to the drug concentration gradient. The coefficient is termed as the intrinsic diffusivity of the drug in the polymer matrix. It is a constant of proportionality or may be a function of concentration. Considerations based on the thermodynamics of irreversible processes indicate that a more fundamental driving force is the gradient of chemical potential of the drug. The resulted Fick's first law has the similar form as the

concentration based one: the drug diffusive flux is proportional to the chemical potential gradient. The coefficient, i.e. the chemical potential based diffusivity, is less dependent on the concentration of the drug [Bird et al., 1960].

A distinction between two types of situations has been made with respect to drug diffusion in polymeric matrix. In one type, the pores in the polymer matrix are smaller than or of the same order magnitude as the mean free path (or average jump distance) of the drug molecules. The molecular network constituting the polymer matrix takes part at the molecular level in frictional interactions with the drug molecules. In the other type, the pores in the polymer matrix are much larger than the mean free path of the drug molecules. The system containing these pores may be regarded as comprising two phases: the polymer phase represents little more than a solid container for the diffusing fluid phase within which the transport process occurs. The polymer phase only serves to define the geometry of the diffusing channels [Mikulecky and Caplan, 1966].

The drug diffusion rate in the polymer matrix depends on the temperature, the molecular structure of the drug and the polymer, the presence of other components and so on. For example, an elevated temperature increases the mobility of the polymer chain and the free volume in the polymer matrix, thereby leading to higher drug diffusivity. Drug molecules of low molecular weight readily pass through polymer molecules of high molecular weight, giving large drug diffusivity. In addition, the presence of a plasticizer increases the drug diffusivity, because it reduces the polymer interchain interactions and can serve as a diluent.

A generic kinetics of drug release from polymeric microspheres has three stages. In the 1st stage, the drug attached to, or loosely entrapped near, the microsphere surface is desorpted and gives rise to a fast release, which is known as initial burst. This is followed by the 2nd stage in which drug mainly diffuses out through the free volume in the polymer matrix. In the subsequent 3rd stage, the polymer degradation mechanism becomes predominant, implying that the drug most likely escapes from the polymer matrix together with the degraded polymer. These three phases are sometimes not so distinctly divided. For instance, certain parameters may be adjusted to make the diffusion faster and desorption slower, thereby leading to more sustained release, which is desirable in most clinical cases [Huang and Braxel, 2001].

1.2.3.2 Control of release site

It is estimated that only less than 1% of the intravenously administered dose of a free drug reaches the desired site, while the remaining majority causes toxicity in various organs [Ceh, 1997]. This fact indicates a large room for research on targeted drug delivery devices which are able to release the drug at selected organs, tissues, cells or even intracellular compartments. Polymeric microspheres can be used as a targeted device.

The particle size of polymeric microspheres is critical to dictate their delivery route and biological fate. Microspheres of particle size less than 10 μm are desirable for intravenous injection. Smaller particle size is required for oral, transdermal, ocular and nasal delivery. Microspheres in size range of 10-100 μm can be used for subcutaneous or intramuscular administration while particle size over 100 μm have been employed for implantation

[Magda et al., 1993]. Once entering systemic circulation, a large portion of the polymeric microspheres is taken by RES. In general, smaller particle size helps to reduce the RES uptake [Willmott and Daly, 1994]. RES targeting is desirable in some cases, however, such as RES diseases and drug delivery avoiding important sites (e.g. heart) to reduce systemic side effects. Besides, microspheres with size range of 20-50 μm are able to provide targeting to tumor via the so-called chemoembolization process. For instance, to treat liver cancer, polymeric microspheres are injected to the liver artery, and are trapped in the microvasculature of the liver. In comparison with the microvasculature of normal tissue, that of tumor is much leakier and will thus trap most of the microspheres [Dass and Burton, 1999].

Surface modification is another means to control the biological fate of the polymeric microspheres. Bioadhesive polymers such as polyanhydride copolymers of fumaric and sebacid acid can increase the absorption of the polymeric microspheres at certain sites [Mathiowitz et al., 1997]. On the other hand, coating the polymeric microspheres with some hydrophilic polymers may avoid the uptake by RES [Stolnik et al., 1995]. Theoretically, a number of ligands can be coupled onto the surface of the microspheres to allow targeting to tumor cells through recognition of proteins involved in receptor-mediated cell entry.

Attempts have also been made to achieve targeting by external forces. For instance, ultra-fine particles of magnetite (Fe_3O_4) have been incorporated into albumin microspheres containing an anticancer drug doxorubicin. The movement of the particles after intra-

arterial administration can be controlled by an externally applied magnetic field [Gupta and Hung, 1989].

1.3 Characterization techniques

Various characterization techniques have been used to obtain a thorough picture of a controlled drug delivery system. This Section summarizes those involved in the present thesis.

1.3.1 Microscopy

Objects of interests to us exist in a very wide range of sizes but the unaided human eyes are unable to see the small ones, including most of controlled drug delivery devices. For two points in an image to be seen as separate, the viewing angle must be big enough so that their images can fall on at least two cells of the retina. Moving an object closer to the eye has the effect of increasing the size of the image on the retina, making the object 'look' bigger. However, because the unaided human eye cannot focus on objects closer than about 250 mm, there is a limit to the extent to which detail perception can be increased by this means. To see more details, the object must be in effect brought 'closer' to the eye than allowed by the nearest distance of distinct vision. The instrument used to achieve this is the microscope [Gage, 1947].

The light microscope is the oldest form of microscopes but still commonly used nowadays due to the ease of use and sample preparations. To obtain a nice contrast in the image of an

object, either transmitted or reflected light, either normal or polarized light, either bright or dark field, either intensity or phase contrast, can be used, making light microscopes a very flexible technique [Amelinckx et al., 1997]. The resolution of a light microscope is governed by its numerical aperture (NA) and the wavelength of the light used. In practice, for an oil-immersion objective of NA 1.4 used with green light, which has a wavelength of 550 nm, the resolution is about 0.25 μm [Bradbury and Bracegirdle, 1997]. Since the practical upper limit of the NA of an objective is about 1.4, further increase in resolution is possible only by the use of shorter wavelengths. Ultraviolet light with its shorter wavelength may be used but the value is limited by the fact that the sensitivity of the eye falls off dramatically as the wavelength becomes shorter. Nowadays for resolution significantly better than 0.25 μm and hence the possibility of greatly increased magnifications, it is common to use radiation of much shorter wavelength, commonly a beam of electrons, in the electron microscope.

The transmission electron microscope (TEM) is arranged much like an ordinary light microscope designed for examining translucent specimens by the transmission of visible light except that magnets are used instead of light-bending lenses to deflect and focus the beam of electrons. The electrons originate in an electron gun that usually has a hot filament (sometimes a cold-cathode emitter) as the source and an arrangement for defining and accelerating a narrow beam of electrons. The accelerated beam is then focused on a tiny area of the specimen by a pair of concentric toroidal electromagnets that act as condensing coils. Since different parts of the specimen absorb electrons differentially, as the projected beam of electrons falls on a fluorescent screen, it shows bright areas where the sample has absorbed less and darker areas where the sample has absorbed more of the

electrons. In another type of electron microscope, scanning electron microscope (SEM), the signals scattered from the surface of the specimen including secondary electrons, backscattered electrons, characteristic X-rays, etc., are received by cathode ray tubes which are synchronized with the scanner probing the surface of the specimen. All electron microscopes require vacuum in the interior since electron beam would be scattered by collision with air molecules [Rochow and Tucker, 1994]. TEM and SEM have become indispensable means to observe drug delivery devices such as polymeric microspheres and liposomes [Honeywell-Nguye et al., 2002, Mandal et al., 2002].

Even better resolution has been achieved by scanning tunneling microscope (STM) and atomic force microscope (AFM), which are based on the finding that atomic-scale images can be obtained when a fine 'tip' is passed very close to a surface [Dvorak, 2003]. In STM, a fine tip is brought close (a few Å) to a sample surface, and the wave functions of the electrons of the tip and those of the surface overlap. By applying a voltage between the tip and the sample, a tunneling current is established through the vacuum barrier. The intensity of this current is a measure of the overlap between these two wave functions and depends very strongly on the tip-sample distance. During a scan of the sample surface, the tunneling current is recorded to produce the atomic-scale topographic map. AFM is similar to STM except that it measures the inter-atomic force between the tip and the sample surface instead of the tunneling current [Stefanis and Tomlinson, 2001]. AFM and STM have been recently applied for visualization of controlled drug delivery devices [Olbrich et al., 2001, Vermette et al., 2002].

Besides increasing resolution, microscopy techniques have undergone great developments in other aspects such as 3-dimentional imaging and chemical analysis. In confocal laser scanning microscope (CLSM), two lenses are arranged to focus on the same point. Because of the pinholes, or apertures, on each side of the specimen, only a very small volume is focused at any time. Light from only the actual plane of focus enters the eyepiece, which eliminates practically all of glare and scattered light. 3-D imaging is possible by the above concept, along with the progress in laser illumination and computerized image analysis [Sheppard and Shotton, 1998]. CLSM technique has been applied to investigate the drug distribution in polymeric microspheres [Yang et al., 2001]. In addition, as mentioned earlier, many signals are generated in SEM when an electron beam is scanned on the specimen surface. Among them is characteristic spectrum of X-rays. Because the intensity of the continuum is a function of atomic number, the chemical composition of the specimen surface can be analyzed by examining these signals. This is what a new microscopy technique, namely SEM-EDX (energy dispersive X-ray spectroscopy), does [Bubert and Jenett, 2002].

1.3.2 Spectroscopy

Spectroscopy is concerned with the interaction of electromagnetic radiation with matter. The consequence of such interaction is that energy is absorbed or emitted by the atoms and molecules in discrete amounts or quanta according to quantum mechanics. A measurement of the radiation frequency gives a value for the change of energy involved, and from a complete investigation it is possible to infer the set of possible discrete energy levels of the matter being studied, which is unique for different matters [Whiffen, 1972].

Experimental methods of spectroscopy began in the more accessible visible region of the electromagnetic spectrum where the eye could be used as the detector. At first, it was discovered that white light was dispersed into a range of colors by a triangular glass prism. The prism was later developed for use as an analytical instrument. Its early application was the observation of the emission spectra of various samples in a flame. Subsequently, the resulting series lines in visible spectrum of atomic hydrogen were fitted to a mathematical formula, beginning the close relationship between experiment and theory in spectroscopy. However theory ran increasingly into trouble so long as it was based on classical Newtonian mechanics until the development of quantum mechanics. The availability of large, fast computers resulted in fewer and fewer approximations required to be made in the theoretical calculations [Hollas, 1996].

Nowadays spectroscopy has become an essential approach to investigate the structure, state, and concentration of matters. Many spectroscopy techniques have been developed in the recent decades, because electromagnetic radiation covers a wide wavelength range from radio waves to γ-rays and the atoms or molecules may be in the gas, liquid, or solid phase or, of great importance in surface chemistry, adsorbed on a solid surface. Three typical spectroscopy techniques, namely, UV-Vis spectrophotometer, X-ray photoelectron spectroscopy (XPS), and fluorescence spectroscopy, are introduced below as examples.

UV-Vis spectrophotometer utilizes the absorption of ultraviolet or visible lights by a sample to determine the sample concentration. Lambert-Beer's Law states that the change

in intensity of light (dI) after passing through a sample is proportional to the path length (b), the sample concentration (c) and the intensity of the incident light (I). That is to say

$$dI/I = -kbc \qquad \text{(Equation 1.1)}$$

where k is a proportionality constant. Thus, the concentration of a sample can be determined by measuring the change of light intensity due to absorption, with the path length kept constant. In the area of controlled release, this technique has been widely used to measure the amount of released agent from the delivery vehicles in the release medium [Meziani et al., 2002, Ruan et al., 2002].

The UV-Vis spectrophotometer often uses two light sources, a deuterium lamp for ultraviolet light and a tungsten lamp for visible light. A diffraction grating which can be rotated allows for a specific wavelength of the light to be selected. One light beam is allowed to pass through a reference cuvette, which contains the solvent only, while the other passes through the sample cuvette. The intensities of the two light beams are then measured and compared at the end [Gillespie, 1994].

XPS is a major tool for surface chemical analysis. XPS analysis involves irradiating a solid sample in vacuum with monoenergetic soft X-rays and analyzing the emitted electrons by energy. These photons have limited penetrating power in a solid on the order of 1-10 micrometers. They interact with atoms in the surface region, causing electrons to

be emitted by the photoelectric effect. The emitted electrons have kinetic energies (*KE*) given by

$$KE = h\nu - BE - \gamma_s \qquad \text{(Equation 1.2)}$$

where *hν* is the energy of the photon. *BE* is the binding energy of the atomic orbital from which the electron originates, and γ_s is the spectrometer work function. Because each element has a unique set of binding energies, XPS can be used to identify and determine the concentration of the elements in the surface. Variations in the elemental binding energies (the chemical shifts) arise from differences in the chemical potential and polarizability of compounds. These chemical shifts can be used to identify the chemical state of the materials being analyzed. Because the mean free path of electrons in solid is very small, the detected electrons originate from only the top few atomic layers, making XPS a unique surface-sensitive technique for chemical analysis. This technique has been applied to investigate the surface chemistry of drug delivery devices, which can be correlated with their interactions with biological environment [Evora et al., 1998, Shakesheff et al., 1997].

In an XPS experiment, the samples are often excited by unmonochromatized K_α lines from aluminum and magnesium sources. The emitted electrons are collected, retarded and analyzed as a function of their *KE* values. The concentric hemispherical analyzer, which is made of two concentric hemispheres, is the most widely used analyzer in XPS. The

spectrum is obtained as a plot of the number of detected electrons per energy interval versus their binding energy [Chastain, 1992].

The phenomenon of fluorescence has been applied for many analytical instruments, including fluorescence spectroscopy. The mechanism of fluorescence can be described as the following: when a substance absorbs light, its molecules are excited from a ground state to a higher energy level. The molecules can then return to their ground state by losing energy, mainly as heat to their surroundings. In some cases, part of the energy absorbed can be reemitted as radiation, usually of longer wavelengths than the exciting light. This process is known as fluorescence [Lakowicz, 1983].

The types of substances that exhibit fluorescence are normally those whose structure contains delocalized electrons present in conjugated double bonds. Fluorescence has the main advantage over other optical techniques of being extremely sensitive. Many applications of fluorescence rely on this fact to label small amounts of sample for detection, often allowing solute in the nanomolar range to be quantified. For example, the amount of released agent from a controlled delivery vehicle may be measured if the agent is fluorescent or it can be labeled with a fluorophore. Furthermore, the fluorescence of many fluorophores, e.g. tryptophan in proteins, is extremely sensitive to their surrounding environments. It enables fluorescence to be used as a sensitive structural probe in many biological systems [Guo et al., 2002].

In the most basic fluorescence spectroscopy experiments the sample is irradiated with light at or close to its absorption maximum, and the resulting fluorescence is measured at a

particular emission wavelength or as a function of emission wavelength (emission spectra). An emission spectrum is also often presented with an excitation spectrum where the fluorescence at a single wavelength is measured as a function of excitation wavelength. These basic measurements, which use a standard commercial fluorescence spectrometer, are often known as steady-state fluorescence measurements, to distinguish them from those of fluorescence lifetime, i.e. the average time the fluorophore spends in the excited state which requires specialized equipment to be measured [Jones et al., 1994].

1.3.3 Chromatography

Chromatography is essentially a physical method of separation in which the components to be separated are distributed between two phases, i.e. the stationary phase and the mobile phase. The mobile phase percolates through the stationary phase in a definite direction. The chromatographic process occurs as a result of repeated sorption/desorption acts during the movement of the sample components along the stationary bed, and the separation is due to differences in the distribution constants of the individual sample components. For applications of analysis, the separated components enter detectors, such as UV-Vis spectrophotometer and fluorescence spectrophotometer, which can measure the amount of components at every moment. The information obtained from a chromatographic experiment is contained in the chromatogram, a record of the concentration or mass profile of the sample components as a function of the movement of the mobile phase. Information readily extracted from the chromatogram includes an indication of sample complexity based on the number of observed peaks, qualitative identification of sample components based on the accurate determination of peak position, quantitative assessment of the

relative concentration or amount of each peak, and an indication of column performance [Heftmann, 1967].

A distinction between the principal chromatographic methods can be made in terms of the properties of the mobile phase. In gas chromatography the mobile phase is an inert gas, in supercritical fluid chromatography the mobile phase is a dense gas (fluid) which is above its critical temperature and pressure, and in liquid chromatography the mobile phase is a liquid of low viscosity. The stationary phase is generally a porous, granular powder in the form of a dense homogeneous bed packed into a tube (column) able to withstand the operating pressures employed. For thin layer chromatography the sorbent is spread as a thin, homogeneous layer on a flat glass or similar inert backing plate [C.F. Poole and S.K. Poole, 1991].

A most commonly used chromatography technique is high pressure liquid chromatography (HPLC). In this technique, a pump provides the high pressure required to push the liquid mobile phase through the chromatographic column. HPLC can be operated at various modes in terms of the dominant interactions between the mobile and stationary phases for the separation. Reversed phase mode utilizes the hydrophobic interactions while normal phase mode hydrogen bonding or polar interactions. The reversed phase is a phrase used to indicate that the stationary phase is less polar than the solvent, and normal phase indicates that the stationary phase is more polar. Reversed phase and normal phase HPLC predominate in the analysis of small organic molecules. For example, the concentration of an anticancer drug paclitaxel has been analyzed by reversed phase HPLC [Richheimer et al., 1992]. Lipids and surfactants can also be analyzed by this method [Swadesh, 1997]. In

the HPLC of size exclusion mode, the stationary phase contains pores of defined size distribution. Analytes larger than the pores are excluded from the pores and pass through the column more rapidly than smaller analytes. Size exclusion HPLC has been used to separate polymer molecules according to different molecular size [Pasch and Trathnigg, 1997]. It can also be used to purify liposomes since the vesicles are much bigger than the encapsulated agents [Kreuter, 1994].

1.3.4 Thermal analysis

Thermal analysis means the analysis of a change in a property of a sample, which is related to an imposed temperature alteration [Brown, 1998]. All thermal analysis instruments have features in common: a sample, contained in a suitable sample pan, is placed in a furnace and subjected to a desired temperature program. During this procedure, one or more properties of the sample are monitored by use of suitable transducers for converting the properties to electrical quantities. The variety of the techniques stems from the variety of properties that can be measured. Measurements are usually continuous and the heating rate is often, but not necessarily, linear with time. The results of such measurements are thermal analysis curves and the features of the curves (peaks, discontinuities, changes of slope, etc.) are related to thermal events in the sample [Hemminger, 1998].

In thermogravimetry analysis (TGA), measurements of changes in sample mass with temperature are made using a thermobalance. The thermobalance is a combination of an electronic microbalance with a furnace, a temperature programmer and a computer for

control, which allows the sample to be simultaneously weighed and heated or cooled in a controlled manner, and the mass, time, temperature data to be captured. In differential thermal analysis (DTA), the difference in temperature between the sample and a reference material is recorded, while both are subjected to the same heating program. On the other hand, in differential scanning calorimetry (DSC), the sample and a reference material are maintained at the same temperature throughout the controlled temperature program, and any difference in the independent supplies of power to the sample and the reference is recorded against the programmed temperature. There are many similarities between DTA and DSC, including the superficial appearance of the thermal analysis curves obtained. Thermal events in the sample appear as deviations from the baselines, in either an endothermic or exothermic direction, depending upon whether the temperature of the sample is lower or higher than that of the reference (DTA), or whether more or less energy has to be supplied to the sample (DSC). Another common thermal analysis technique is thermomechanometry analysis (TMA), in which the expansion or contraction of the sample is measured as a function of temperature, while the sample is under compression, or tension, or negligible loads [Brown, 2001].

Thermal analysis has been applied on the study of chemical reaction kinetics, materials, pharmaceutical production and so on [Ford and Timmins, 1989, Hatakeyama and Quinn, 1999, Speyer, 1994]. For drug delivery studies, as examples, (1) DSC/DTA can be used to determine the glass transition temperature of poly(esters), which represents a measure of the flexibility of the polymer chain and may be an indication of the ease of hydrolysis of the ester bonds [Baker, 1992]; (2) DSC/DTA are useful in evaluating the form of the drug in the polymeric delivery systems. The absence of an endotherm corresponding to drug

fusion is indicative of the drug being dispersed as either a molecular dispersion or a solid solution [Dubernet, 1995]; (3) Many microspheres are very porous and thermal analysis has proved useful in characterizing their internal pore structure. For instance, the endotherm of water at -15ºC in the polyamide microspheres was attributed to the melting of highly structured water within and around the microspheres [Dubernet, 1995]; (4) Castelli et al. found that the lowering of the phase transition temperature of lipid vesicles was proportional to the concentration of a drug diclofenac within the vesicle membranes. Thus, they claimed that it was feasible to monitor the release of the drug from polymeric microspheres to multilamellar lipid vesicles as a model biomembrane system by DSC [Castelli et al., 2001].

1.3.5 Light scattering

Light that incidents on an ensemble of particles, such as crystals, aerosols, molecules, atoms, is partially deflected (scattered). The evaluation of the scattered light with regard to its intensity, wavelength, and direction often yields valuable information about the matter scattering light. Many different light scattering processes may occur and be used for analysis purpose. These processes may be classified to elastic and inelastic scattering. An elastic scattering process refers to an interaction without a permanent exchange of energy between the light and the matter. This restriction of energy exchange does not prohibit a change in direction, but prohibits a change in frequency. An inelastic process, on the other hand, leads to a permanent energy exchange and a change of frequency of the emitted radiation [Mayinger and Feldmann, 2001].

Light scattering has been applied to analyze molecular weight, size and surface charge of colloid particles, diffusion coefficient, etc [Chia et al., 2001, Feng et al., 2002]. For example, the dynamic light scattering analysis utilizes the fact that the scattering particles undergo rapid thermal motions. These movements cause short-term fluctuations in the intensity of the scattered light. The intensity fluctuations (dynamics) of the scattered light are determined and analyzed. The results can be correlated to the above-mentioned parameters of colloid or polymer solution [Sun, 1994].

1.4 Thesis organization

As discussed earlier, polymeric microspheres can be used to encapsulate and release both water-insoluble and water-soluble drugs. In Chapter 3 and Chapter 4 of the present thesis, polymeric microspheres were used for the controlled release of an anticancer agent, paclitaxel, which is water-insoluble, and a protein human serum albumin (HSA), which is water-soluble, respectively. Novel materials, such as polymers, organic solvents and additives, were applied and their effects on the physicochemical properties of the resulted microspheres were investigated. In Chapter 5, polymeric microspheres were used to encapsulate another major controlled drug delivery system, namely liposomes, to form a novel device: liposomes-in-microsphere (LIM) of biodegradable polymers. It was then extensively characterized in vitro.

CHAPTER 2 MATERIALS AND METHODS

2.1 Chemicals

Polymers:

PLGA (L/G ratio=50:50, Mw=40000-75000) was from Sigma (USA);

Poly(lactic acid)-poly(ethylene glycol)-poly(lactic acid) (PLA-PEG-PLA, 10% PEG, Mw=80000 or 50000) was a gift from Prof. X.M. Deng, Chengdu Institute of Organic Chemistry, Chinese Academy of Science, PR China;

PEG (Mw=40000) was from Fluka (Germany);

Poly(vinyl alcohol) (PVA, Mw=30000-70000) was from Sigma (USA);

Chitosan (hydrochloride salt, Cat. No. FLA-40) was a gift from Koyo Chemical (Japan).

Lipids:

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC),

1,2-Dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DPPG),

and Cholesterol were from Sigma (USA);

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG 2000) was from Avanti Lipids (USA).

Organic solvents:

Dichloride methane (DCM) was from Tedia (USA);

Ethyl acetate,

Cyclohexane,

and Acetone were from Merck (Germany);

Chloroform,

and Methanol were from Fisher (UK).

Others:

Paclitaxel was from Hande Biotechnology Inc., PR China;

HSA was from CSL (Australia);

D-alpha tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS) was from Eastman (USA);

Calcein,

Triton X-100,

Sephadex gel (G-50-80),

Phosphate buffered saline (PBS),

and Phosphotungstic acid were from Sigma (USA);

Sodium azide,

and Sodium chloride were from Merck (Germany);

Protein assay kit (Cat. No. 500-0002) was from Bio-Rad (USA);

Milli-Q water and deionized water were produced by a Millipore water purification system (Millipore Corporation, USA).

All the above chemicals were of the commercially highest grade.

2.2 Instruments

Mechanical stirrer: Eurostar digital, IKA, Germany;

Vortex mixer: Maxi Mix II, Thermolyne, USA;

Homogenizer: Ultra-Turrax TP18/10, IKA, Germany;

Probe sonicator: Microson™, Misonix, USA;

Bath sonicator: Transsonic 460/H, Elma-Hans Schmidbauer GmbH & Co KG, Germany;

Membrane extruder: LiposoFast™, Avestin, Canada;

Centrifuge A: 5810R, Eppendorf, Germany;

Centrifuge B: 2001, Kubota, Japan;

Freeze dryer: Christ Alpha 1-2, Martin Christ, Germany;

Shaker: GFL® 1086, Gesellschaft fur Labortechnik mbH, Germany;

Transmission light microscope: Axiovert 135M, Carl Zeiss, Germany;

SEM: JSM-5600LV, Jeol, Japan;

Platinum coating machine: JFC-1300, Jeol, Japan;

Cross-sectioning machine: CM3050, Leica, Austria;

UV-VIS scanning spectrophotometer: UV-3101 PC, Shimadzu, Japan;

XPS: AXIS His-165 Ultra, Shimadzu, Japan;

Fluorescence spectrometer: Quantamaster™ GL-3300, Photon Technology International, USA;

HPLC: LC 1100, Agilent, USA;

HPLC column: reverse phase Inersil® ODS-3 column with inner diameter 150×4.6 mm and pore size 5 μm, GL Science, Japan;

DSC: DSC 822e, Mettler Toledo, Switzerland;

Laser light scattering particle sizer: 90 Plus, Brookhaven Instruments, USA;

Zeta potential analyzer: Zeta Plus, Brookhaven Instruments Corporation, USA.

2.3 Single emulsion process

The single emulsion process was used to prepare paclitaxel-loaded microspheres at 25°C and the atmospheric pressure. Certain amount of paclitaxel was dissolved in the organic solvent (DCM, in some cases mixed with acetone, 5 ml) with the polymer (PLA-PEG-PLA or PLGA, 100 mg), which was then poured rapidly into 100 ml of PVA solution (2% w/v) whilst stirred at 800 rpm for 2 hrs (mechanical stirrer). The resulted o/w emulsion was further stirred overnight at 400 rpm to completely extract/evaporate the organic solvent, leaving behind solid microspheres. Afterwards the final product was obtained by centrifugation (centrifuge A, 8000-9000 rpm, 10 min), washing and freeze drying. Because the presence of emulsifier on the microsphere surface might disturb the detection of PEG in the surface chemistry analysis, it was necessary to completely remove PVA in the washing step. To do so, the above washing process was carefully repeated for at least 5 times.

2.4 Double emulsion process

The double emulsion process was used to prepare HSA-loaded microspheres at 25°C and the atmospheric pressure. HSA (100 µl) aqueous solution (50 mg/ml) was dispersed into the organic phase, which consists of 150 mg polymer (PLGA or PLA-PEG-PLA) dissolved in 2 ml organic solvent (DCM, ethyl acetate or acetone), by probe sonication for

30 s at an energy output of 7 W, resulting in a water-in-oil (w/o) emulsion. When applicable, an additive was dissolved in either the water (for PEG) or the oil phase (for Vitamin E TPGS) before the emulsification. The w/o emulsion was injected drop by drop, with a 3 ml dipper, into 50 ml 2% PVA aqueous solution and underwent either mechanical stirring (800 rpm or 1200 rpm, 2 min) or homogenization (3 pulses). The obtained w/o/w emulsion was poured into 450 ml 2% PVA aqueous solution. Mechanical stirring at 400 rpm was performed for 2.5 h to extract/evaporate the organic solvent, and solid microspheres were formed. Final product was obtained after filtration, washing and freeze-drying. To monitor the preparation process, samples at various stages were collected and observed with a transmission light microscope.

2.5 Modified double emulsion process

As we will discuss in detail in Chapter 5, a 'modified double emulsion process' was used to avoid the damage caused by the sonication treatment to the encapsulated fragile agents such as liposomes. Compared with the above-mentioned double emulsion process, the modified process is different in the following two points: (1) vortex mixing (2000 rpm, 10 min) was used instead of sonication to form the w/o emulsion, (2) because insufficient mechanical treatment such as vortex mixing could cause unstable w/o emulsion, one or more of the following newer materials were used to maintain the stability of the w/o emulsion: PLA-PEG-PLA instead of PLGA as the polymer, ethyl acetate and acetone (or their mixtures with DCM) instead of DCM as the organic solvent, emulsifiers used in the formation of w/o emulsion such as PVA (2% w/v, dissolved in the inner water phase) or

Vitamin E TPGS (10% w/w, dissolved in the oil phase). The w/o/w emulsion was formed by mechanical stirring at 800 rpm.

2.6 Preparation of liposomes

Liposomes were prepared by the film hydration method (multilamellar vesicles, MLVs) followed by membrane extrusion (large unilamellar vesicles, or LUVs). Briefly, certain amount of lipids dissolved in organic solvents (chloroform or its mixture with methanol) were transferred to a small glass vial, and dried by nitrogen gas, to form a thin lipid film on the wall of the vial. After the film was stored in vacuum at 4°C overnight to remove the residual organic solvents, it was hydrated with an appropriate volume of calcein solution (180 mM) for 5 h. Vortex mixing (2000 rpm, 10 min) was then performed in the presence of a few glass beads to form MLVs. LUVs were obtained by downsizing the MLVs with a membrane extruder with the liposome size controlled by the membrane pore size. Gel filtration was carried out to separate the MLVs or LUVs from the non-encapsulated calcein.

2.7 Coating of liposomes

Hydrophilic polymer coating was used to protect liposomes from organic solvents when they were encapsulated into polymeric microspheres. To coat liposomes with PEG, DSPE-PEG 2000 was mixed with the other lipids before they were dissolved in organic solvents for liposome preparation. The concentration of DSPE-PEG 2000 in the total lipid amount was 5%. To coat liposomes with PVA or chitosan, 2 ml liposomes were mixed with 6 ml

aqueous solution of PVA or chitosan and incubated at 4°C overnight. The final concentration of PVA or chitosan in the aqueous phase was 1%, unless stated otherwise.

2.8 SEM

The surface morphology of microspheres was examined by SEM, after the samples were coated with platinum. The platinum coating machine came together with the SEM. According to the manufacturer, platinum has advantages over gold. It can result in higher conductivity of coated sample surface, which can lead to higher quality of the SEM image. For each sample the diameter of at least 100 microspheres was measured and averaged by a computer program SMILE VIEW, which came together with the SEM equipment. To view the interior of the microspheres, the samples were cross-sectioned and SEM was then performed.

To visualize the liposomes, a drop of liposome solution of around 20 μl was dispersed onto a conductive tape which adhered to the top surface of a copper stud. It was then frozen in liquid nitrogen for 10 min and dried in a freeze drier. After negative-stained with 1% phosphotungstic acid and coated with platinum, the sample was viewed with SEM.

2.9 UV-Vis spectrophotometer

The HSA concentration in aqueous solution was determined using a UV-Vis spectrophotometer by the Bradford method, a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of proteins [Bradford,

1976]. The protocol used was the Bio-Rad microassay procedure [Bio-Rad company]. Briefly, 800 µl of each sample solution was mixed with 200 µl of dye reagent concentrate and incubated at room temperature for at least 5 min but no more than 1 h. The absorbance was then measured at 595 nm.

2.10 XPS

XPS was used to analyze the surface chemistry of the microspheres. A full spectrum scan was conducted over a binding energy of 0 to 1100 eV at a passing energy of 80 eV. Detailed analysis for C1s, O1s, N1s of the samples was conducted over 279-292 eV, 525-537 eV and 392-404 eV, respectively with a pass energy of 40 eV. Curve fitting was performed by a computer program XPSpeak provided by the equipment manufacturer. This software allows manipulation of the peak positions, full width at half maximum values, peak area and the Guassian-Lorentzian function.

2.11 Fluorescence spectrometer

Calcein is commonly used as a fluorescence probe of the aqueous core of liposomes due to its special self-quenching property [Gregoriadis, 1984b]. Below a certain concentration (around 2 µM), nice linear relationship exists between the fluorescence intensity and calcein concentration (excitation wavelength = 495 nm, emission wavelength = 516 nm). If the calcein concentration is high enough, however, the interactions among the molecules will cause fluorescence quenching, i.e. dramatic decrease of fluorescence intensity. In particular, when the concentration goes up to 20 mM, the fluorescence intensity will

become negligible. Therefore, in our case, the calcein inside the liposomes (180 mM) wouldn't lead to appreciable fluorescence. However, when the calcein was released from the liposomes, it would be diluted, leading to the appearance of fluorescence. By adjusting the concentration of released calcein to the linear range, the amount of released calcein from the liposomes could be analyzed by a fluorescence spectrometer. The encapsulated calcein inside the liposomes was analyzed by measuring the fluorescence intensity after breaking the liposomes with Triton X-100.

2.12 HPLC

Paclitaxel concentration in aqueous solution was determined by HPLC. The mobile phase consisting of a mixture of acetonitrile and Milli-Q water (50/50, v/v) was delivered at a flow rate of 1 ml/min. A 50 µl aliquot of the samples was injected with an autosampler. The column effluent was detected at 227 nm with a variable wavelength detector.

2.13 DSC

The physical state of paclitaxel inside the microspheres was characterized by DSC. The samples were sealed in aluminum pans with lids. The samples were purged with pure dry nitrogen at a flow rate of 2 ml/min. A temperature ramp speed was set at 10 K/min and the heat flow was recorded from 273-523 K. Indium was used as the standard reference material to calibrate the temperature and energy scales of the DSC instrument.

2.14 Laser light scattering for particle sizing

The liposome samples of 300 μl were diluted to 3 ml before the measurements. Three parameters were recorded: effective diameter, which indicated the particle size; polydispersity, which indicated the particle size distribution; and counting rate, which indicated the number of liposomes.

2.15 Zeta potential analysis

Zeta potential is an indicator of the surface charge of particles in a colloid dispersion, which affects the velocity of the particle movement and can also be analyzed by the method of light scattering [Morrison and Ross, 2002]. Surface charge of the microspheres and liposomes was examined by a zeta potential analyzer. For the microspheres, the samples were dispersed in PBS buffer (pH=7.4) at the concentration of 1.5±0.3 mg/ml and slightly sonicated in a bath sonicator for 1 min before the analysis. For the liposomes, the samples of 200 μl were diluted to 2ml before the analysis.

2.16 Integrity of liposomes

To investigate the integrity of liposomes after the treatment by an organic solvent, the solvent (5 ml) was poured to 1 ml emulsion of calcein-loaded liposomes and they were mixed by a vortex mixer (2000 rpm, 10 min). The water phase was collected after it was completely separated from the organic phase. Two properties, namely the liposome number and the calcein inside the liposomes, were analyzed before and after the treatment

by the organic solvent by laser light scattering and fluorescence spectrometer, respectively. The change of these two properties indicated the damage of the liposomes by the organic solvent. As described earlier, the number of liposomes was indicated by the counting rate in the light scattering sizing measurement. Higher counting rate implies more vesicles present. The calcein remaining in the liposomes after the treatment of the organic solvent was analyzed by subtracting the calcein release caused by liposome breakage, which was calculated by the fluorescence intensity, from the total calcein amount inside the liposomes without the treatment by organic solvents.

The integrity of liposomes in LIMs was examined by SEM, light scattering and fluorescence spectrometer. The liposomes inside LIMs were visualized by SEM after the polymer matrix was cut apart. The particle size and calcein leakage after the liposomes were released from LIMs were measured by light scattering and fluorescence spectrometer respectively. The results were compared with those obtained before the liposomes were encapsulated.

2.17 Encapsulation efficiency

2.17.1 Encapsulation efficiency of paclitaxel in microspheres

The encapsulation efficiency is defined as the ratio of the amount of encapsulated drug to that of the drug used for microsphere preparation. To determine the paclitaxel content in the microspheres, 3 mg of paclitaxel-loaded microspheres were dissolved in 1 ml DCM. A mixture of acetonitrile and Milli-Q water (50/50, v/v) was then added. A nitrogen gas

stream was introduced to evaporate DCM until a clear solution was obtained. The solution was analyzed by HPLC for paclitaxel concentration. The above measurements were performed in duplicate. The deviation between the two results was found to be less than 10%.

2.17.2 Encapsulation efficiency of HSA in microspheres

Microspheres (5 mg) were dissolved in 0.25 ml DCM, which was then transferred into an Eppendorf tube. Milli-Q water (0.5 ml) was added and the tube was vortexed for 5 min to extract the HSA. Subsequently the system underwent centrifugation (centrifuge B) at 3000 rpm for 5 min to separate the oil and water phases. The latter was collected for protein assay by the Bradford method. The processes of extraction and protein assay were repeated 4 times. The encapsulation efficiency of microspheres, which is defined by the following equation, was determined in duplicate. The deviation between the two results was found to be less than 10%.

$$EE = \frac{\sum_{i=1}^{4} C_i \times V_i}{W_{MS}} \times \frac{W_{HSA} + W_{polymer} + W_{additive}}{W_{HSA}} \times 100\% \qquad \text{(Equation 2.1)}$$

EE – HSA encapsulation efficiency inside microspheres

C_i – HSA concentration in the water phase of each extraction

V_i – volume of water phase of each extraction

W_{MS} – weight of microspheres used for EE analysis

W_{HSA} – weight of HSA used in the microsphere preparation process

$W_{polymer}$ – weight of polymer used in the microsphere preparation process

$W_{additive}$ – weight of additive used in the microsphere preparation process

2.17.3 Encapsulation efficiency of liposomes in LIMs

The liposome encapsulation efficiency inside LIMs was calculated by subtracting the amount of liposome loss during the modified double emulsion process from the total amount of liposomes used for LIM preparation and dividing the difference by the latter. Duplicate measurements were performed. The deviation between the two results was found to be less than 10%. To measure the amount of liposomes not encapsulated in the microspheres, the outer water phase in the w/o/w emulsion and the water used for solvent removal and washing were collected after the LIMs were obtained. These liposomes were treated with 2% Triton X-100 at 60°C for 1 h to break the liposomes to make the calcein released. The amount of liposomes not encapsulated was then obtained by measuring the fluorescence intensity. Two experiments were further performed to ensure that no appreciable calcein was released from the liposomes before the treatment of Triton X-100 and that calcein was completely released after the treatment.

2.18 In vitro release

2.18.1 In vitro release of paclitaxel from microspheres

The experiments were performed in duplicate. The deviation between the two results was found to be less than 10%. Microspheres (10 mg) were placed in Eppendorf tubes and

incubated in 10 ml release medium (PBS buffer, pH=7.4) under agitation in a shaker (37 °C, 110/min). At desirable time intervals, the microsphere suspensions were centrifuged at 10000 rpm for 10 min (centrifuge A). The supernatant (10 ml) was withdrawn and replaced with 10 ml fresh release medium. The amount of paclitaxel released was determined by measuring drug concentration in the supernatant. To determine the drug content in the supernatant, 1 ml DCM was added to extract paclitaxel. The organic phase was collected and left in a fume cupboard to evaporate DCM. This was followed by adding 3 ml of mixture of acetonitrile and water (50/50, v/v) to dissolve the drug. HPLC was then performed to determine the quantity of the drug.

To determine the extraction efficiency of paclitaxel from the release medium to DCM, a known amount of paclitaxel was subjected to the same procedure as above. The extraction efficiency obtained here was then used as a correction factor for the data obtained with the microspheres.

2.18.2 In vitro release of HSA from microspheres

The experiments were performed in duplicate. The deviation between the two results was found to be less than 10%. Microspheres (10 mg) were placed in Eppendorf tubes and incubated in 1 ml release medium (PBS buffer, 0.02% sodium azide, pH=7.4) under agitation in a shaker (37°C, 110/min). At desirable time intervals, the microsphere suspensions were centrifuged at 3000 rpm for 10 min (centrifuge B). The supernatant (1 ml) was withdrawn and replaced with 1 ml fresh release medium. The amount of protein

released was determined by measuring protein concentration in the supernatant using the Bradford method.

2.18.3 In vitro release of liposomes from LIMs

The experiments were performed in duplicate. The deviation between the two results was found to be less than 10%. LIMs (10 mg) were placed in Eppendorf tubes and incubated in 1 ml release medium (PBS buffer, 0.02% sodium azide, pH=7.4) under agitation in a shaker (110/min, 37°C). At desirable time intervals, the LIM suspensions were centrifuged at 3000 rpm for 10 min (centrifuge B). The supernatant (1 ml) was withdrawn and replaced with 1 ml fresh release medium. The liposome amount in the release medium was determined by measuring the fluorescence of calcein after treating the liposomes with 2% Triton X-100 at 60°C for 1h as described in Sec 2.17.3.

CHAPTER 3 PACLITAXEL-LOADED MICROSPHERES

3.1 Introduction

Cancer is a group of diseases which affect the cells of the body, causing them to grow too rapidly and without order, forming tumors. Tumors are either benign, meaning that they stay confined to the part of the body where they formed, or they are malignant, invading surrounding tissue and destroying healthy cells. Such malignant tumors may result in cells breaking off and spreading through the bloodstream to other parts of the body to create new tumors. This process of metastasis can have fatal results [Chabner and Longo, 1996]. At present, cancer is the number one cause of death in Singapore and the number two in the United States [Chia, 1996].

Since the former US president Richard Nixon declared war on cancer 30 years ago, numerous anticancer agents have been developed. Among them, paclitaxel is believed to be one of the best discovered from nature (see Fig. 3.1 for its molecular structure). No other chemotherapeutic agent except penicillin has generated so much interest [Panchagnula, 1998]. It has been widely applied to treat various cancers, especially breast cancer and ovarian cancer [Huizing, 1995, Lopes et al., 1993]. Its unique action mechanism involves binding to microtubules, forming dysfunctional microtubules and thus leading to cell death [Horwitz, 1992]. In this Chapter, paclitaxel was used as a prototype of anticancer drugs.

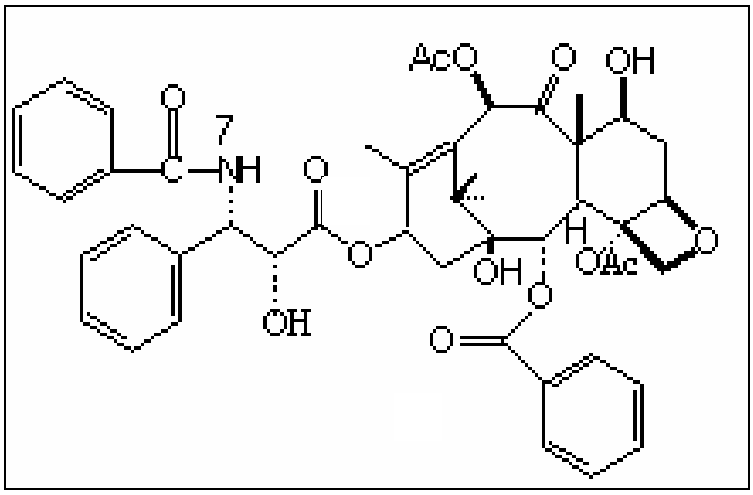


Figure 3.1 Molecular structure of paclitaxel. The number 7 near the nitrogen atom corresponds to the peak number in the XPS analysis of Sec. 3.2.3.

There are two major hurdles for clinical application of paclitaxel. One is its availability. Paclitaxel was originally extracted from the bark of a rare and slowly growing Pacific yew tree or Western yew tree (Taxus Bevifolia). For the chemotherapy of each patient, four trees of more than 100 years old would have to be sacrificed to obtain two grams of the drug. This is not affordable for the nature. Total synthesis of paclitaxel has been achieved, which is however too complicated and its price is thus extremely high [Holton et al., 1994, Nicolaou et al., 1994b]. A most promising solution is semi-synthesis, in which the drug is produced from needles and twigs of more abundant English yew trees or Chinese red bean yew trees [Colin et al., 1990]. Another hurdle to apply paclitaxel to treat cancers is the difficulty in its clinical administration. Due to the low aqueous solubility of the drug, an adjuvant Cremophor EL has to be used. Paclitaxel in Cremophor EL called Taxol® is the only available clinical form of paclitaxel and has been reported to be responsible for various serious side effects such as hypersensitive reactions, nephrotoxicity, neurotoxicity and cardiotoxicity [Liebmann et al., 1993]. In recent years, various controlled delivery forms, such as polymeric micro/nanospheres [Dordunoo et al., 1995], liposomes [Sharma

and Straubinger, 1994], micelles [Onyuksel et al., 1994], parenteral emulsion [Lundberg, 1997], cyclodextrins [Sharma et al., 1995], and prodrugs [Nicolaou et al., 1994a], have been investigated to increase the solubility, to minimize the side effects as well as to avoid the use of the toxic adjuvant. Among these alternative delivery systems, polymeric micro/nanospheres have attracted a lot of interests.

As discussed earlier, the single emulsion technique is commonly used to encapsulate water-insoluble drugs such as paclitaxel into polymeric microspheres. In most of the published papers, PLGA and paclitaxel were dissolved in the organic solvent DCM to form the oil phase. The oil phase was then dispersed into a large volume of water phase in the presence of the emulsifier PVA. After the organic solvent was removed by extraction/evaporation, solid microspheres were formed with the drug encapsulated [Harper et al., 1999, Liggins et al., 2000, Wang et al., 1996]. Our group has been pursuing to improve the above recipe and process. In the previous work, our group has applied natural emulsifiers such as phospholipids and Vitamin E TPGS in place of PVA to improve the biocompatibility and the encapsulation efficiency of the delivery vehicles [Feng and Huang, 2001, Feng et al., 2002, Mu and Feng, 2002], and the spray drying technique in place of solvent extraction/evaporation for larger-scale production of micro/nanospheres [Mu and Feng, 2001]. The focus of this Chapter was to use a new kind of polymer, the triblock copolymer PLA-PEG-PLA, to replace the commonly used PLGA. The molecular structure of PLA-PEG-PLA is shown in Fig. 3.2. We expected some advantages of PLA-PEG-PLA over PLGA.

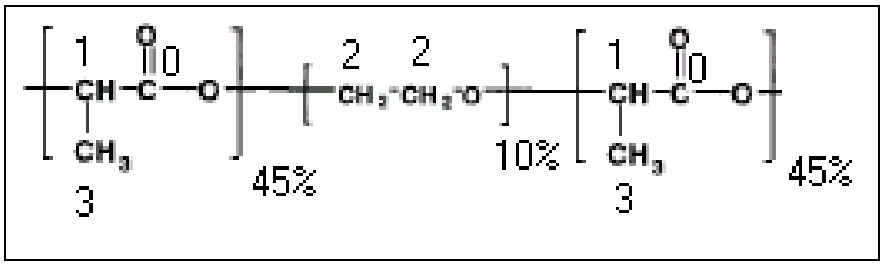


Figure 3.2 Molecular structure of PLA-PEG-PLA. The numbers 0, 1, 2, 3 near the carbon atoms correspond to the peak numbers in the XPS analysis of Sec. 3.2.3.

The release of paclitaxel from PLGA micro/nanospheres was found extremely slow, because this drug is highly hydrophobic and PLGA is also hydrophobic. Our group previously reported that only 50% of the drug could be released within 3 months [Feng and Huang, 2001, Mu and Feng, 2001]. However, the continuous release of an anticancer agent from a controlled delivery device for one week to one month is usually required for effective treatment of the cancer [Wang et al., 1996]. We expected that incorporation of a hydrophilic segment such as PEG into the hydrophobic poly(lactic acid) (PLA) chain, which forms the triblock copolymer PLA-PEG-PLA, would greatly facilitate the drug release. Li et al. reported the use of PLA-PEG-PLA for vaccine delivery [Li et al., 2000]. Nevertheless, it has not yet been applied for controlled release of hydrophobic drugs. Another potential advantage of PLA-PEG-PLA would be the presence of hydrophilic segment PEG on the surface of the microspheres formed. It is believed that increase of surface hydrophilicity of delivery vehicles normally leads to better biocompatibility, because most of the biological environment is hydrophilic in nature and biocompatibility appears to be correlated directly with the degree of hydrophilicity that a surface exhibits [LaPorte, 1997]. In this Chapter, we were to prepare paclitaxel-loaded PLA-PEG-PLA microspheres by the single emulsion process, to characterize the product for its

physicochemical properties and in vitro release behavior, and to investigate the effects of various material parameters.

3.2 Results and discussions

3.2.1 Preparation of microspheres

Six microsphere samples of different compositions were prepared by the single emulsion method (Table 3.1). In order to examine the effects of different polymers, PLGA (L/G ratio 50/50, Mw 40000-75000), PLA-PEG-PLA (10% PEG, Mw 50000) and PLA-PEG-PLA (10% PEG, Mw 80000) were used in Samples Pm1, Pm2 and Pm3, respectively. The L/G ratio of 50/50 was selected for Pm1 because it is well documented that PLGA at this L/G ratio possesses the fastest degradation rate and results in fastest drug release rate from the microspheres [Anderson and Shive, 1997]. Different drug loading amount and organic solvents were used in samples Pm4 to Pm6 to examine their effects on the properties of the microspheres formed.

3.2.2 Particle size, encapsulation efficiency and colloidal stability

In Table 3.1 the particle size, encapsulation efficiency and zeta potential of the microsphere samples are listed against the polymer, the organic solvent used and the drug loading. It can be seen that the average particle size of all samples was 13-23 μm, which meets the requirement of chemoembolization for particle size [Dass and Burton, 1999]. In

fact, the chemoembolization of PLGA microspheres has been shown to effectively blockade the blood supply of a tumor [Wang et al., 1997].

Table 3.1 Samples of paclitaxel-loaded microspheres. The drug loading is defined as the ratio of the drug weight and the sample weight.

Sample number	Composition (polymer/solvent/drug loading w/w)	Particle size (μm)	Encapsulation efficiency (%)	Zeta potential (mV)
Pm1	PLGA/DCM/5%	13.72±5.93	74.18	-36.37±2.85
Pm2	PLA-PEG-PLA50000/DCM/5%	22.14±10.00	64.94	-0.21±0.00
Pm3	PLA-PEG-PLA80000/DCM/5%	21.18±7.47	73.28	-28.95±0.90
Pm4	PLA-PEG-PLA80000/DCM/8%	22.15±10.40	81.55	-30.91±0.77
Pm5	PLA-PEG-PLA80000/DCM/10%	21.84±10.20	81.53	-30.87±1.00
Pm6	PLA-PEG-PLA80000/ 50% DCM+50% acetone/5%	18.42±10.40	73.93	-22.39±1.41

Shown in Fig. 3.3 is the calibration curve of paclitaxel concentration vs. peak area in HPLC analysis, which was used in the measurement of encapsulation efficiency and in vitro release of the drug. The linear range is from 49.6 ng/ml to 496 μg/ml. Within this range 10 data points were recorded. The R^2 value is 0.9999998.

The encapsulation efficiency of the microspheres ranged from 64% to 82%. In general, microencapsulation of hydrophobic drugs such as paclitaxel is relatively easy with hydrophobic polymers such as PLGA and PLA-PEG-PLA since loss of the drugs to the water phase occurs less likely in comparison with hydrophilic drugs.

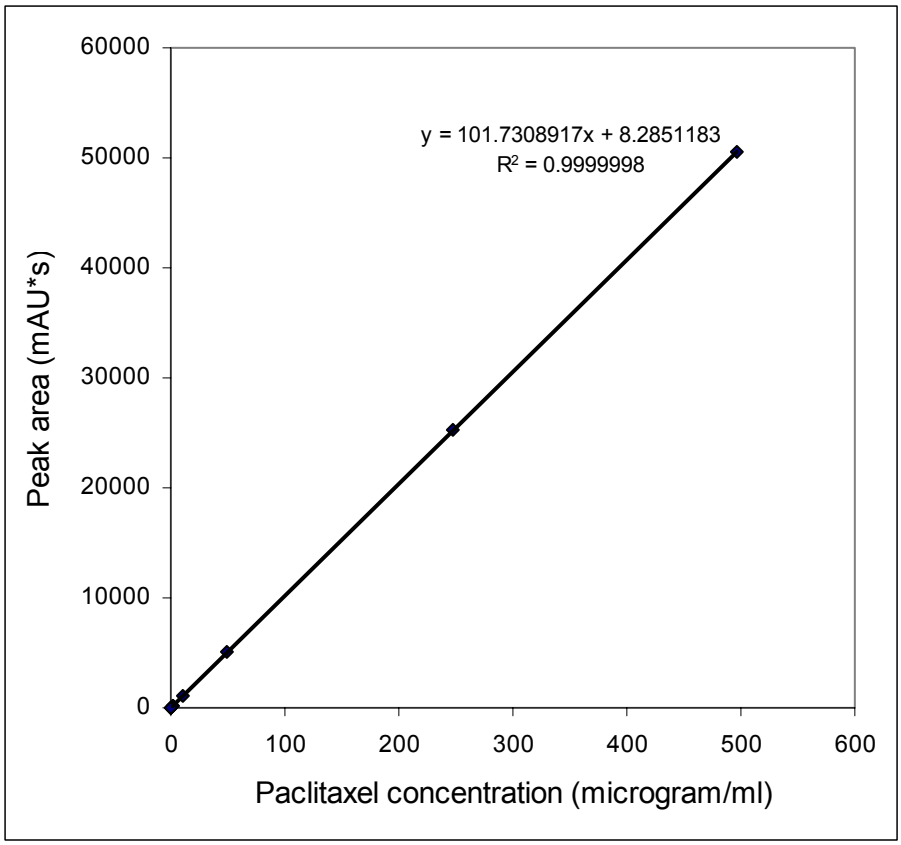


Figure 3.3 Calibration curve of HPLC analysis of paclitaxel

Colloidal stability of the microspheres is important because they are supposed to be delivered as a colloidal form. The colloidal stability was analyzed by measuring zeta potential of the microspheres. Larger absolute value of zeta potential indicates better colloidal stability [Sernelius, 2001]. As can be seen from Table 3.1, the zeta potentials of all samples were negative, implying that the microsphere surface was negatively charged. This should be attributed to the presence of ionized carboxyl groups, such as those from lactic acid or glycolic acid, on the microsphere surface since PEG is actually uncharged. In general, the zeta potential of PLA-PEG-PLA microspheres (Pm2 to Pm6) was less negative than that of PLGA microspheres (Pm1), presumably because in PLGA microspheres both the lactic acid and glycolic acid segments contribute to the surface

charge while in PLA-PEG-PLA microspheres, only the lactic acid segments lead to negative charge. Surprisingly enough, the sample of PLA-PEG-PLA with lower Mw had an especially low absolute value of zeta potential (sample Pm2), implying very low colloid stability. In fact, serious aggregation was observed by SEM for this sample (image not shown). Therefore, this sample was not selected in the following experiments. Nevertheless, the PLA-PEG-PLA microsphere samples having Mw 80000 were sufficiently stable for drug delivery application (Pm3 to Pm6) due to the fact that their zeta potential was between –31 mV and -22 mV. In general, these values are considered to be associated with stable colloid [Dobias, 1993].

3.2.3 Surface chemistry

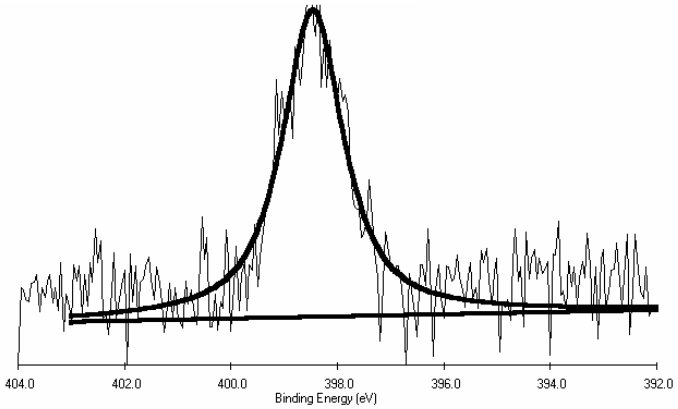
As shown in Fig. 3.1 and 3.2, paclitaxel has nitrogen element in its chemical structure while PLA-PEG-PLA doesn't have any. Therefore, the N1s region of XPS data was used to determine whether the drug was present on the surface of the paclitaxel-loaded PLA-PEG-PLA microspheres. The XPS N1s region of the pure paclitaxel and the PLA-PEG-PLA microspheres is shown in Fig. 3.4 (A) and (B), respectively. It is clear that the characteristic peak of paclitaxel (peak number 7, binding energy 398.5 eV) was not detected on the surface of PLA-PEG-PLA microspheres, which implies that the drug present on the microsphere surface was negligible.

The XPS C1s regions were used to determine the distribution of PEG segment on the microsphere surface. Thermodynamically, the hydrophilic PEG segment tends to migrate to the surface of particles formed by copolymers between PEG and PLA, to minimize the

contact between hydrophilic and hydrophobic segments, forming a micellar structure with the particle surface all covered by PEG. However, the actual percentage of PEG on the particle surface depends on many factors such as the configuration and composition of the copolymers and the size of the particles. Fig. 3.4 (C) and (D) show the XPS C1s region of the pure material of PLA-PEG-PLA and the paclitaxel-loaded PLA-PEG-PLA microspheres respectively. It can be seen that the shape of XPS spectra of the pure polymer and the microspheres is virtually identical. Curve fitting by the software XPSpeak showed that the percentage of area for the characteristic peak of PEG (peak number 2, binding energy around 284.9 eV) was approximately 13%. These results imply that the PLA-PEG-PLA microspheres didn't form the micellar structure, since otherwise the percentage of characteristic PEG peak would be 100%. In other words, part of the PEG segment remained in the interior of the microspheres while the other located on the surface. In contrast to our results described above, it has been reported that the diblock PLA-PEG nanoparticles resulted in the micellar structure [Kim et al., 1999, Kim et al., 2001]. This might be because the diblock polymer and small particle size allowed PEG to readily migrate to the surface of the particles. The presence of PEG in the interior of our microspheres might be advantageous for fast drug release, as will be discussed in the next Section.

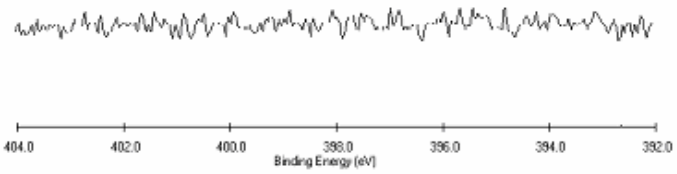
The surface modification of the microspheres caused by PEG could improve the biocompatibility of the delivery vehicle as discussed in Sec. 3.1. It should be reasonable to predict that increasing the amount of PEG monomer in the synthesis of the PLA-PEG-PLA copolymer could increase the amount of PEG on the microsphere surface, leading to better biocompatibility.

Peak number	Binding energy (eV)	Percentage of peak area (%)
7	398.457	100

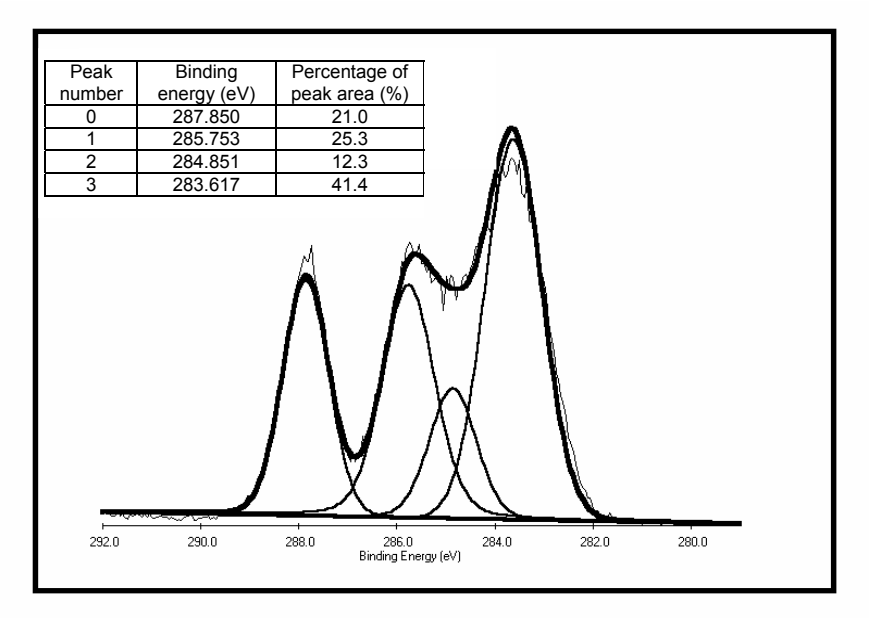


(A) XPS N1s region of paclitaxel

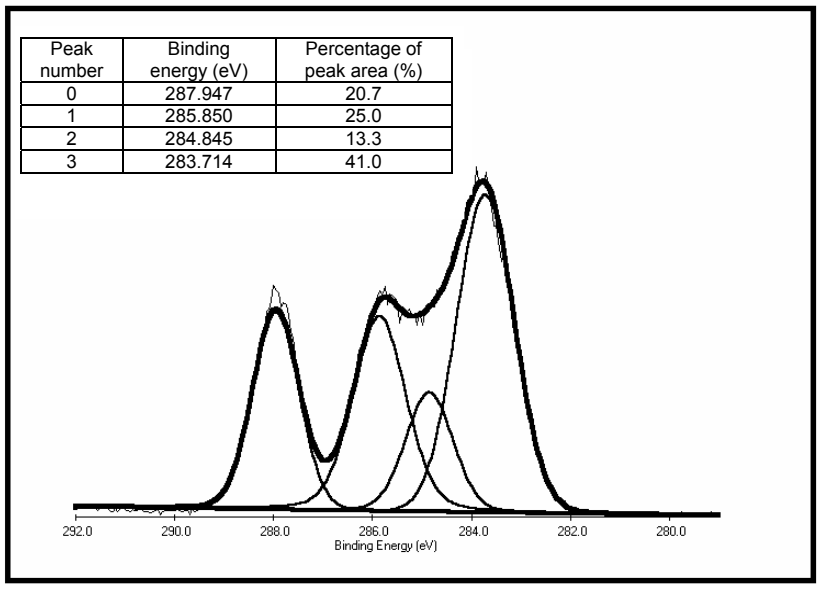
Peak number	Binding energy (eV)	Percentage of peak area (%)
7	398.457	0



(B) XPS N1s region of paclitaxel-loaded PLA-PEG-PLA microspheres (Pm3)



(C) XPS C1s region of PLA-PEG-PLA polymer



(D) XPS C1s region of paclitaxel-loaded PLA-PEG-PLA microspheres (Pm3)

Figure 3.4 XPS spectra of the pure materials and paclitaxel-loaded microspheres. The table in each figure presents the percentage of the peak area. The peak numbers in the tables correspond to the numbers in Fig. 3.1 and 3.2. The XPS data of all PLA-PEG-PLA microsphere samples (Pm2-Pm6) were found to be virtually the same.

3.2.4 Surface and internal morphology

The morphology of polymeric microspheres is well-known to be a critical factor to affect the drug release kinetics [Fan and Singh, 1989]. It was found that the presence of PEG segment within the PLA chains could significantly alter the surface and internal morphology of the microspheres. As shown in Fig. 3.5 (1) and (2), the PLGA microspheres possess smooth surface and dense interior. This is understandable since the o/w emulsion with the oil core formed by the water-insoluble polymer, the water-insoluble drug and the organic solvent should lead to dense particles formed after the organic solvent is removed. In contrast, the surface of the PLA-PEG-PLA microspheres was coarse. Pores were seen inside the microspheres and distributed more or less evenly across the entire cross-section (Fig. 3.5 (3) and (4)). This could be attributed to the presence of the PEG segments in the interior of PLA-PEG-PLA microspheres as shown by XPS analysis (Fig. 3.4), which allows the water phase to enter the PLA-PEG-PLA microspheres during the emulsification and solvent removal process.

The porosity of the PLA-PEG-PLA microspheres could be further adjusted by mixing DCM with a water-soluble solvent acetone. As shown in Fig. 3.5 (5) and (6), the presence of acetone caused coarser surface and more porous interior of the microspheres, presumably due to a ‘local explosion’ phenomenon caused by fast extraction of the water-soluble acetone to the water phase in the microsphere preparation process [Arshady, 1991]. In addition, the SEM results showed that different drug loading did not significantly alter the morphology of the microspheres (images not shown).

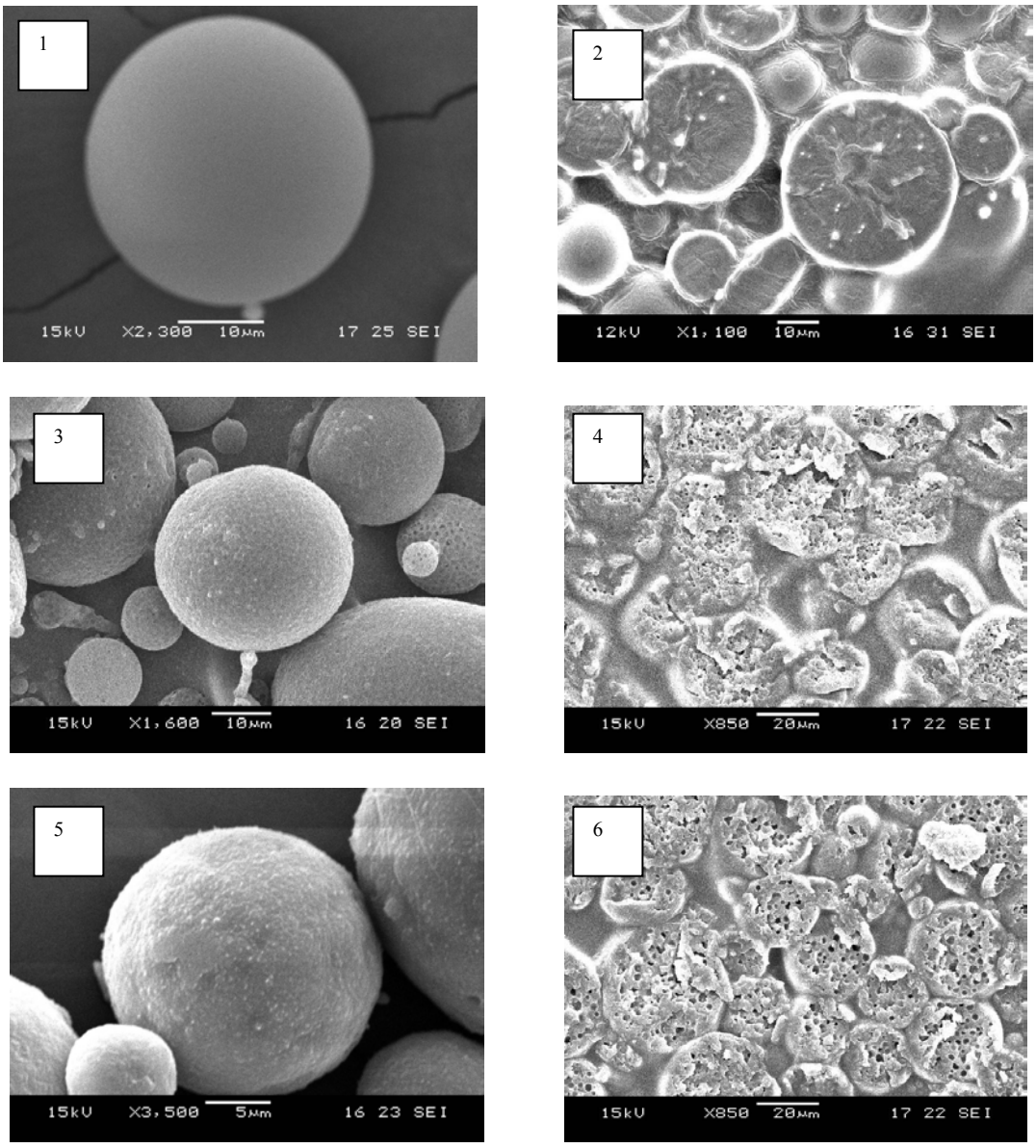


Figure 3.5 Surface and internal morphology of paclitaxel-loaded microspheres. (1) (2): Pm1, PLGA/100% DCM; (3) (4): Pm3, PLA-PEG-PLA80000/100% DCM; (5) (6): Pm6, PLA-PEG-PLA80000/50% DCM + 50% acetone.

3.2.5 Drug state in microspheres

DSC was used to analyze the physical state of paclitaxel inside microspheres. As shown in Fig. 3.6, the melting peak of paclitaxel crystal appeared at 496.2K (thermogram 1), which was consistent with the result of Liggins et al. [Liggins et al., 1997]. This peak was also

detected when the drug amount was reduced to 0.4 mg (thermogram 2), which was the amount of paclitaxel found in 4 mg microsphere sample Pm5. However, after paclitaxel was encapsulated into the microspheres (sample Pm5), its melting peak disappeared, indicating that in the microspheres the drug existed as amorphous form rather than crystal (thermogram 3). This was also the case for the other microsphere samples (Pm1, Pm3, Pm4, Pm6). Therefore, among the three common drug release mechanisms from polymeric microspheres [Fan and Singh, 1989], drug dissolution in the polymer matrix was negligible compared with drug diffusion and polymer degradation in the paclitaxel-loaded microspheres.

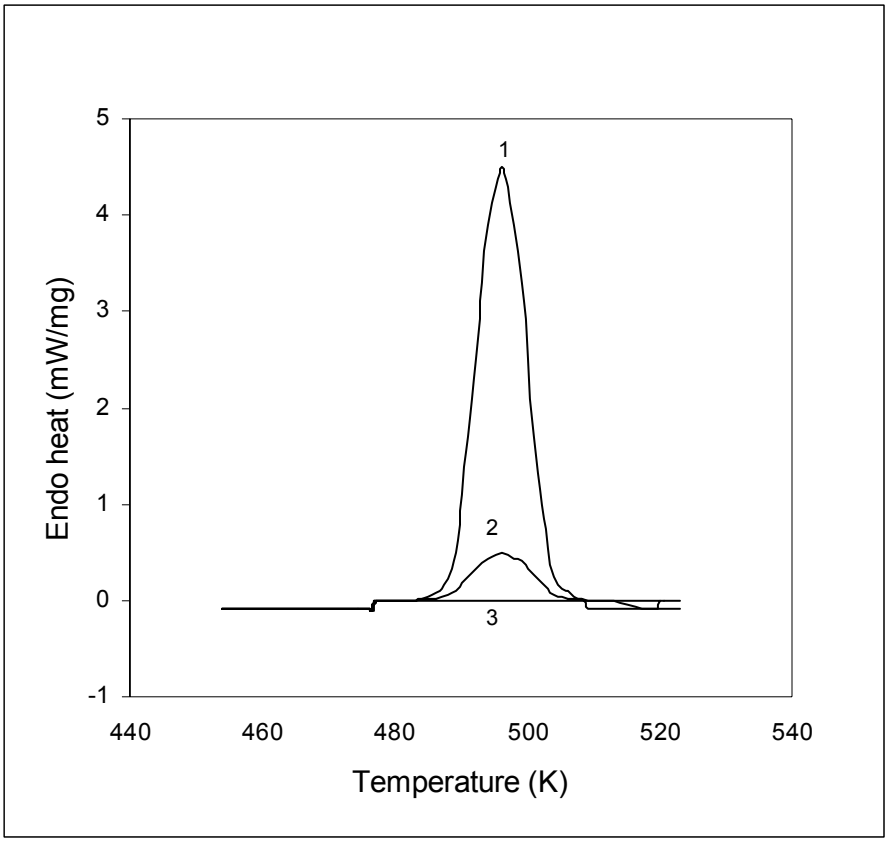


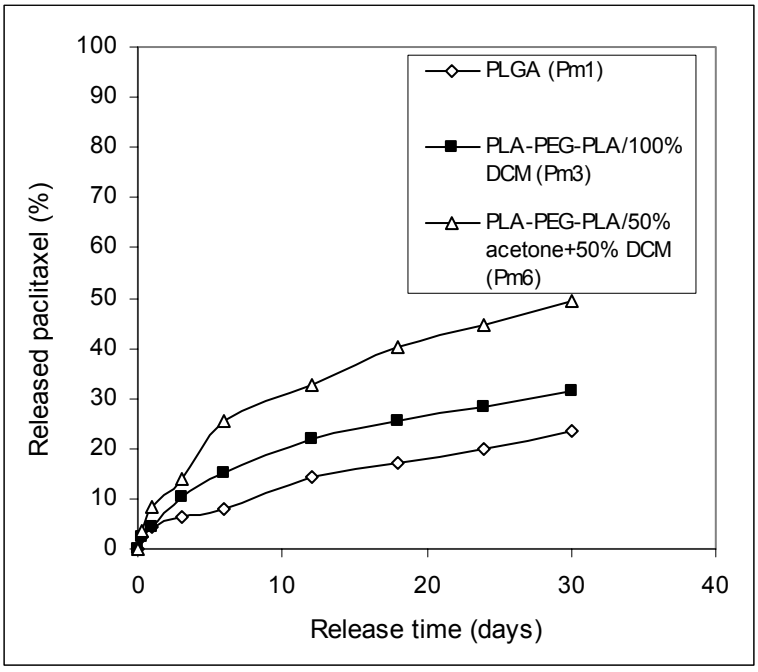
Figure 3.6 DSC thermograms of (1) 4 mg paclitaxel, (2) 0.4 mg paclitaxel, and (3) 4 mg PLA-PEG-PLA microspheres containing 10% paclitaxel (Pm5).

3.2.6 In vitro release

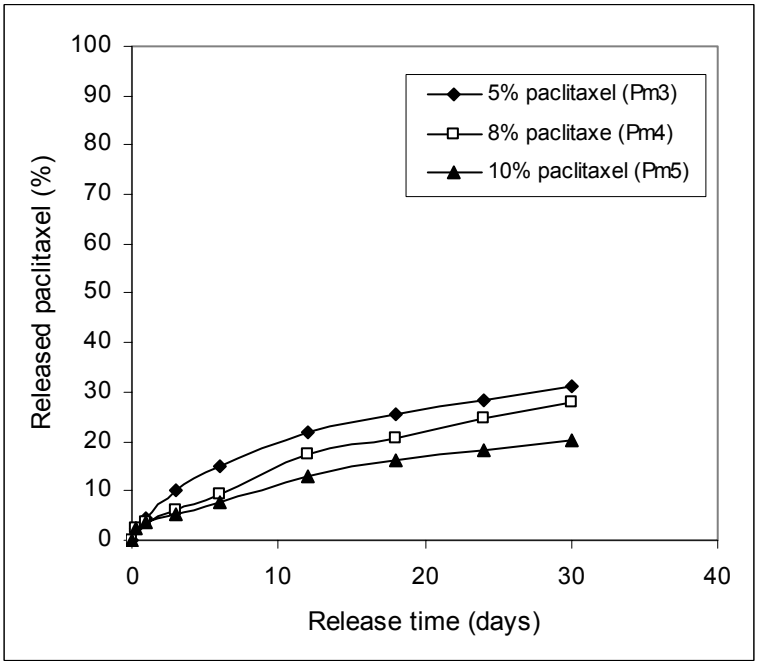
PLA-PEG-PLA microspheres resulted in significantly faster release of the drug from the microspheres compared with that from PLGA samples (Fig. 3.7 (A)). This might be, at least partially, due to the porous morphology of the PLA-PEG-PLA microspheres (Fig. 3.5). Their porous structure appeared to be able to further facilitate the paclitaxel release with addition of acetone into the organic solvent, which has been shown earlier to increase the microsphere porosity (Fig. 3.5) and thus lead to faster release. The paclitaxel release level of sample Pm6 (50% acetone) reached 49.6% after 30 days of sustained release.

The effect of drug loading amount on the drug release rate from the microspheres appeared to vary depending on the drugs and polymers used. Even in the case of the same system, i.e. paclitaxel-encapsulated PLA/PLGA microspheres, the results reported from different laboratories could be different. Wang et al. reported decreasing release rate when drug loading was increased [Wang et al., 1996], while Liggins et al. reported an opposite trend [Liggins et al., 2000]. Fig. 3.7 (B) shows the in vitro release curves of our paclitaxel loaded PLA-PEG-PLA microsphere samples Pm3-Pm5, which had different paclitaxel loading amount. It can be seen that a higher drug loading resulted in a slower release. Because the drug dissolution in the PLA-PEG-PLA microspheres was negligible, as shown by the DSC results, and the polymer degradation should be similar for these three samples, the effect of drug loading amount on the in vitro release profile could be attributed to the change of drug diffusion rate caused by the different drug loading levels. In the single emulsion process, paclitaxel is completely soluble in DCM and evenly distributed in the polymer matrix. Different amount of paclitaxel might affect the oil

droplet formation and solvent removal, thereby leading to different tortuosity inside the microspheres, which can thus give rise to different drug diffusion rate.



(A) Effect of polymers and solvents



(B) Effect of drug loading

Figure 3.7 In vitro release of paclitaxel from polymeric microspheres

3.3 Summary

In this Chapter, paclitaxel-loaded microspheres made of the biocompatible and biodegradable polymer PLA-PEG-PLA were prepared by the single emulsion technique. The microspheres of various compositions were characterized with regard to the particle size, the encapsulation efficiency, the colloidal stability, the surface chemistry, the surface and internal morphology, the physical state of the drug in the polymeric matrix and the in vitro release kinetics. The physicochemical properties of the PLA-PEG-PLA microspheres were compared with those of the conventional PLGA microspheres. The effects of molecular weight of PLA-PEG-PLA, the presence of acetone in the organic solvent and the drug loading amount were investigated. It was found that PLA-PEG-PLA of Mw 80000 could form microspheres of particle size of around 20 μm, which is suitable for chemoembolization, satisfactory encapsulation efficiency and stable colloid dispersion in water, while PLA-PEG-PLA of Mw 50000 led to serious aggregation of the microspheres. Paclitaxel was found to exist in an amorphous form in the microspheres. The hydrophilic segment PEG was distributed both on the surface and in the interior of the PLA-PEG-PLA microspheres, resulting in the formation of evenly distributed pores in the microspheres, which, in comparison with the PLGA microspheres, led to significantly faster in vitro release of the highly hydrophobic drug. Mixing DCM with a water-soluble solvent acetone could result in more porous morphology of the microspheres and further enhance the paclitaxel release. About 50% sustained release within 1 month was achieved. It was also found that higher drug loading amount could result in slower drug release from the microspheres, which might be due to the decreased drug diffusion in the polymeric matrix.

To conclude, PLA-PEG-PLA microspheres could be a promising controlled delivery system for clinical administration of paclitaxel and other anticancer drugs.

CHAPTER 4 HSA-LOADED MICROSPHERES

4.1 Introduction

Most of the current drugs, such as penicillin and paclitaxel, were developed by screening a large number of synthetic or natural compounds [Perum and Propst, 1989]. Although it doesn't need much information about the molecular mechanism of the diseases, the screening approach suffers from inherent problems such as (1) it is time and money consuming, (2) there is high possibility of neglecting important compounds, and (3) the compounds discovered by this approach frequently don't have the optimal structure. A series of breakthroughs in molecular biology has led to the so-called molecular medicine, a new generation of disease treatment [Dill, 1999, FASEB, 1998]. This means that now scientists are able to develop drugs at molecular level, with the following knowledge and skills: what life is composed of (human genome sequence, structure of proteins and DNA/RNA), how lives work (DNA code is transcribed to RNA, which serves as a template for protein synthesis. Proteins manipulate a variety of biological functions.), how to investigate drug molecules (resolution of molecular structure by X-ray crystallography and NMR spectroscopy, structure-activity relationship study), and how to design and produce drug molecules (computer-aided molecular design, production of proteins by recombinant gene technology, protein engineering and chemical synthesis). The advantage of this approach is obvious: it allows us to deal with the underlying causes of diseases, and thus, theoretically, the opportunity to obtain drugs with highly specific activity is greatly increased. The drugs developed by this approach are often called molecular drugs, which,

in the present definition, are mainly macromolecules like proteins/peptides, as it appears that nature needs molecules of complicated structure to store the large amount of information and regulate the complex biological functions [Buckel, 1996].

Proteins are very large molecules composed of amino acids joined by peptide bonds. The vast majority of the proteins found in living organisms are composed of only 20 different kinds of amino acids, repeated many times and strung together in a particular order. Protein molecules are very complicated, having up to four levels of structure: the sequence of amino acids is known as the primary structure (Fig. 4.1); Hydrogen bonds between amino acids cause the amino acid chain to assume a helical secondary structure; the chains loop and fold back upon themselves, forming the tertiary structure; some proteins, such as hemoglobins, are composed of more than one protein subunit, and the spatial conformation of these chains is known as the quaternary structure (Fig. 4.2) [Campbell and Shawn, 2003].

With more and more therapeutic proteins entering the market, their controlled delivery has become a hot topic [Cleland and Langer, 1994]. Initially, however, the feasibility of using polymeric microspheres to release large molecules like proteins was doubted, because it seemed to be unlikely for drug molecules to permeate through the barrier formed by polymer molecules with the same or even smaller molecular weight. Later, it was found that the protein molecules, which were water-soluble, could form pores inside the microspheres, through which drug release became possible [MIT News Office, 1998]. As discussed earlier, to encapsulate water-soluble drugs into polymeric microspheres, the double emulsion technique is often used. This process has been applied to produce Lupron

Depot®, the first commercialized product of polymeric microsphere formulation of proteins [Sanders and Hendren, 1997].

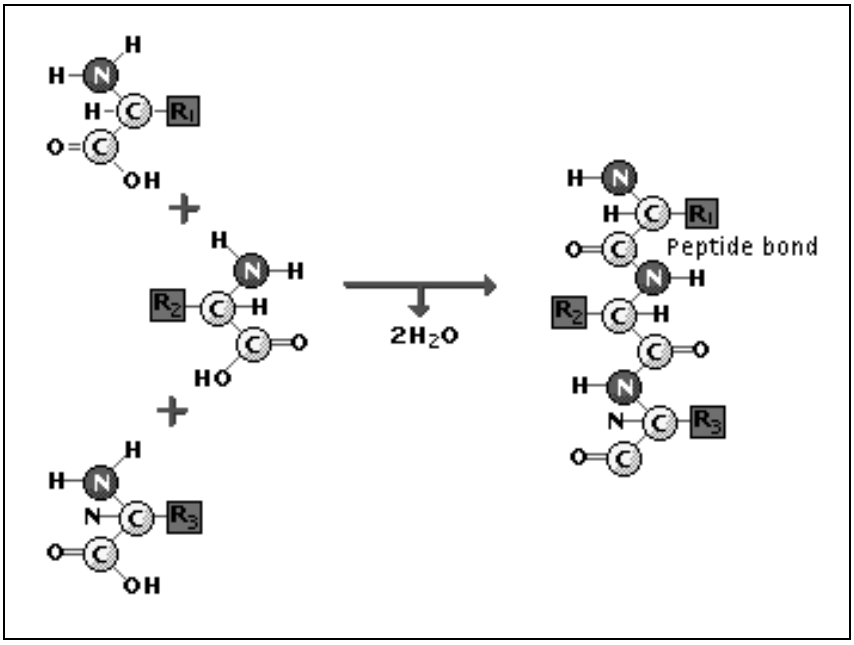


Figure 4.1 Primary structure of proteins [Britannica Encyclopedia On-line]

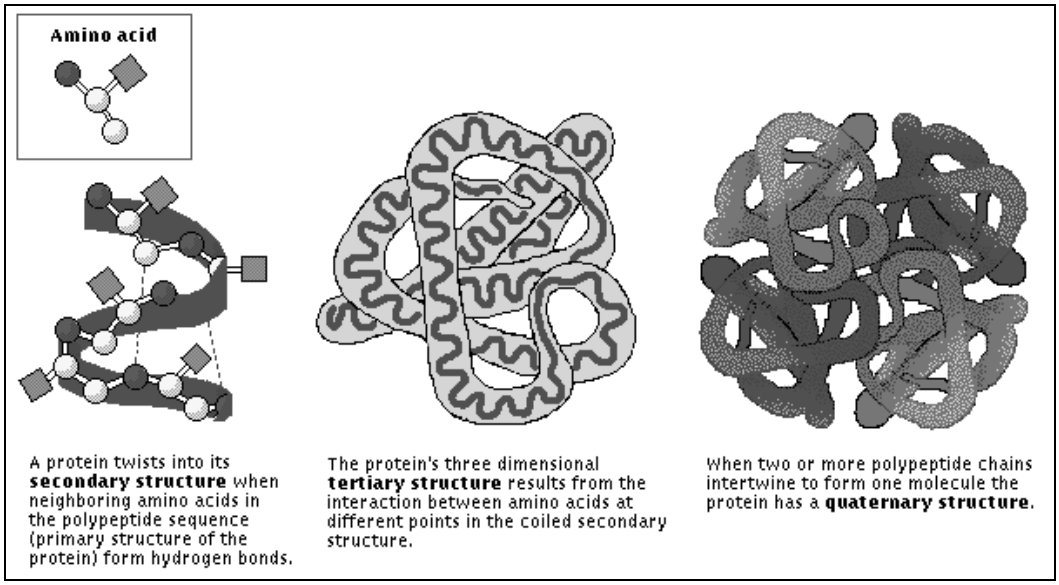


Figure 4.2 Secondary, tertiary and quaternary structure of proteins [Britannica Encyclopedia On-line]

In this Chapter, the double emulsion process was utilized to encapsulate a model protein HSA, which is fairly cheap, stable, and easy for analysis, into polymeric microspheres. Considering the fact that in the last Chapter varying the polymer hydrophobicity has shown interesting results for the controlled release of a water-insoluble drug, here we used some materials of different hydrophobicity and their effects on HSA-loaded microspheres were investigated: (1) PLGA and PLA-PEG-PLA as the polymers. PLGA is routinely used and hydrophobic while PLA-PEG-PLA is newer and has a hydrophilic PEG segment between the hydrophobic PLA segments. Only the PLA-PEG-PLA with Mw 80000 was used here because the one with Mw 50000 exhibited serious aggregation in the resulting microspheres as shown in the last Chapter. From the point of view of its molecular structure, PLA-PEG-PLA is believed to have many advantages over PLGA such as friendliness to protein activity, longer blood circulation half-life, better biocompatibility, higher capability for non-invasive drug delivery, reduced amount of emulsifier to be used, and less drug readsorption to the microsphere surface [Deng et al., 1990, Deng et al., 1999, Tobio et al., 1998]. (2) DCM, ethyl acetate and acetone as the solvents. Their water solubility increases in the order of DCM < ethyl acetate << acetone [Smallwood, 1996.]. Currently DCM is routinely used but it suffers from the drawback of high toxicity, as chlorinated solvents in general are hazardous [Sah et al., 1996]. In contrast, ethyl acetate and acetone have much lower toxicity [Smallwood, 1996] and could thus be good alternatives. (3) PEG and Vitamin E TPGS as the additives. Hydrophilic PEG is a conventional additive to adjust the morphology of the microspheres. Vitamin E TPGS is essentially a conjugate between a hydrophilic segment PEG and a hydrophobic segment Vitamin E [Yu et al., 1999]. Considering the fact that Vitamin E TPGS has a number of clinical applications such as enhancing absorption and increasing the cytotoxicity of

anticancer drugs, recently our group has been pursuing to apply it in the preparation of controlled drug delivery systems. We previously reported that as an emulsifier Vitamin E TPGS could significantly improve the encapsulation efficiency of paclitaxel within polymeric nanospheres [Mu and Feng, 2002]. Here, it was mixed with the polymer and we expected that it would improve the encapsulation efficiency of proteins and affect the other properties of the microspheres.

4.2 Results and discussions

4.2.1 Preparation of microspheres

The double emulsion process is more difficult than the single emulsion process to handle, because the w/o/w emulsion is a rather delicate structure. Inappropriate operations could lead to broken emulsion, non-spherical spheres, etc. In practice, we used optical microscope to monitor the double emulsion process, to ensure that the operations were performed appropriately. Typical images captured during the process are shown in Fig. 4.3.

4.2.2 Particle size

The strength of mechanical forces used to obtain the w/o/w emulsion could be applied to control the particle size of the polymeric microspheres. As shown in Fig. 4.4, it is obvious that stronger emulsification strength resulted in smaller particle sizes.

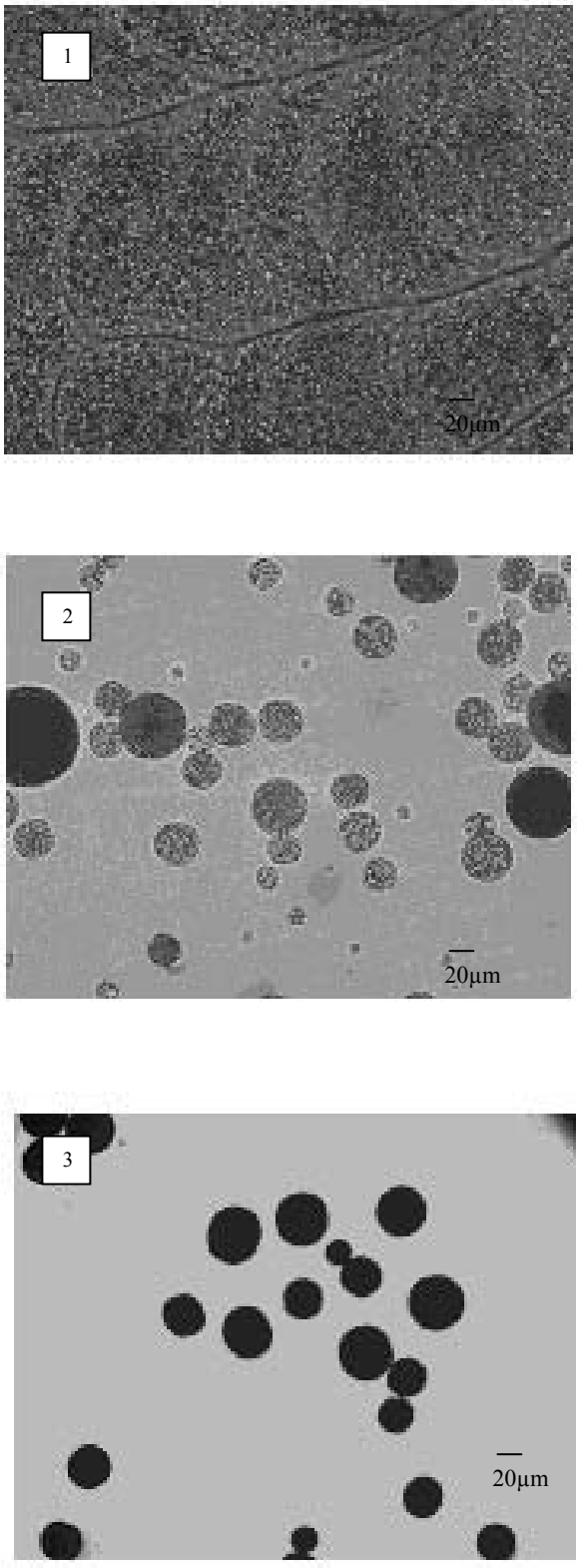


Figure 4.3 Optical microscopy images of well formed (1) w/o emulsion, (2) w/o/w emulsion, (3) solid microspheres. PLGA and DCM were used as the polymer and organic solvent respectively. No additive was used and the w/o/w emulsion was obtained by mechanical stirring at 800 rpm.

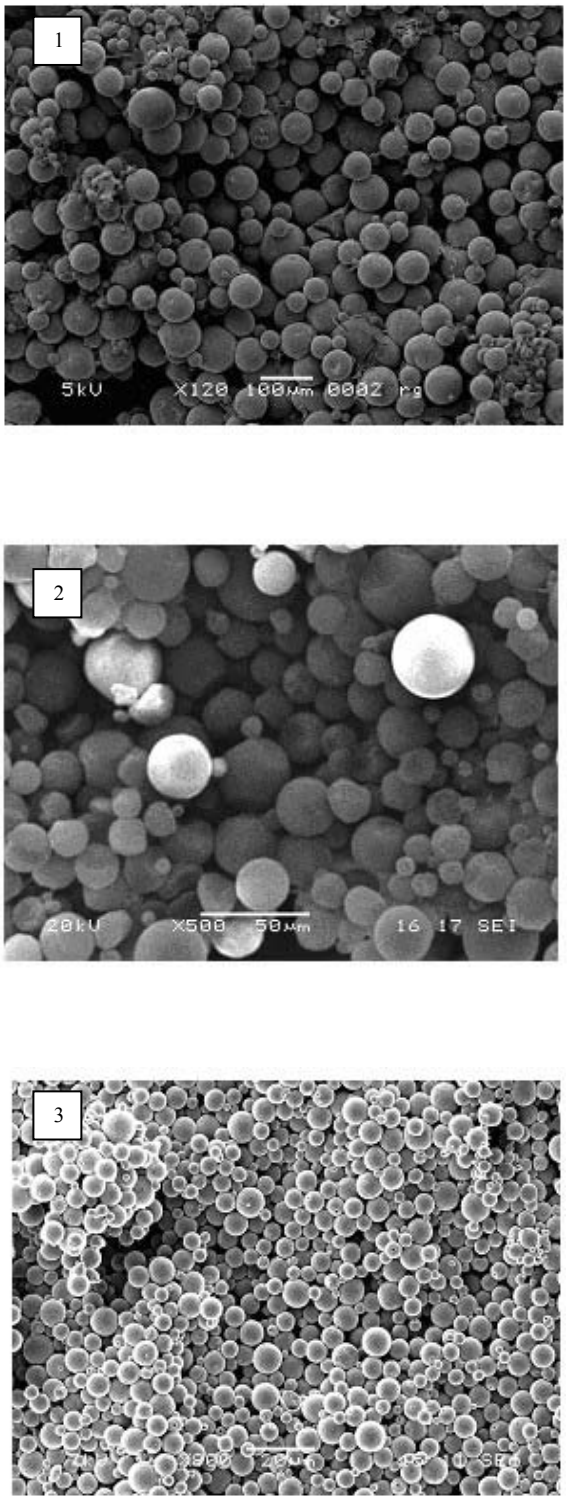


Figure 4.4 SEM images of HSA-loaded PLGA microspheres with the w/o/w emulsion obtained at different emulsification strengths. DCM was used as the solvent and no additive was used. (1) microspheres of diameter (55.3±11.3) µm, formed by mechanical stirring at 800 rpm; (2) microspheres of diameter (24.8±6.8) µm, formed by mechanical stirring at 1200 rpm; (3) microspheres of diameter (6.7±1.4) µm, formed by homogenizing at 20000 rpm. Note the difference in bar length.

The particle size of the polymeric microspheres could also be affected by the materials used. As shown in Fig. 4.5, at similar emulsification strengths, the particle size of PLA-PEG-PLA microspheres was slightly smaller than that of PLGA microspheres. This was likely to be due to the fact that liquid droplets containing PLA-PEG-PLA, which is more hydrophilic than PLGA, were easier to break in the emulsification process within the aqueous environment.

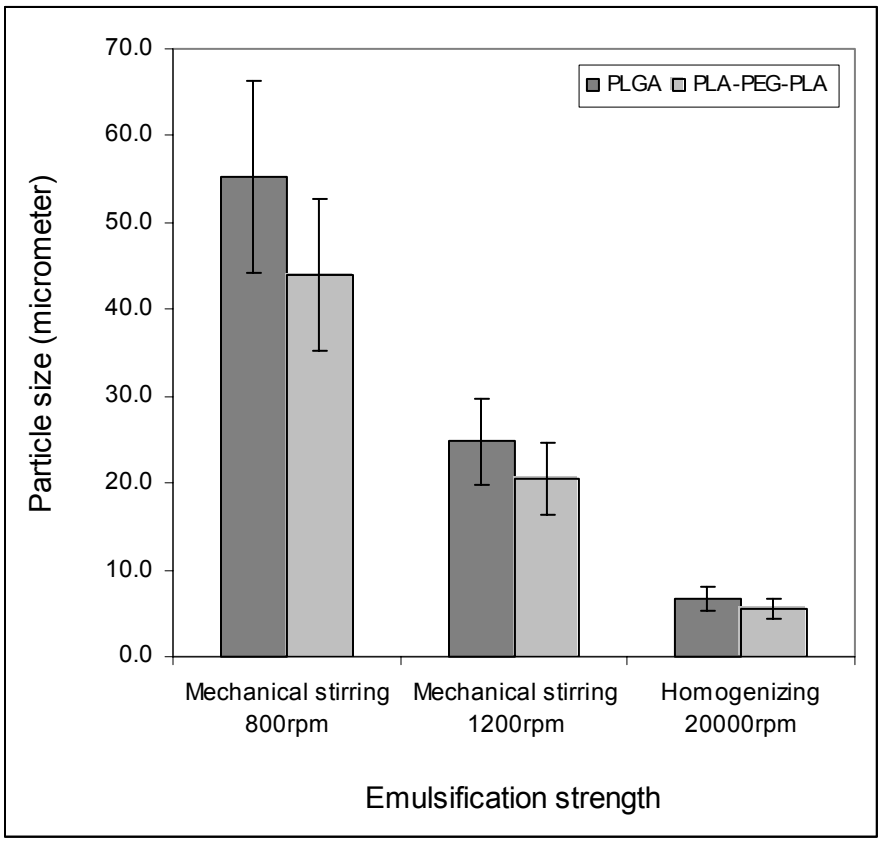


Figure 4.5 Effect of polymers on particle size of HSA-loaded microspheres. DCM was used as the solvent and no additive was used.

In contrast to polymers, the properties of solvents affect the microsphere properties mainly by influencing the removal rate of the solvents. Solid microspheres are formed essentially in two steps, i.e. solvent extraction followed by solvent evaporation, in both processes the

solvent properties play key roles. The solubility of the solvent in water determines the extraction rate, while the evaporation rate depends on its boiling point. Generally, faster extraction rate, indicated by higher solubility in water, and faster evaporation rate, indicated by lower boiling point, leads to faster solvent removal and faster microsphere formation. As a result, it will be more difficult to break the polymer droplets, and larger particle size is thus obtained. According to the water solubility and boiling point of the solvents, the extraction rate should decrease in the order of acetone >> ethyl acetate > DCM, while the evaporation rate of DCM should be the highest. In our experiments, either ethyl acetate or acetone was mixed with the conventional solvent DCM at different ratios. Fig. 4.6 showed that higher percentage of ethyl acetate or acetone in the solvent mixture generally resulted in larger particle sizes, suggesting that solvent extraction rate was directly related to the particle size of the microspheres formed. This implies that, under our experimental conditions, solvent extraction was predominant in the solvent removal process compared with solvent evaporation.

It has been found that the effect of additives (PEG and Vitamin E TPGS) on the particle size of microspheres was not significant. Apparently, 2% of the additives resulted in slightly larger particle size compared with microspheres formed in the absence of any additives, while increasing the additive concentration to 10% led to a small decrease in the particle size. It should be pointed out that all these differences in particle size caused by additives were less than 7 μm, which was smaller than the standard deviation of the measurement (10 μm).

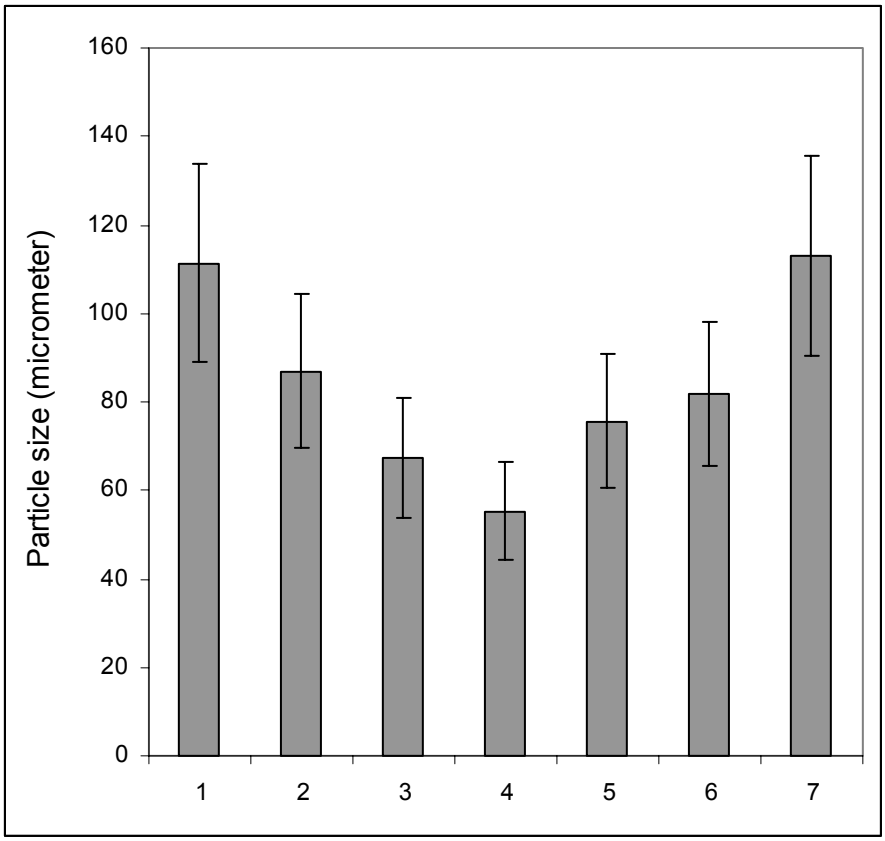


Figure 4.6 Effect of organic solvents on particle size of HSA-loaded PLGA microspheres. No additive was used and the w/o/w emulsion was obtained by mechanical stirring at 800 rpm. (1) 70% acetone+30% DCM; (2) 30% acetone+70% DCM; (3) 10% acetone+90% DCM; (4) 100% DCM; (5) 50% ethyl acetate+50% DCM; (6) 80% ethyl acetate+20% DCM; (7) 100% ethyl acetate.

4.2.3 Surface and internal morphology

PLA-PEG-PLA led to coarser surface, compared with PLGA, and the microspheres tended to stick together (Fig. 4.7). However, PLA-PEG-PLA (Mw 80000) microspheres were well-dispersed after treatment by bath sonication for a few minutes. In addition, more porous internal morphology was observed in PLA-PEG-PLA microspheres, and the pores were more homogeneous than those of PLGA microspheres (Fig. 4.8). These results were similar to those of the single emulsion process (Sec. 3.2.4), except that HSA-loaded PLGA microspheres had porous internal structure given by the w/o emulsion while paclitaxel-

loaded PLGA microspheres didn't. The different morphology of the PLA-PEG-PLA microspheres, compared with that of PLGA ones, can be explained by the presence of PEG segment in the copolymer. This hydrophilic segment may cause heterogeneous microsphere surface in terms of hydrophobicity, leading to the coarse surface morphology. Furthermore, it could help water to enter the interior of the microspheres and thus lead to more porous internal structure. The more homogeneous distribution of the pores could be due to the more stable w/o emulsion given by PLA-PEG-PLA.

Opposite effects on particle size of HSA-loaded microspheres can be caused by different rate of solvent removal. On one hand, fast removal can result in rapid polymer precipitation and thus smoother surface and denser inner structure of the microspheres, as seen in the results of paclitaxel-loaded microspheres (Sec. 3.2.4). On the other hand, local explosion inside the polymer droplets may occur upon rapid solvent removal and lead to formation of porous structure and coarse surface [Arshady, 1991]. Consequently, the trend of morphology change given by different solvents was rather complicated. Ethyl acetate generally resulted in smoother surface and denser internal morphology at all mixing ratios with DCM. At 100%, however, ethyl acetate led to distorted microsphere shape, presumably due to too rapid solvent extraction. When water-soluble acetone alone was used as the solvent, no microsphere could be formed. At other mixing ratios of acetone to DCM, either smooth or coarse surface, dense or porous interior could occur. For example, 70% and 10% acetone led to smoother surface compared with pure DCM, while 30% acetone led to more porous surface, suggesting that acetone concentration in the middle range might result in porous surface. In terms of internal morphology, DCM mixed with 10% and 30% acetone resulted in denser interior compared with pure DCM, while

increasing the acetone ratio to 70% resulted in more porous inner structure (SEM images not shown).

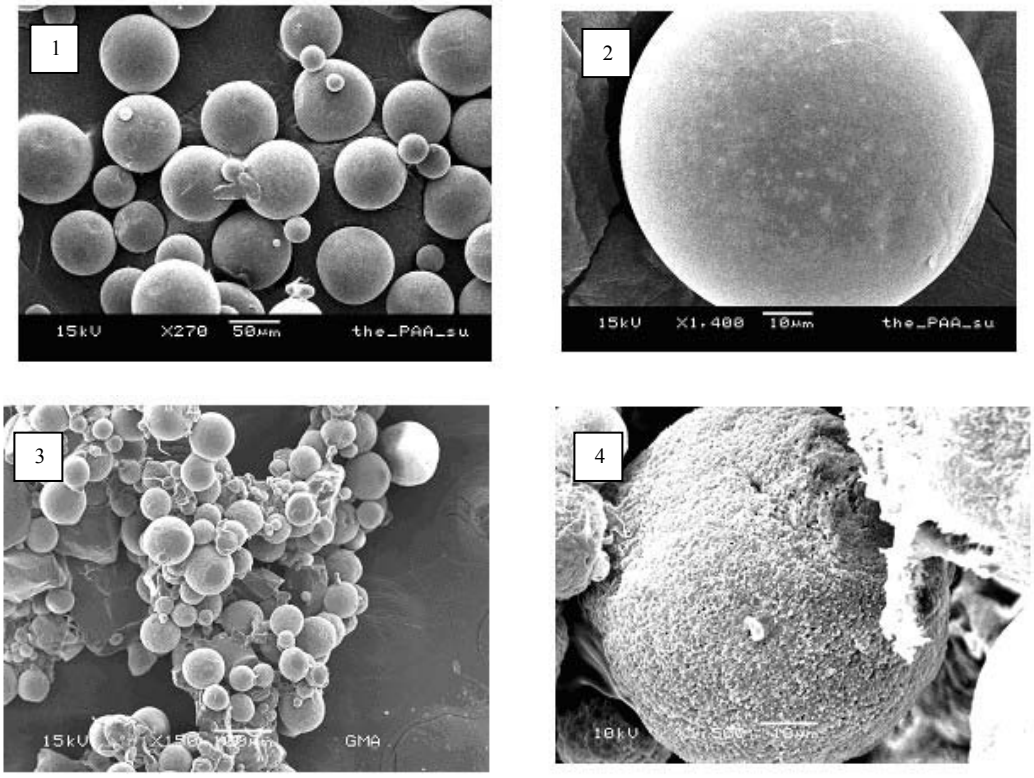


Figure 4.7 Surface morphology of HSA-loaded PLGA and PLA-PEG-PLA microspheres. DCM was used as the solvent. No additive was used. The w/o/w emulsion was obtained by mechanical stirring at 800 rpm. (1, 2) PLGA; (3, 4) PLA-PEG-PLA.

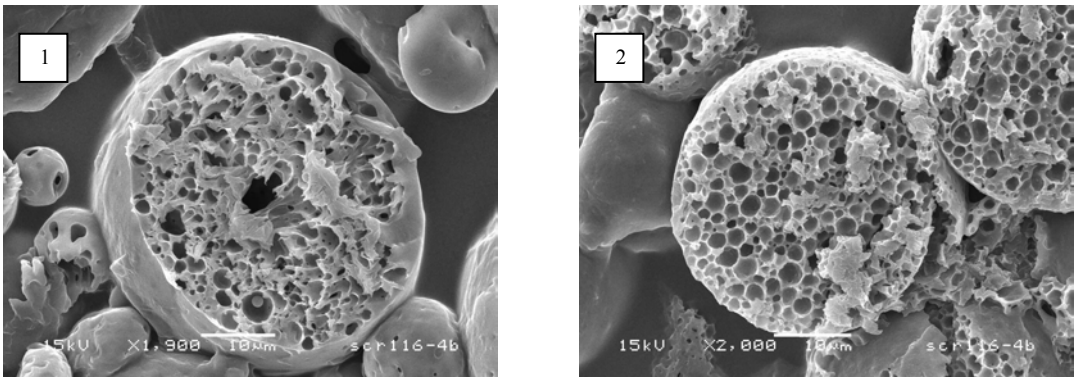


Figure 4.8 Internal morphology of HSA-loaded (1) PLGA and (2) PLA-PEG-PLA microspheres. DCM was used as the solvent. No additive was used. The w/o/w emulsion was obtained by mechanical stirring at 800 rpm.

PEG has been reported to be a pore-forming agent [Sah et al., 1994]. Our results showed that 2% PEG was not enough to have this function. In fact, the surface and interior of the microspheres with the use of 2% PEG appeared to be even slightly less porous than those without the use of additives. Increasing the PEG content to 10% led to remarkable increase in pores both on the surface and in the interior of the microspheres (SEM images not shown). In contrast, Vitamin E TPGS had different effect on the microsphere morphology. A low concentration of 2% led to porous structure, presumably because this amphiphilic additive could enhance the interaction of the oil and water phases so that the water could enter the microspheres more easily, forming pores inside the microspheres. However, 10% Vitamin E TPGS resulted in decreased porosity. This might be because high concentration of this additive increased the viscosity of the microsphere interior so remarkably that the influx of water phase was retarded (Fig. 4.9).

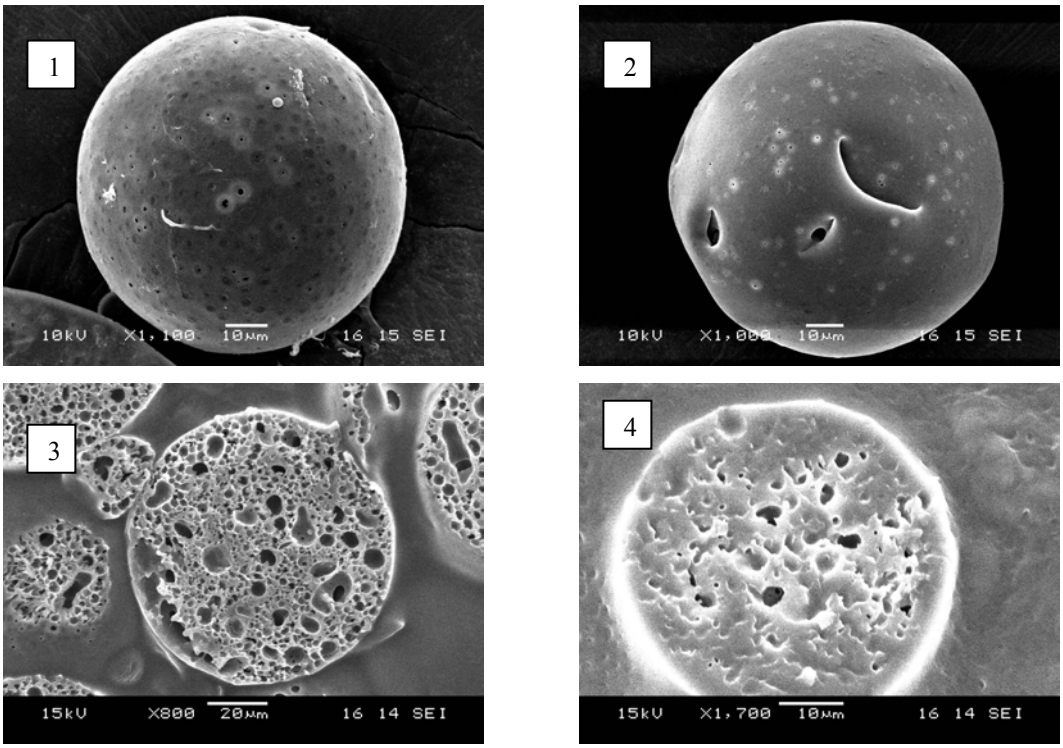


Figure 4.9 Surface and internal morphology of HSA-loaded PLGA microspheres. DCM was used as the solvent. 2% (1, 3) or 10% (2, 4) of Vitamin E TPGS was used as the additive. The w/o/w emulsion was obtained by mechanical stirring at 800 rpm.

4.2.4 Encapsulation efficiency

Shown in Figure 4.10 is the calibration curve of protein concentration vs. absorption (ABS) in UV-Vis spectrophotometer analysis, which was used in the measurement of encapsulation efficiency and in vitro release of the microspheres. The linear range is from 1.2 μg/ml to 10 μg/ml. Within this range 7 data points were recorded. The R^2 value was 0.9994.

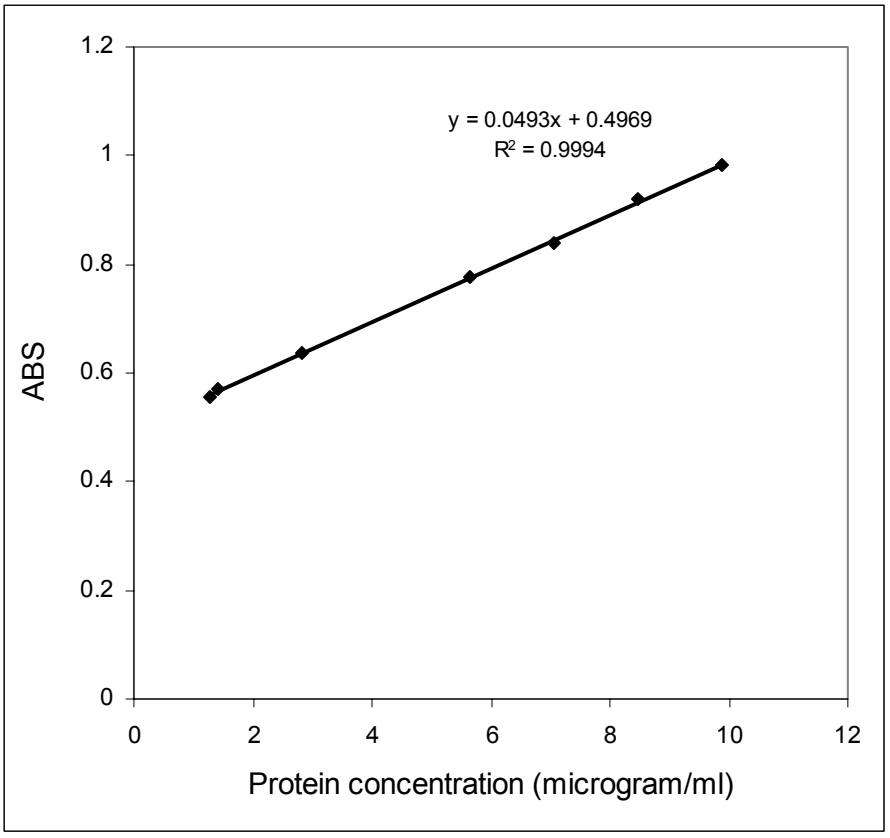


Figure 4.10 Calibration curve of UV-Vis spectrophotometer analysis of HSA

Different from the case of the single emulsion process, loss of the encapsulated protein during the double emulsion process is practically inevitable because the protein in the inner water phase tends to merge with the outer water phase. Thus, the encapsulation efficiency of water-soluble agents such as HSA is normally lower than that of water-insoluble agents such as paclitaxel. The encapsulation efficiency value for the double emulsion process can be affected by the following factors: (1) stability of the w/o and w/o/w emulsions, (2) the solvent removal rate, (3) the interactions among polymer, drug, solvent and additive, and (4) particle size [Freytag et al., 2000, Nihant et al., 1994, Rosa et al., 2000], all influenced by the hydrophobicity of the materials used for microsphere preparation. Therefore, choice of polymer, solvent and additive with suitable hydrophobicity can help to improve the encapsulation of proteins in the microspheres. Our results, with HSA as the encapsulated agent, showed that PLA-PEG-PLA microspheres possessed higher encapsulation efficiency as compared to their PLGA counterparts (Fig. 4.11). At similar particle size, the encapsulation efficiency value of HSA in PLA-PEG-PLA microspheres was about 10% higher than that in PLGA microspheres. This can be explained by the fact that PLA-PEG-PLA can improve the w/o emulsion stability.

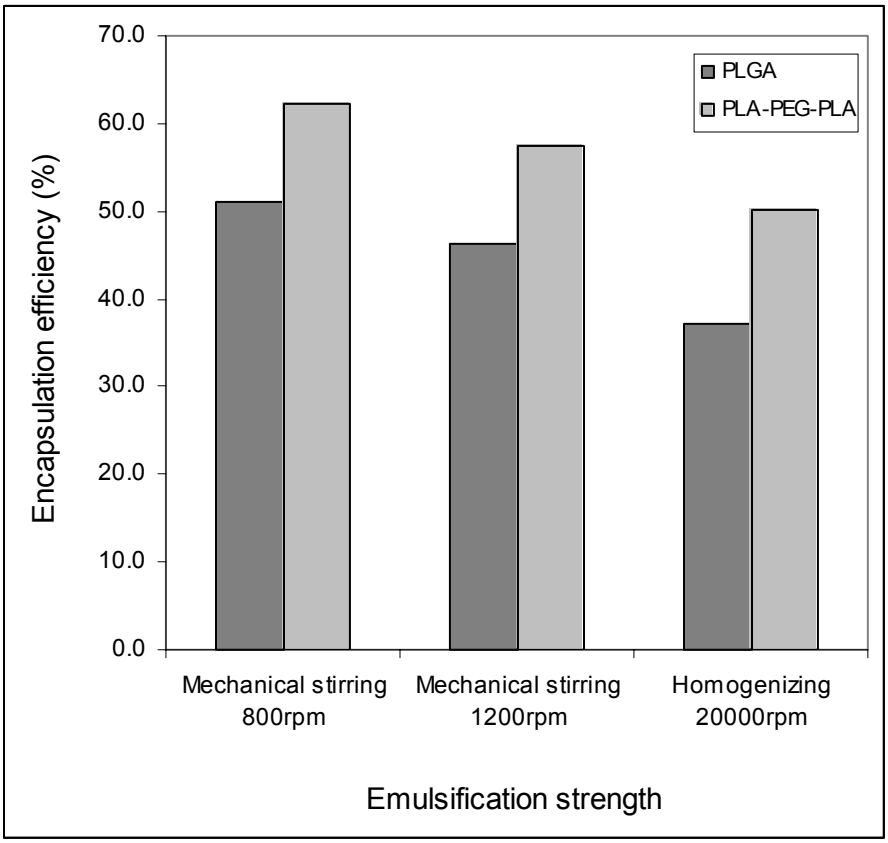


Figure 4.11 Effect of polymers on encapsulation efficiency of HSA in microspheres. DCM was used as the solvent and no additive was present.

Similar to the case of particle size and morphology, the effect of organic solvents on encapsulation efficiency appeared to be complex. Except certain ratios of acetone or ethyl acetate to DCM (70% acetone or 100% ethyl acetate), all the other mixing ratios resulted in lower encapsulation efficiency compared with DCM alone. DCM mixed with 70% acetone or 100% ethyl acetate resulted in 69.09% and 65.33% of encapsulation efficiency value respectively. Both were significantly higher than that achieved by using DCM alone (51.1%). Pean et al. also reported the observation that there was an optimized mixing ratio of DCM and acetone to yield high encapsulation efficiency [Pean et al., 1998].

We expected a distinctly different effect of PEG and Vitamin E TPGS on encapsulation efficiency, i.e. Vitamin E TPGS would improve encapsulation efficiency, based on the hypothesis that, as a surfactant, Vitamin E TPGS could improve the w/o emulsion stability while PEG could not. In addition, our group previously reported that Vitamin E TPGS helped to achieve 100% encapsulation efficiency of paclitaxel in the single emulsion process [Mu and Feng, 2002]. However, our experimental data showed that both PEG and Vitamin E TPGS had negative effect on encapsulation efficiency when w/o/w emulsion solvent extraction/evaporation process was used to microencapsulate the hydrophilic compound HSA. The encapsulation efficiency values of using either low (2%) or high concentrations (10%) of these two additives were around 25% lower than that obtained without the use of additives. Rosa et al. also observed that coencapsulation of surfactants reduced encapsulation efficiency of insulin in PLGA microspheres. They attributed the result to displacement of the drug molecules from w/o emulsion interface by the surfactants, eventually decreasing the amount of drug located within the w/o emulsion [Rosa et al., 2000].

4.2.5 In vitro release

As expected, the release rate of water-soluble HSA (Fig. 4.12 and Fig. 4.13) was remarkably faster than that of water-insoluble paclitaxel (Fig. 3.7) from polymeric microspheres. Similar to the case of the single emulsion process, compared with PLGA, the polymer PLA-PEG-PLA led to faster HSA release at the two particle sizes tested (Fig.

4.12), probably due to its more porous matrix, faster drug diffusion and polymer degradation caused by higher hydrophilicity. More than 90% of the encapsulated HSA was released from PLA-PEG-PLA microspheres within 13 days. This is an interesting phenomenon in the sense that this polymer, as compared with PLGA, exhibited ability to increase both the encapsulation efficiency value and the release rate (Fig. 4.11 and 4.12, respectively). In general, it is well-known that higher release rate is related to lower encapsulation efficiency value.

Since the effects of organic solvents on many microsphere properties such as particle size, morphology and encapsulation efficiency were complex and the in vitro HSA release was actually affected by these properties, the effect of solvents on the in vitro HSA release was found to be even more complicated. No clear-cut trend of release rate could be observed as the co-solvent ratio was altered (data not shown). We thus suggested that it might not be wise to control the HSA release rate by varying the solvent mixing ratio, although some other microsphere properties, such as biocompatibility, particle size, morphology and encapsulation efficiency, could be improved or adjusted by appropriately selecting the mixing ratio of the solvents, as discussed earlier.

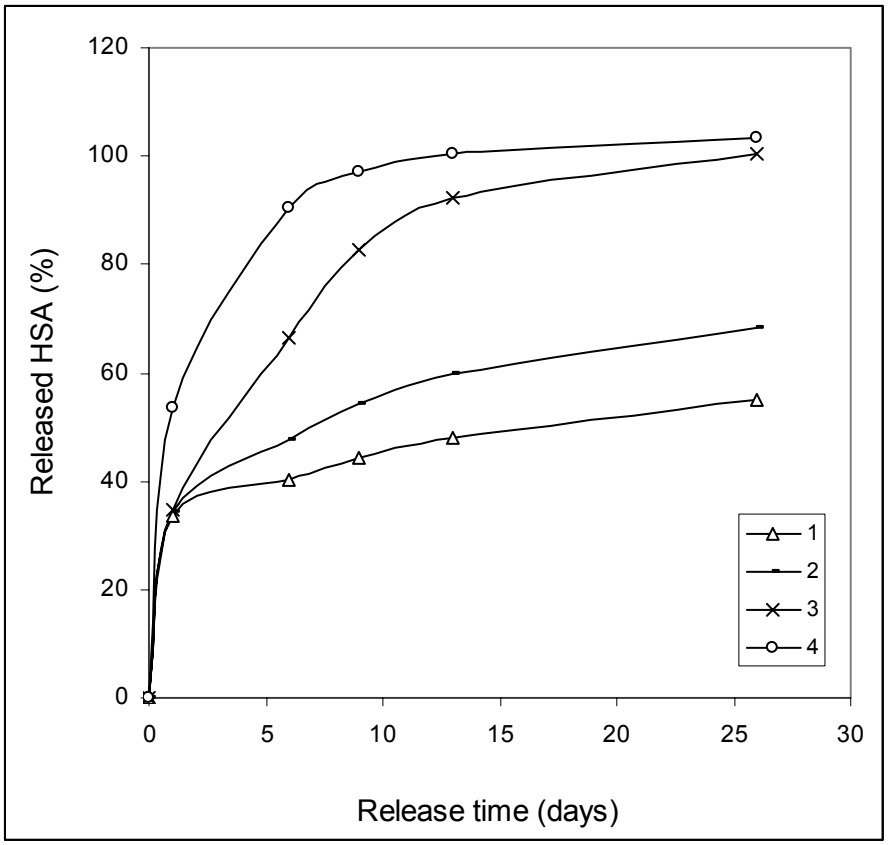


Figure 4.12 Effect of polymers on in vitro release of HSA from microspheres. DCM was used as the solvent and no additive was used. (1) PLGA mechanical stirring 1200 rpm, particle size 24.8 μm; (2) PLGA homogenizing 20000 rpm, particle size 6.7 μm; (3) PLA-PEG-PLA mechanical stirring 1200 rpm, particle size 20.5 μm; (4) PLA-PEG-PLA homogenizing 20000 rpm, particle size 5.5 μm.

The effect of additives on HSA release corresponded well to the morphology of polymeric microspheres (Fig. 4.12). A low concentration of 2% PEG resulted in denser morphology (both surface and internal) and slightly slower release rate, probably because of the slower drug diffusion rate. Higher PEG concentration (10%) resulted in more porous structure and slightly faster HSA release. Compared with PEG, the effect of Vitamin E TPGS was more significant. Remarkably faster HSA release (and also more porous morphology) was caused by low Vitamin E TPGS concentration at 2%, while 10% of Vitamin E TPGS significantly reduced the release of HSA and also resulted in denser morphology of the microspheres.

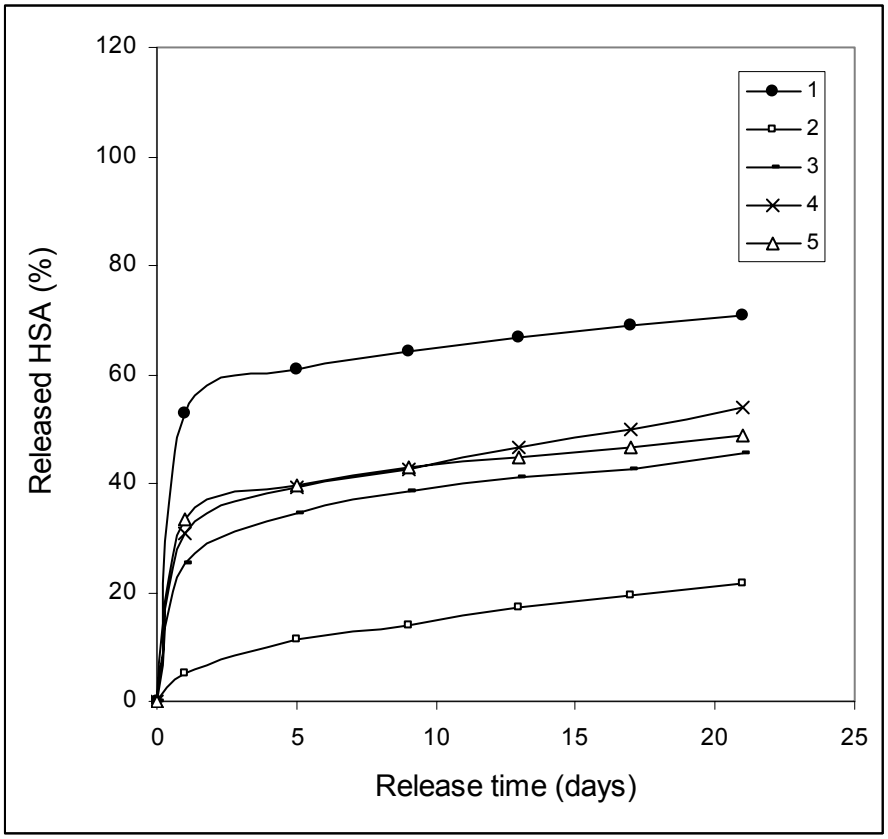


Figure 4.13 Effect of additives on in vitro release of HSA from PLGA microspheres. DCM was used as the solvent and the w/o/w emulsion was obtained by mechanical stirring at 800 rpm. (1) 2% Vitamin E TPGS, (2) 10% Vitamin E TPGS, (3) 2% PEG, (4) 10% PEG, (5) no additive.

4.3 Summary

In this Chapter, HSA-loaded polymeric microspheres were prepared by the double emulsion process. The effects of materials with different hydrophobicity from their conventional counterparts, such as PLA-PEG-PLA as the polymer, ethyl acetate/acetone as the (co)solvent and Vitamin E TPGS as the additive, on the physical properties of HSA-loaded microspheres were investigated. It has been found that these materials resulted in significantly distinct characteristics of the microspheres. PLA-PEG-PLA and lower

percentage of ethyl acetate/acetone led to smaller particle size. PLA-PEG-PLA, high concentration of PEG and low concentration of Vitamin E TPGS led to more porous morphology while ethyl acetate and high concentration of Vitamin E TPGS led to denser microspheres. PLA-PEG-PLA and certain percentage of ethyl acetate/acetone led to higher encapsulation efficiency. PLA-PEG-PLA, high concentration of PEG and low concentration of Vitamin E TPGS led to faster in vitro HSA release. The materials that we applied could render the protein-loaded polymeric microspheres with interesting applications. Furthermore, the information obtained in the present and the previous Chapters about the solvent extraction/evaporation technique would be useful for encapsulating other drug delivery devices, such as liposomes, into polymeric microspheres, which is the topic of the next Chapter.

CHAPTER 5 LIPOSOMES-IN-MICROSPHERE

5.1 Introduction

As mentioned earlier, in most, if not all, of the controlled drug delivery systems, the drug is incorporated in or attached to either polymers or lipids. Thus, these systems can be categorized into two groups, namely the polymer-based and the lipid-based systems [Langer, 1998]. Probably because polymer molecules are normally much larger than those of lipids, the former usually form solid phase, such as polymeric microspheres or nanoparticles, films, pellets, etc., while the latter form liquid or liquid crystalline phase, such as liposomes [Harrington, 2001], micelles [Mahato et al., 1997], emulsions [Benita and Levy, 1993] and microemulsions [Bagwe et al., 2001], etc., for controlled drug delivery. One can find many common points between the polymer-based and lipid-based systems with regard to preparations, characterizations and applications. On the other hand, they have many differences. For example, the lipid-based systems often suffer from the problem of instability while the polymer-based systems are normally rather stable; the lipid-based systems are normally more biocompatible than the polymer-based ones as lipids are part of the cell membranes. We propose that an appropriate combination of the polymer-based and the lipid-based systems could integrate their advantages, avoid their disadvantages, and lead to new applications. Some systems in the literature may be considered as the consequence of this concept. For example, in stealth liposomes, which have been most successful for delivery of an anticancer agent doxorubicin, a polymer is coated on the surface of liposomes to improve their stability [Lasic and Martin, 1995].

Also, Feng and Hung utilized phospholipids, instead of the conventional PVA, as the emulsifier in the preparation of polymeric nanoparticles for clinical administration of anticancer drugs. The lipids were found to be coated on the nanoparticles to improve the biocompatibility [Feng and Hung, 2001]. In this Chapter, we fabricated and characterized a novel drug delivery device, i.e. liposomes-in-microsphere (LIM) of biodegradable polymers, based on the concept of combining the polymer-based and lipid-based systems. In this novel system, drugs are firstly loaded into liposomes, which are then encapsulated into polymeric microspheres. Currently polymeric microspheres and liposomes are probably the most successful polymer-based and lipid-based systems respectively [Langer, 1998]. Polymeric microspheres have been reviewed in Sec. 1.2 and the following is a brief introduction to liposomes.

Each phospholipid molecule contains one hydrophilic headgroup and two hydrophobic chains. After dispersed in the aqueous environment, phospholipids tend to adopt the structure of liposomes, or phospholipid vesicles, in which the hydrophilic headgroups are positioned outward to the water phase and the hydrophobic chains inward tail to tail, in order to minimize the contact of the hydrophobic parts of the phospholipids with water. The vesicles may consist of one or several concentric membranes with their size ranging from 20 nm to several dozen μm. The thickness of each membrane bilayer is approximately 4 nm. Liposomes exist in a unique physical state, i.e. liquid crystalline state, in which the matter exhibits simultaneously some properties of liquid state, such as low viscosity, and some properties of crystalline state, such as anisotropy of mechanical, optical, electrical and magnetic properties. Both hydrophilic and hydrophobic drugs can be

loaded to the liposomes: the former is entrapped in the aqueous core while the latter is dissolved in the lipid bilayers (Fig. 5.1) [Lasic, 1993].

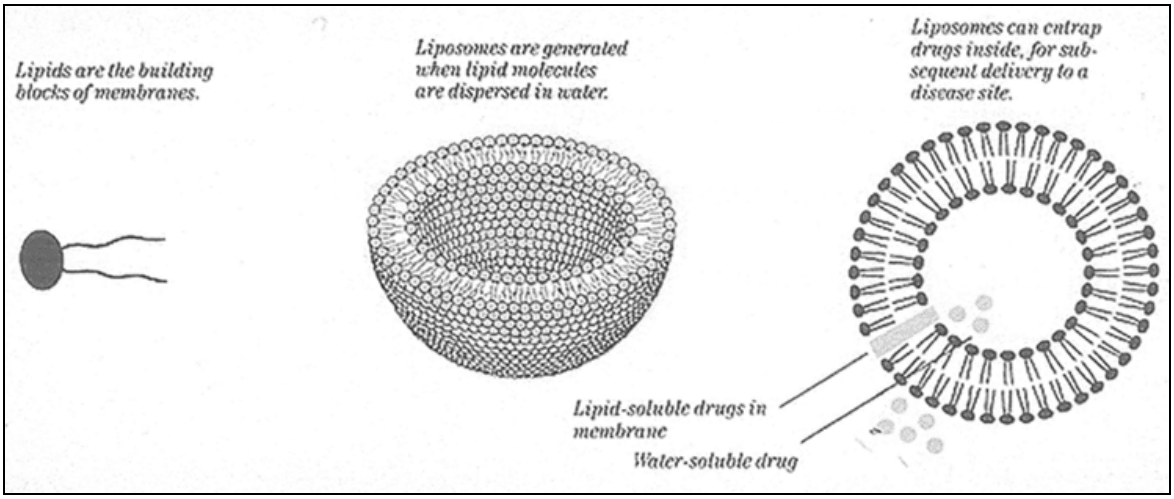


Figure 5.1 Structure of phospholipids and liposomes [Britannica Encyclopedia On-line]

Film hydration followed by extrusion/sonication is often used to prepare liposomes. At first, a thin lipid film is formed by drying a lipid solution, which may contain hydrophobic drugs, on a glass surface. The film is then hydrated with an aqueous solution, which may contain hydrophilic drugs. A gentle mechanical treatment (e.g. vortex mixing) is then conducted to form multilamellar vesicles (MLVs). To reduce the particle size and lamellar number of the liposomes, membrane extrusion or sonication can follow to obtain large unilamellar vesicles (LUVs) or small unilamellar vesicles (SUVs), respectively [New, 1990]. The preparation of liposomes represents a different strategy to produce micro/nano-size devices from the solvent extraction/evaporation technique for polymer systems. The former may be called the ‘down-to-up’ strategy in that the micro/nano-size is formed by self-assembling individual molecules, while the latter may be called the ‘up-to-down’

strategy in that the micro/nano-size is obtained by breaking macro-systems. In general, the 'down-to-up' strategy results in smaller size of the devices formed.

Drugs are released from the liposomes mainly by diffusion. Diffusivity is different for different liposome contents. Some general rules are (1) The diffusivity of macromolecules is smaller than that of small molecules; (2) the diffusivity of ions is larger than that of neutral molecules; (3) the diffusivity of anions is larger than that of cations. The behavior of the lipid bilayers also significantly affects the drug release kinetics. For example, Long-chain and unsaturated phospholipids and high cholesterol content result in rigid and tightly packed membrane, and thus low drug release rate [Packer and Fleischer, 1997]. Drug targeting can be achieved by liposomes via similar mechanisms as those for polymeric microspheres. One exception is chemoembolization, which cannot be done by liposomes due to their limit in particle size. In addition, some special external parameters, such as pH and temperature, have been used to realize liposome-mediated drug targeting. For example, in temperature-sensitive liposomes, the phase transition temperature of the lipid bilayers (Tc) is adjusted to that of a certain site in human body. As such, the drug will be mainly released at the desired site because dramatically more defects form in the lipid membrane during the phase transition [Barenholz, 2001, Crommelin et al., 1997].

One can think of many potential advantages/applications for the novel drug delivery system LIMs, which is formed by encapsulating liposomes into polymeric microspheres. Some of them are outlined below.

(1) In LIMs, the microspheres could form a matrix to protect the liposomes while the biocompatibility of the polymer matrix could be improved by the existence of part of the lipids on the surface.

(2) LIMs could improve the drug loading and encapsulation efficiency, compared with liposomes or microspheres alone, due to the fact that both the lipid layers and the polymer matrix would block the drug leakage.

(3) More flexible drug release kinetics could be achieved by LIMs compared with that by microspheres or liposomes alone, meaning that LIMs could be made to meet various requirements for the release profile. For instance, the release of extremely water-soluble drugs such as etanidazole from microspheres is too fast for clinical applications [Wang and Wang, 2002]. By encapsulating these drugs into the LIM system, both the lipid membranes and the polymer matrix would retard the drug release, thereby making sustained release possible.

(4) LIMs could be applied for tumor-targeted gene delivery, if gene-loaded liposomes are encapsulated inside the microspheres of 20~50μm in size. This application combines the tumor-targeted capability of microspheres and the capability of liposomes to transfer genes into cells. After LIMs are delivered intra-arterially to the upstream of a tumor, they would be entrapped in the tumor vasculature due to the chemoembolization effect, since 20~50 μm is a suitable size for this effect to occur, as discussed earlier. The liposomes carrying therapeutic genes would then be released from the polymer matrix and enter the tumor cells.

(5) One of the biggest problems in controlled delivery of therapeutic proteins/peptides using polymeric microspheres is denaturation of the drugs during the fabrication and storage processes of the delivery system, and after they are delivered into

human body [Fu et al., 2000]. In LIM system, the lipid bilayers of the liposomes could protect the loaded drug from the harsh conditions involved in the subsequent microsphere fabrication process. Furthermore, the acidic microenvironment frequently existing inside the microspheres is believed to be harmful to the stability of many proteins/peptides. After incorporated into liposomes, however, these drugs could be protected by the lipid bilayers. Compared with the strategy of using certain chemicals co-encapsulated with the proteins/peptides to protect their activity, which are often termed as 'additives' or 'stabilizers' [Cleland and Jones, 1996], our approach could be advantageous because a physical rather than chemical protection would be obtained. Potentially, the physical protection could work for all drugs.

The key issue to fabricate the LIM system is how to encapsulate liposomes into microspheres, since loading drugs, either water-soluble or water-insoluble, to liposomes has become routine operations [New, 1990]. Because liposomes normally exist in a water phase, we intended to make use of the double emulsion process, which is commonly used for microencapsulation of water-soluble agents, to achieve this goal. In order to apply the double emulsion process to fabricate LIMs, we could simply treat the liposomes as the water-soluble agents. However, two problems had to be solved first: the two harsh conditions involved in the double emulsion process, namely (1) sonication for the production of the w/o emulsion and (2) the organic solvents used are harmful for the integrity of liposomes. These two problems were solved in Sec. 5.2 and 5.3 respectively by the following strategies: (1) the conventional double emulsion process was modified to avoid the sonication treatment, and (2) the liposomes were coated with a hydrophilic

polymer to protect them from organic solvents. Subsequently, in Sec. 5.4 the novel device LIMs was successfully fabricated and characterized.

5.2 Modification of double emulsion process

In the first step of the double emulsion process, the w/o emulsion is routinely produced by a highly energetic emulsification method, namely sonication, because forming a stable w/o emulsion is crucial for good encapsulation effects. Unstable w/o emulsion leads to substantial loss of the encapsulated agents during the microencapsulation process, resulting in low encapsulation efficiency. Another consequence of unstable w/o emulsion is the existence of a large portion of the encapsulated agents on or near the microsphere surface, leading to high initial burst in the release kinetics [Igartua et al., 1997, Sah et al., 1995]. Despite being critical to obtain stable w/o emulsion and satisfactory encapsulation effects, the highly energetic emulsification methods such as sonication were identified to be one of the major culprits of destroying the encapsulated agents. Sonication can degrade the encapsulated agents through large pressure and temperature gradients, high shear forces, or by generating free radicals. In addition, sonication exposes the encapsulated agents to the degrading action of organic solvent across a very large interfacial area [Capan et al., 1999, Krishnamurthy et al., 2000, Mclean and Mortimer, 1988, Misik et al., 1995]. Moreover, it was reported that sonication could also result in degradation of the polymer matrix [Reich, 1998]. Some countermeasures that can prevent the degradation of encapsulated agents have been developed, most of which involve using additives (often called ‘stabilizers’) such as dimethyl sulfoxide [Krishnamurthy et al., 2000], sucrose [Sturesson and Carlfors, 2000], and zinc acetate [Lam et al., 2001]. However, an inherent

disadvantage associated with this strategy is that particular stabilizers can work only for particular encapsulated agents. Also, the effectiveness of this strategy for liposomes is questionable, since previously the encapsulated agents have only included individual molecules such as proteins. To solve the problem caused by sonication, we used an alternative approach, i.e. using mild emulsification procedures such as vortex mixing to replace sonication, with the introduction of certain materials that can help to obtain stable w/o emulsion, which is critical for high encapsulation efficiency and low initial burst.

The following materials were applied to improve the w/o emulsion stability: (1) A partially hydrophilic polymer PLA-PEG-PLA, which is more hydrophilic than the conventional polymer PLGA, due to the presence of a hydrophilic PEG segment in its molecular structure. Only the PLA-PEG-PLA with Mw 80000 was used here because the one with Mw 50000 exhibited serious aggregation in the resulted microspheres as discussed in Chapter 3. (2) More hydrophilic organic solvents. Ethyl acetate or acetone was mixed with the conventional solvent DCM. Ethyl acetate and acetone have higher water solubility than DCM, and their density gap with water is smaller. Another advantage of these two alternative solvents is their low toxicity. (3) Emulsifiers in the w/o emulsion. Conventionally the w/o emulsion is formed in the absence of emulsifier, probably because highly energetic mechanical treatments such as sonication are considered to be sufficient to form stable w/o emulsion without using any emulsifier. Two emulsifiers were applied here, i.e. PVA, which is water-soluble, and Vitamin E TPGS, which is both water and oil soluble [Mu et al., 2001]. We were to assess the feasibility of our strategy by investigating the effects of these materials on encapsulation of HSA under mild emulsification conditions. The reason why we did not directly use liposomes as the encapsulated agent is

that at this stage the problem of preventing the liposomes from the damage caused by organic solvents had not been solved yet. In contrast to liposomes, HSA is a rather stable compound. Moreover, the protein assay method that we would use, i.e. the Bradford method, does not distinguish between denatured and intact proteins. Therefore, if we could show that under the mild emulsification treatment the encapsulation effect of HSA into polymeric microspheres was acceptable with the aid of the above mentioned materials, the 'modified double emulsion process' should be considered to be feasible. We would further examine the effect of this process on liposomes in Sec. 5.4, after the problem of organic solvent was solved.

Nine batches of microsphere samples were prepared and compared in terms of internal morphology, encapsulation efficiency, initial burst and other properties. As shown in Table 5.1, SA1 and SA2 were controls prepared by sonication and vortex mixing respectively. In other words, sonication and vortex mixing were used in these two samples respectively to form w/o emulsion, and in both of them all materials used were conventional, i.e. PLGA as the polymer, DCM as the solvent, no emulsifier used in the w/o emulsion formation. In each of the samples SA3 to SA9, vortex mixing was used and one new material was applied to replace the corresponding conventional counterpart: polymer PLA-PEG-PLA was used in SA3 to replace PLGA; Solvent mixtures of DCM and ethyl acetate or acetone having different mixing ratios were used in SA4 to SA7 to replace DCM; Emulsifiers PVA and Vitamin E TPGS were used in w/o emulsion in SA8 and SA9.

Table 5.1 Samples of HSA-loaded microspheres

Sample number	Sample name	Method to form w/o emulsion	Polymer	Solvent	Emulsifier in w/o emulsion
SA1	Sonication control	Sonication	PLGA	DCM	No
SA2	Vortex control	Vortexing	PLGA	DCM	No
SA3	PLA-PEG-PLA	Vortexing	PLA-PEG-PLA	DCM	No
SA4	30% ethyl acetate	Vortexing	PLGA	DCM:ethyl acetate=70:30	No
SA5	70% ethyl acetate	Vortexing	PLGA	DCM:ethyl acetate=30:70	No
SA6	30% acetone	Vortexing	PLGA	DCM:acetone =70:30	No
SA7	70% acetone	Vortexing	PLGA	DCM:acetone =30:70	No
SA8	PVA	Vortexing	PLGA	DCM	PVA
SA9	Vitamin E TPGS	Vortexing	PLGA	DCM	Vitamin E TPGS

Homogeneous internal morphology of microspheres is indicative of stable w/o emulsion in the double emulsion process, because the appearance of the w/o emulsion is shown in the internal morphology of the microspheres after the removal of organic solvents. Fig. 5.2 shows the internal morphology of the microsphere samples. By comparing the images of samples SA1 and SA2 it can be seen that, as expected, vortex mixing in the absence of the newer materials resulted in heterogeneous internal structure, indicating the formation of unstable w/o emulsion (sample SA2). As a result, the encapsulation efficiency of the

vortex control sample (sample SA2) was very low (23.4%, Fig. 5.3) and its initial burst was very high (89.9%, Fig. 5.4).

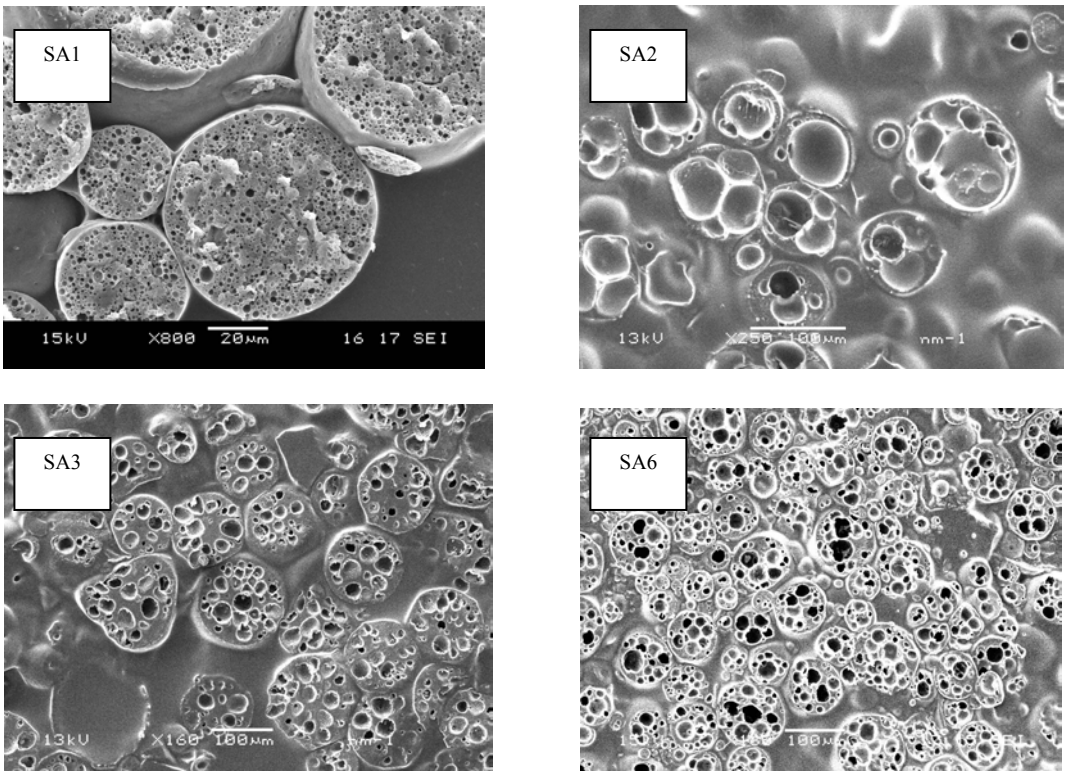


Figure 5.2 Internal morphology of HSA-loaded microspheres. SA1: sonication control; SA2: vortex control; SA3: PLA-PEG-PLA; SA6: 30% acetone.

All the new materials that we tested exhibited positive effects, more or less, on improving w/o emulsion stability, as the internal structure of all samples became significantly more homogeneous than that of the vortex control sample SA2. Shown in Fig. 5.2 are some typical images. Substantial increase of encapsulation efficiency and decrease of initial burst in these samples were observed consequently (Fig. 5.3 and 5.4). In fact, many of them had better or comparable results than the sonication control sample SA1. Thus, we would adopt the modified double emulsion process to encapsulate liposomes into polymeric microspheres, and examine the encapsulation effects for liposomes in Sec. 5.4.

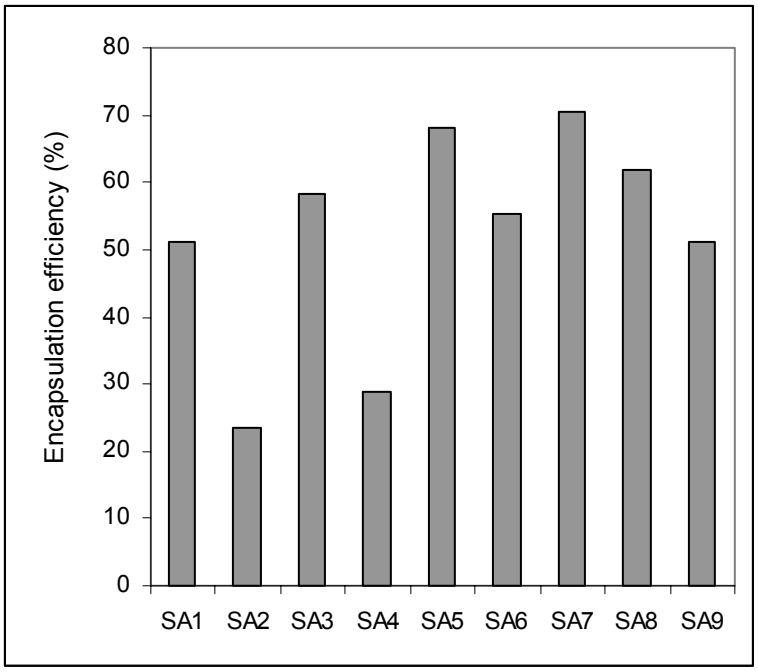


Figure 5.3 Encapsulation efficiency of HSA-loaded microspheres. SA1: sonication control; SA2: vortex control; SA3: PLA-PEG-PLA; SA4: 30% ethyl acetate; SA5: 70% ethyl acetate; SA6: 30% acetone; SA7: 70% acetone; SA8: PVA; SA9: Vitamin E TPGS.

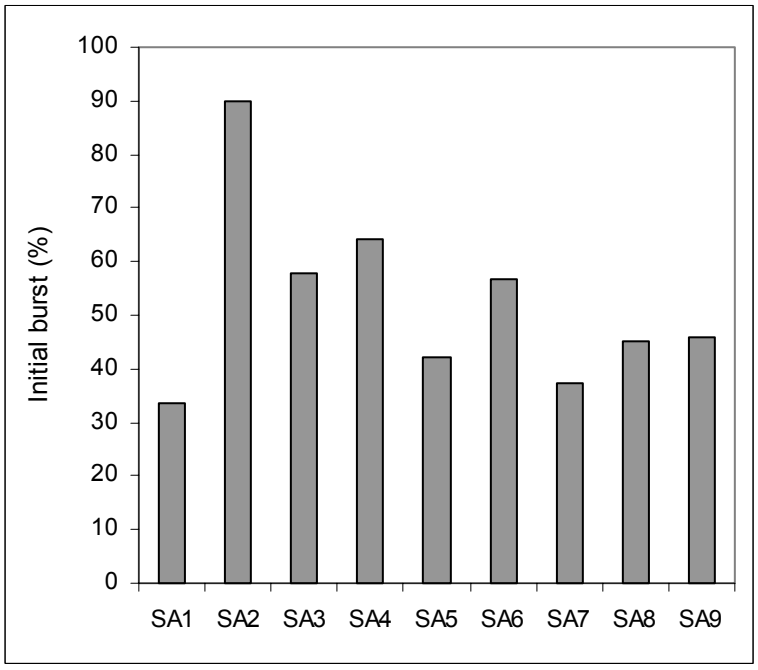


Figure 5.4 Initial burst of HSA-loaded microspheres. SA1: sonication control; SA2: vortex control; SA3: PLA-PEG-PLA; SA4: 30% ethyl acetate; SA5: 70% ethyl acetate; SA6: 30% acetone; SA7: 70% acetone; SA8: PVA; SA9: Vitamin E TPGS.

The effects of these materials can be explained as following. A more hydrophilic polymer like PLA-PEG-PLA was helpful for the interaction between water and oil phases [Li et al., 1999]. The improvement caused by ethyl acetate and acetone was probably due to the fact that (1) as compared with DCM, they were more water-soluble and their density difference with water was smaller, thereby leading to better mixing between water and oil phases; (2) their extraction rate was greater, thus the polymer precipitated more rapidly and HSA had less opportunity to diffuse out [Bodmeier and McGinity, 1988]. These explanations were further supported by the observation that higher ratio of ethyl acetate/acetone resulted in higher encapsulation efficiency and lower initial burst (Fig. 5.3 and 5.4: SA4-7). Emulsifiers were able to improve the w/o emulsion stability because their amphiphilic structure facilitated the oil-water interaction [Conway and Alpar, 1996]. Under our experimental conditions, the effects of PVA appeared to be slightly better than Vitamin E TPGS (Fig. 5.3 and 5.4: SA8 and 9).

We noticed that some of the above results seemed to be ‘contradictory’ to some of those in the previous Chapter. For example, in the previous Chapter the effect of organic solvents on HSA encapsulation efficiency was complex, while here all the mixtures of DCM with ethyl acetate or acetone resulted in better encapsulation effects than DCM alone. This ‘contradiction’ is actually understandable, because here vortex mixing rather than sonication was used to form the w/o emulsion and maintenance of the w/o emulsion stability was thus the key to obtain good encapsulation effects, and any means to improve the w/o emulsion stability, such as more hydrophilic solvents, could lead to better encapsulation effects.

Characterizations were also carried out on other microsphere properties such as surface morphology, particle size and kinetics of in vitro release. It was found that all samples had nice spherical shape and most had smooth surface morphology. Several typical SEM images are shown in Fig. 5.5. PLA-PEG-PLA resulted in coarse microsphere surface, a result in agreement with those of previous Chapters (samples SA3).

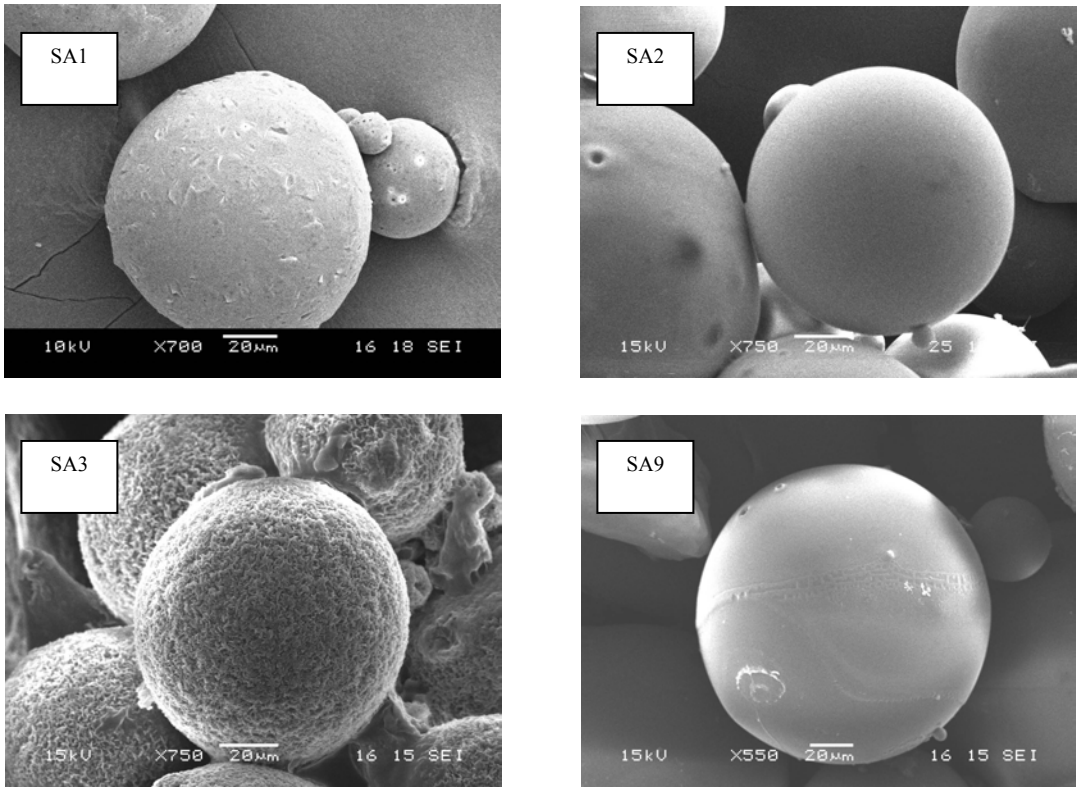


Figure 5.5 Surface morphology of HSA-loaded microspheres. SA1: sonication control; SA2: vortexing control; SA3: PLA-PEG-PLA; SA9: Vitamin E TPGS.

As shown in Fig. 5.6, vortex mixing led to slightly smaller particle size (SA1 vs. SA2), probably due to partial loss of the inner water phase (Figure 5.2). The particle size of all the other samples appeared to be larger than that of vortex control sample SA2, presumably due to the improvement of w/o emulsion stability and thus less loss of inner water phase. Higher ratio of ethyl acetate/acetone as the cosolvent led to larger size. The

reason may be that these two solvents were extracted to the outer water phase more rapidly than DCM, thus the polymer droplets were harder to be broken if the solvent mixture contained more ethyl acetate/acetone.

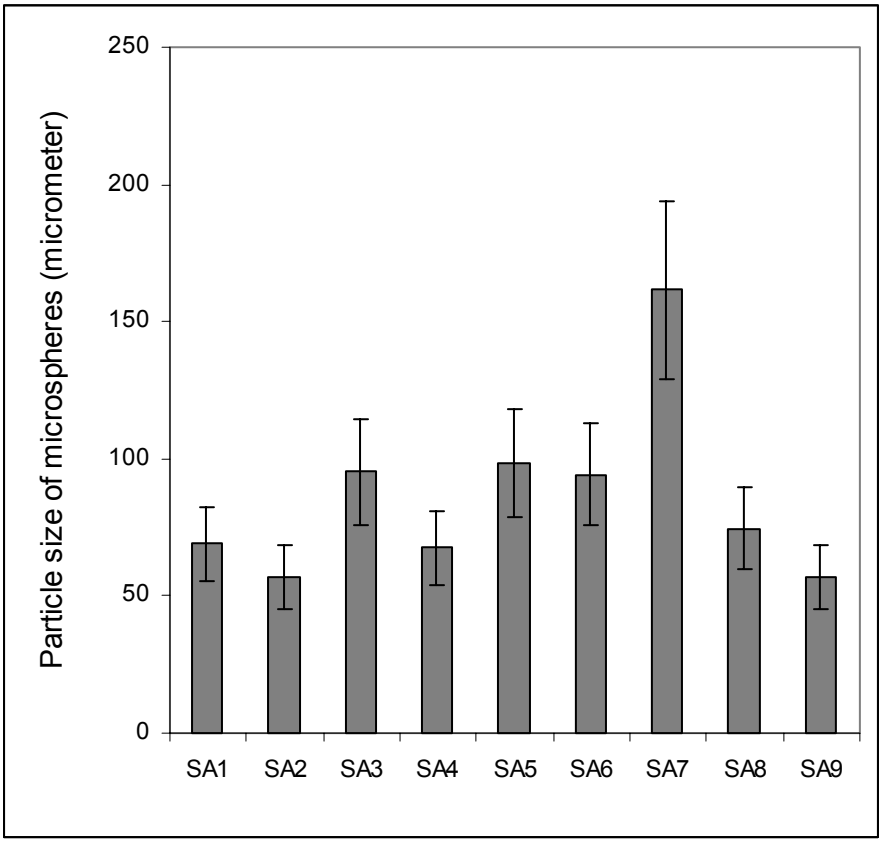


Figure 5.6 Particle size of HSA-loaded microspheres. SA1: sonication control; SA2: vortex control; SA3: PLA-PEG-PLA; SA4: 30% ethyl acetate; SA5: 70% ethyl acetate; SA6: 30% acetone; SA7: 70% acetone; SA8: PVA; SA9: Vitamin E TPGS.

All samples tested had sustained release pattern for the encapsulated protein after the initial burst (Fig. 5.7). The release rate given by samples prepared by vortex mixing was generally greater than that of the control sample prepared by sonication, perhaps partially due to the more porous internal morphology observed in the former. The partially hydrophilic matrix of PLA-PEG-PLA microspheres led to fast HSA diffusion and polymer

degradation, thus the release rate was very fast (samples SA3). HSA release from samples prepared with higher ratio of more hydrophilic cosolvent (ethyl acetate or acetone) was slower, probably due to their larger particle size.

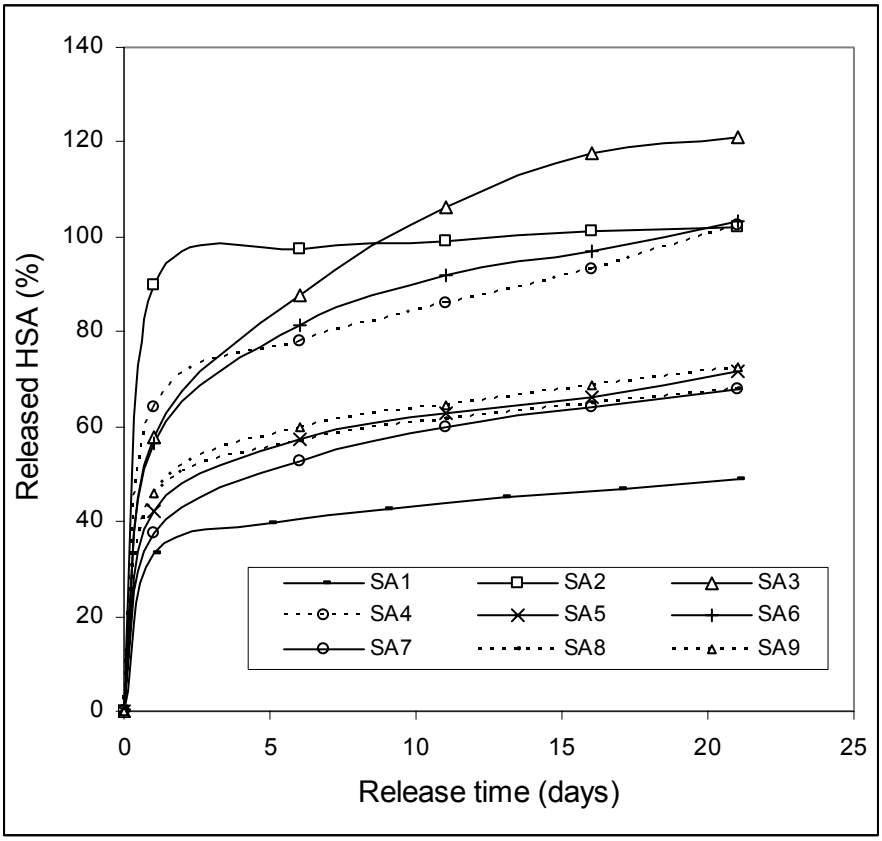


Figure 5.7 In vitro release of HSA from microspheres. SA1: sonication control; SA2: vortex control; SA3: PLA-PEG-PLA; SA4: 30% ethyl acetate; SA5: 70% ethyl acetate; SA6: 30% acetone; SA7: 70% acetone; SA8: PVA; SA9: Vitamin E TPGS.

5.3 Coating of liposomes

In the lipid bilayers of liposomes, hydrophilic head groups of the phospholipid molecules are positioned outward to the water phase and the hydrophobic chains inward tail to tail. Since phospholipids are soluble in organic solvents, liposomes could readily be damaged

by the organic solvent used for LIM preparation, although the hydrophilic head groups could have some protective effect. There have been some successful attempts in the literature to protect the liposomes from organic solvents by cross linking the phospholipid molecules in the lipid bilayers [Gregoriadis, 1984a]. However, the polymerization of phospholipids involves complex operations, e.g., UV radiation. Here we used a hydrophilic polymer coating technique, which is much simpler in operation, to achieve the same goal. To the best of our knowledge, no similar report can be found from the literature for this purpose, although there are numerous reports on the effect of coating to preserve the liposome stability against reticuloendothelia system (RES) [Lasic and Martin, 1995].

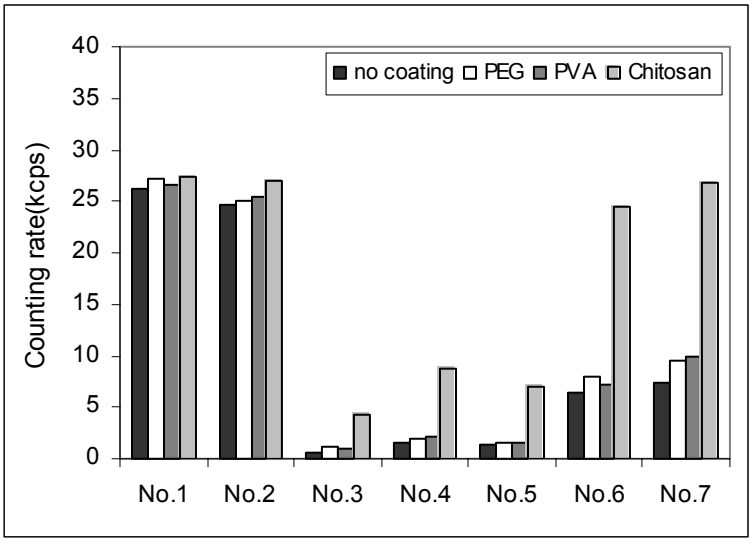
It could be anticipated that the protective effect of hydrophilic polymer coating would depend on (1) solubility of the polymer in the organic solvent, (2) the adhesive ability of the polymer on the lipid bilayers, (3) the ability of the organic solvent to penetrate the lipid layers, and (4) the ability of the organic solvent to dissolve the phospholipids. We tested the effect of three polymer materials, which have been reported in the literature as liposome coating, namely PEG, chitosan and PVA, and five organic solvents, which have been used in the literature for preparation of polymeric microspheres, namely chloroform, DCM, acetone, ethyl acetate and cyclohexane.

Fig. 5.8 shows the effects of different coating materials on the change of liposome properties, including vesicle number (Fig. 5.8 (A)) and amount of calcein inside the liposomes (Fig. 5.8 (B)), after the treatment with different organic solvents. In the absence of organic solvents, vortex mixing slightly reduced the vesicle number and the amount of calcein inside the liposomes (No. 2). In the presence of organic solvents, however, the

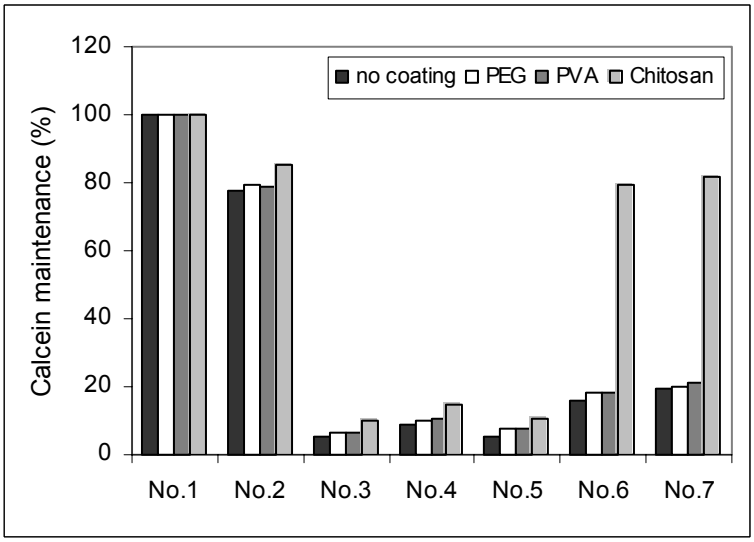
change of the above two properties became very dramatic, indicating significant liposome breakage. The change of liposome properties depended on the choice of coating materials and organic solvents. It can be seen from Fig. 5.8 that the damaging effect of different organic solvents increased in the order of cyclohexane < ethyl acetate << DCM < the mixture of DCM and acetone ≈ chloroform, in terms of either the vesicle number or the calcein maintenance. The observation that acetone had great damaging effect might be explained by its readiness to get across the barrier formed by hydrophilic head groups in the lipid bilayers, since it is water-soluble. Chloroform damaged the liposomes easily because phospholipids readily dissolve in chloroform. In fact, chloroform is the most commonly used solvent for phospholipids. Furthermore, among the three coating materials that we used, chitosan appeared to have the best effect to protect liposomes from damage by the organic solvents. In particular, when cyclohexane or ethyl acetate was used as the organic solvent and chitosan as the coating material, the integrity of the liposomes was mostly preserved after the treatment with the organic solvent.

Chitosan is the principal derivative of chitin, one of the most abundant polysaccharides in nature. It has been applied for many biomedical applications. Main industrial sources of chitin are the shell wastes of shrimp, lobster and crab. Alkaline deacetylation of chitin forms chitosan [Felt et al., 1998]. The excellent protective effect of chitosan in our case could be due to its two characteristics: (1) Chitosan is practically insoluble in all common organic solvents [Kumar, 2000]. In contrast, PEG is soluble in a number of organic solvents. (2) It is one of the few natural polymers that bear cationic charges. The cationic nature enables it to establish a strong attractive force with the negative charged lipid bilayers [Takeuchi et al., 1996]. Therefore, the chitosan coating is difficult to be removed

from the liposome surface. In contrast, PVA is easy to detach from the liposomes because the force to maintain the attachment is purely by adsorption.



(A) The change of vesicle number after the treatment of organic solvents

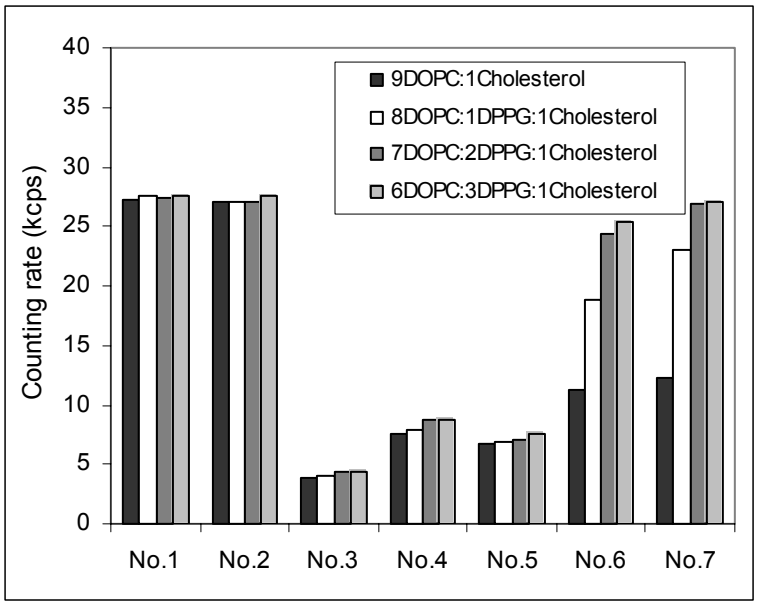


(B) The maintenance of calcein in the liposomes after the treatment of organic solvents

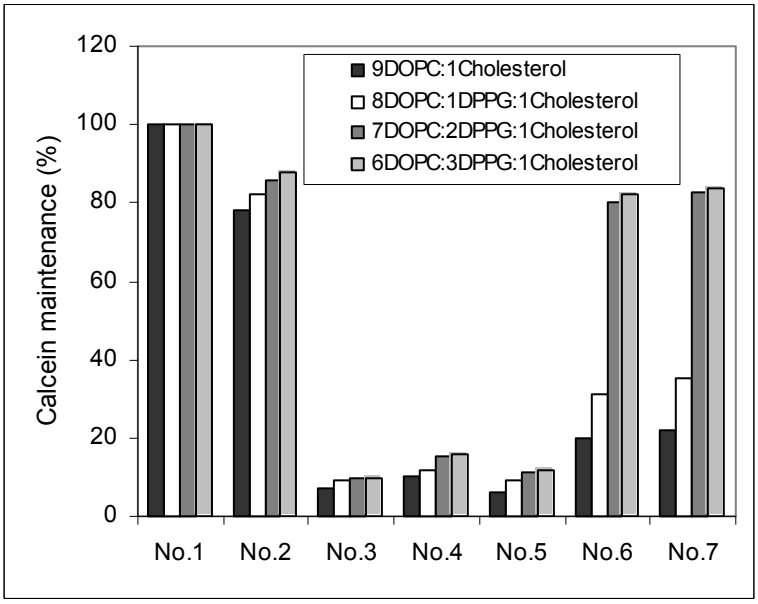
Figure 5.8 Change of liposome properties after the treatment of organic solvents: effect of different coating materials and organic solvents. The liposomes used were LUVs with lipid composition DOPC:DPPG:cholesterol=7:2:1 and total lipid concentration 2 mM, and produced by membranes of pore size 200 nm. No.1 (control 1): no solvent treatment, no vortex mixing; No. 2 (control 2): no solvent treatment, treated with vortex mixing; No. 3: treated with chloroform and vortex mixing; No. 4: treated with DCM and vortex mixing; No. 5: treated with the mixture of DCM (90%) and acetone (10%) and vortex mixing; No. 6: treated with ethyl acetate and vortex mixing; No. 7: treated with cyclohexane and vortex mixing.

As shown in Fig. 5.9, the presence and amount of negative charge played a critical role in the protective effect of chitosan coating. In the absence of DPPG, a negative charged phospholipid, the protective effect of chitosan was marginal, presumably because only adsorption force was involved in attaching the coating to the liposome surface in this case. Addition of 10% DPPG in the lipid composition led to significant improvement of the protective effect, probably due to the presence of the electrical attractive force between the cationic coating and the anionic phospholipid. Further increase of the DPPG amount led to further improvement of the stability of the liposomes. Nevertheless, compared with 20% DPPG, the improvement given by 30% DPPG was trivial, implying that 20% DPPG allowed nearly complete attraction of the positive charges in the chitosan coating.

The effects of chitosan coating on the properties of liposomes, including surface charge, particle size and in vitro release rate of calcein, were investigated. As shown in Fig. 5.10 (A), coating the negative-charged surface of liposomes with the cationic polymer chitosan shifted the zeta potential, which is an indicator of surface charge, from negative value to positive value. This is why chitosan-coated liposomes have been used as mucoadhesive drug delivery systems as their positively charged surface can help them adhere to the cells, whose membranes are usually negatively charged [Takeuchi et al., 2001]. Fig. 5.10 (B) shows the results of particle size. It can be seen that chitosan coating slightly increased the particle size of liposomes. In vitro release of calcein from liposomes was carried out at 25°C without shaking. As we can see from Fig. 5.10 (C), the chitosan on the liposome surface slightly reduced the calcein release rate.

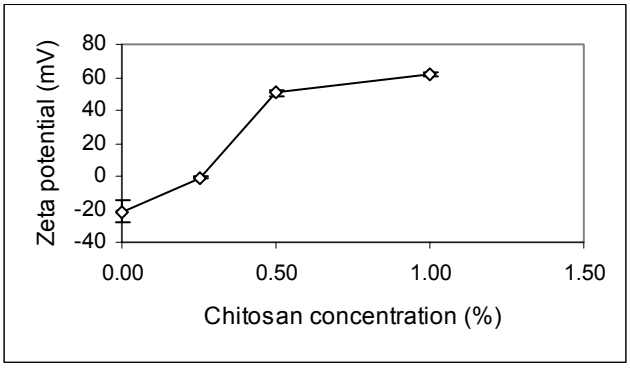


(A) The change of vesicle number after the treatment of organic solvents

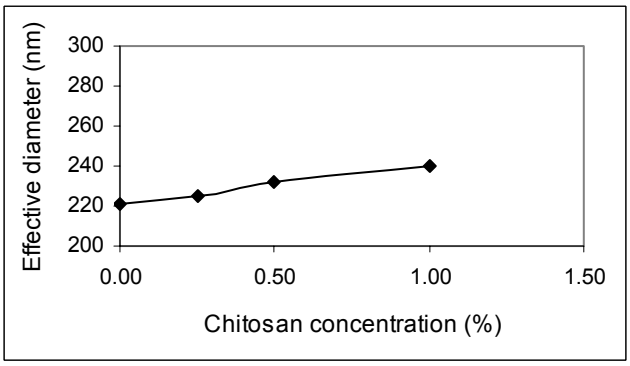


(B) The maintenance of calcein in the liposomes after the treatment of organic solvents

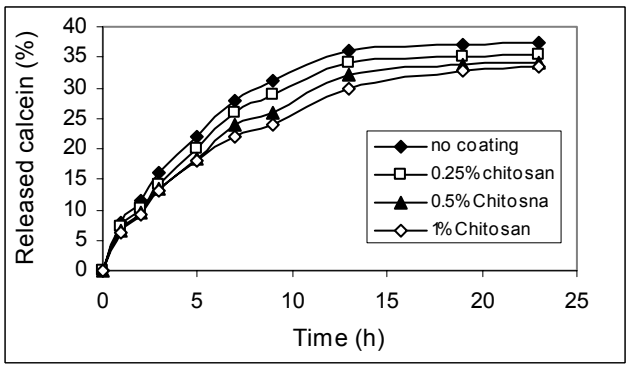
Figure 5.9 Change of the properties of chitosan coated liposomes after the treatment of organic solvents: effect of lipid charge. The liposomes used were LUVs with total lipid concentration 2 mM, produced by membranes of pore size 200 nm, and coated with 1% chitosan. No.1 (control 1): no solvent treatment, no vortex mixing; No. 2 (control 2): no solvent treatment, treated with vortex mixing; No. 3: treated with chloroform and vortex mixing; No. 4: treated with DCM and vortex mixing; No. 5: treated with the mixture of DCM (90%) and acetone (10%) and vortex mixing; No. 6: treated with ethyl acetate and vortex mixing; No. 7: treated with cyclohexane and vortex mixing.



(A) Surface charge of liposomes



(B) Particle size of liposomes



(C) Calcein release from liposomes

Figure 5.10 Effects of chitosan coating on the properties of liposomes. The liposomes used were LUVs with lipid composition DOPC:DPPG:cholesterol=7:2:1 and total lipid concentration 2 mM, and produced by membranes of pore size 200 nm.

5.4 Fabrication and characterizations of LIMs

5.4.1 Fabrication

After the problems of possible damage to the liposomes by sonication and organic solvents were solved by modifying the double emulsion process and the liposome structure respectively, we should be able to encapsulate liposomes into the polymeric microspheres. The key points in the preparation of LIMs can be summarized as the following: (1) the modified double emulsion method was implemented, in which vortex mixing was used to produce the w/o emulsion. (2) PLA-PEG-PLA (Mw 80000) and ethyl acetate were used as the polymer and the organic solvent, respectively. (3) Negative charged liposomes were coated with chitosan. It should be pointed out that emulsifiers were not used in the formation of w/o emulsion for the LIM preparation because they might have damaging effects on liposomes [S.C Basu and M. Basu, 2002]. In addition, cyclohexane, one of the solvents that showed good effect for liposome protection as discussed in the previous Section, was not used in the preparation of LIMs because we found that PLA-PEG-PLA does not dissolve readily in it. Six LIM samples (Table 5.2, LIM1 – LIM6), which had different particle size of liposomes and porosity of polymer matrix, were prepared. A blank microsphere sample was also prepared for comparison. The integrity of liposomes after being encapsulated and released from the microspheres was examined. The particle size, morphology and encapsulation efficiency of the LIMs were characterized. The feasibility of controlling the in vitro release kinetics of LIMs by the properties of liposomes and polymer matrix was then demonstrated.

Table 5.2 Samples of LIMs. Liposomes were LUVs or MLVs with lipid composition DOPC:DPPG:cholesterol=5:2:5 and total lipid concentration of 2 mM, and coated with 1% chitosan. PEG was used as a pore forming additive in the polymer matrix. It was mixed with the polymer before the preparation of LIMs.

Sample number	Liposome size (nm)	PEG concentration (%)	LIM size (μm)	Released liposome size (nm)	LIM encapsulation efficiency (%)
LIM1	185.5 (LUVs)	0	66.5±12.2	192.3	50.3
LIM2	255.8 (LUVs)	0	60.8±13.6	240.1	52.5
LIM3	438.2 (LUVs)	0	67.9±11.5	411.8	53.1
LIM4	1382.2 (MLVs)	0	62.8±13.3	1278.5	56.5
LIM5	185.5 (LUVs)	5	63.1±13.2	171.2	48.2
LIM6	185.5 (LUVs)	10	69.8±15.1	182.7	46.8

5.4.2 Integrity of liposomes

The integrity of liposomes after being encapsulated into microspheres and released from LIMs was confirmed by three different approaches, namely direct visualization, particle size measurement and fluorescence analysis. Fig. 5.11 shows the SEM images of the liposomes before the microencapsulation, the interior of the blank microspheres, and the liposomes inside LIMs after the microencapsulation process. MLVs (LIM4, Table 5.2) were used because we wanted to observe the liposomes and polymer matrix under the same magnification. It can be seen from Fig. 5.11 (C) that the spherical shape of the liposomes was intact.

Particle size of the liposomes after they were released from LIMs was measured. It can be seen from Table 5.2 that, for all the six LIM samples, the released liposomes had particle sizes close to those before they were encapsulated into the polymeric microspheres.

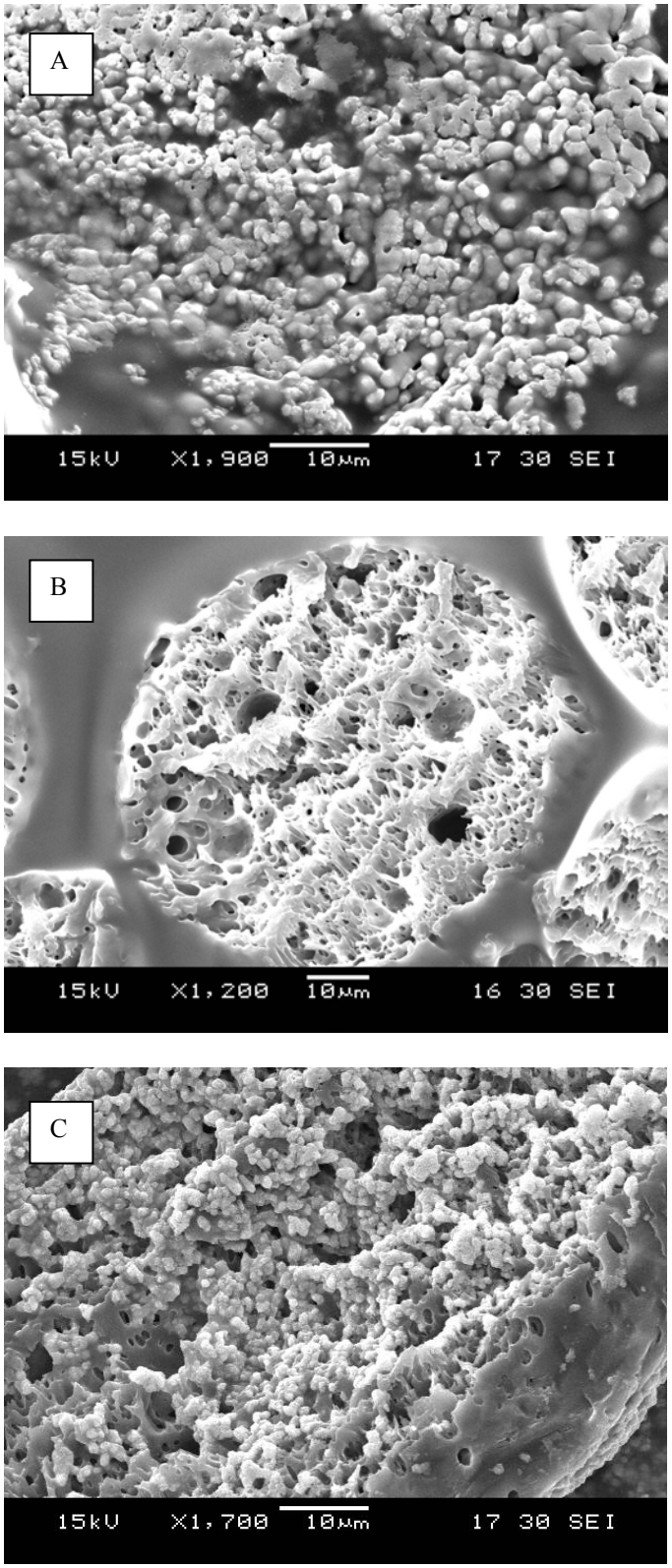


Figure 5.11 SEM images of (A) freeze-dried liposomes, and the interior of (B) blank microspheres and (C) LIMs. The liposomes were MLVs with particle size 1382.2 nm, lipid composition DOPC:DPPG:cholesterol=5:2:5 and total lipid concentration of 2 mM, and coated with chitosan.

In order to quantitatively confirm the integrity of liposomes, we examined the fluorescence intensity after the liposomes were released. It should be noted that the composition of the liposomes used for the samples shown in Table 5.2 was different from that shown in Fig. 5.10. A higher cholesterol concentration was used here, because we wished to have negligible calcein leakage from the liposomes after they only underwent through vortex mixing so that the amount of liposomes could be measured by the fluorescence intensity of calcein. Incorporating cholesterol in the liposome composition is well known to make the lipid bilayers more rigid. The experimental results showed that no appreciable calcein (less than 10%) was leaked out in this case. Subsequently we measured the calcein leakage after the liposomes were released from the LIMs. It was found that the level of leakage was also less than 10%. Therefore, based on the results of the above three different approaches, it can be concluded that the integrity of liposomes after microencapsulation and release was largely preserved.

5.4.3 Particle size, surface morphology and encapsulation efficiency

As shown in Table 5.2, the particle size of all LIM samples was between 60 μm and 70 μm, which was close to that of blank microspheres, i.e. 60.8±10.7 μm. This implies that encapsulation of liposomes didn't affect the size of the polymeric matrix significantly. Fig. 5.12 illustrates the surface morphology of LIMs. All samples had rather spherical shape. When no PEG was included in the polymer matrix, the surface of both the blank microspheres and the LIMs was smooth. The SEM images of the blank microspheres and the sample LIM1 are shown as examples. As expected, addition of the pore forming

additive PEG increased the porosity of LIMs. A lot of pores were visible on the surface of samples LIM5 and LIM6. Apparently, the latter, which had higher PEG concentration, was more porous than the former. The results of encapsulation efficiency of liposomes in LIMs are shown in Table 5.2. It can be seen that the encapsulation efficiency of all samples was around 50%. Furthermore, increasing the particle size of liposomes and decreasing the pore size of polymer matrix appeared to increase the encapsulation efficiency slightly. This is probably because in the LIM fabrication process, the liposomes will be more difficult to migrate to the outer water phase in the case of larger liposomes and smaller pores in the polymer matrix.

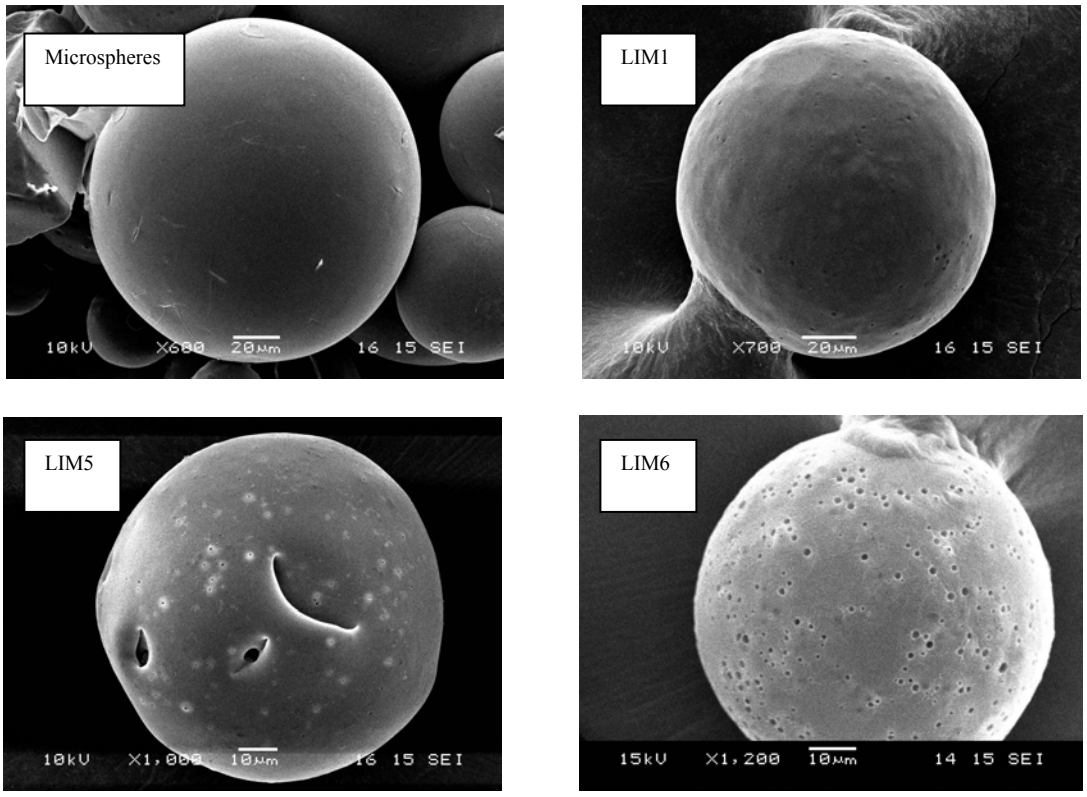


Figure 5.12 Surface morphology of blank microspheres and LIMs

5.4.4 In vitro release

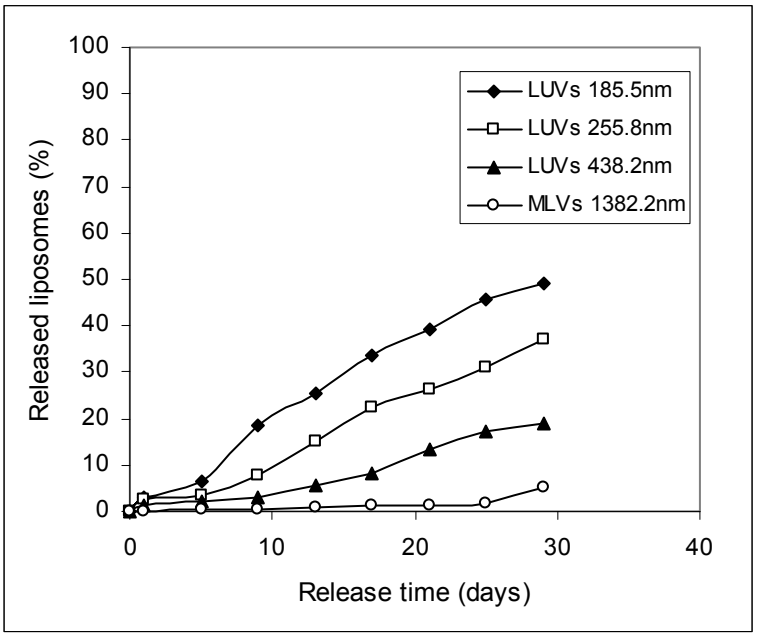
Fig. 5.13 shows the in vitro release kinetics of liposomes from the LIMs. As can be seen from Fig. 5.13 (A), when the pore forming additive PEG was not used, the liposome release showed a delayed pattern, meaning that the liposomes had an off-release period before they began to be released at a constant rate. We assumed that both diffusion of the liposomes and degradation of the polymer matrix played important roles in the release mechanism. The interior of LIMs contained many pores interconnected by tortuous water channels. The liposomes had to diffuse through these channels before they could be released from the polymer matrix. Initially, the particle size of liposomes was too big to pass through the water channels so that the release rate during this period was very low. As time went on, the degradation of the polymer matrix caused expansion of the pores and channels, thereby leading to a sustained release of liposomes.

This hypothesis was supported by the observation that smaller liposome size led to shorter off-release period, since smaller liposomes would be easier to diffuse through the water channels (Fig. 5.13 (A)). The MLVs with particle size of 1382.2 nm showed a significant release from LIMs only after 25 days, while a sustained release of LUVs with particle size of 185.5 nm started from as early as around the 5th day. Furthermore, the release rate during the sustained release period increased with decreasing particle size of the liposomes. Nearly half of the LUVs with particle size of 185.5 nm were released after 29 days, while in the case of the MLVs with particle size of 1382.2 nm only 5.1% of the liposomes were released during the same period. In order to obtain a sustained release pattern throughout the whole release period, PEG was included in the polymer matrix to facilitate the

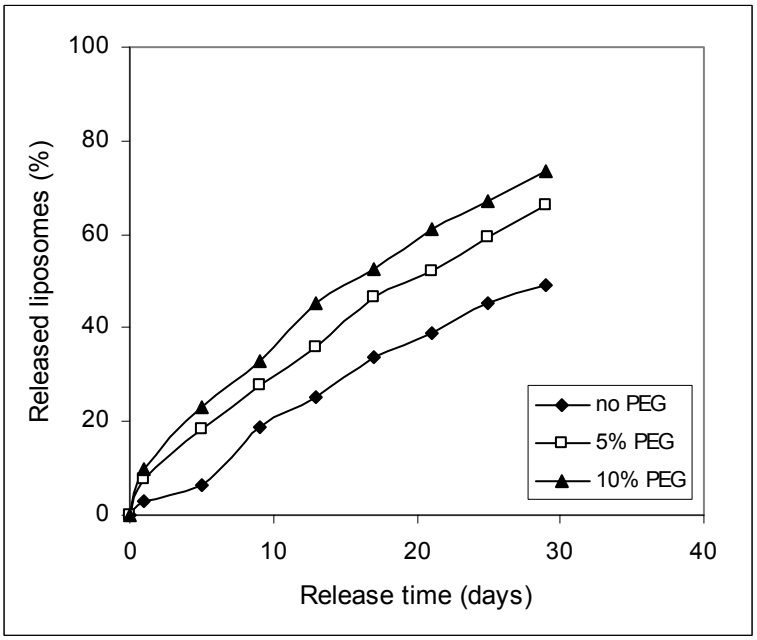
formation of pores and water channels. As evidenced from Fig. 5.13 (B), the off-release period disappeared after PEG was added. Higher PEG concentration resulted in faster release rate, as the LIMs had greater porosity in this case. Up to 73.6% of the liposomes were released after 29 days when 10% PEG was used. In general, by adjusting the properties of liposomes and polymer matrix, different release patterns can be achieved and the release rate can be controlled.

5.5 Summary

A novel drug delivery system, liposomes-in-microsphere (LIM) of biodegradable polymers, which is based on the concept of combination of the polymer-based and the lipid-based controlled drug delivery systems, was successfully developed by modifying the double emulsion process and coating the liposomes with chitosan. It was found that the liposomes encapsulated in and released from the polymeric microspheres were intact. The encapsulation efficiency of liposomes in the polymeric microspheres was about 50%. The encapsulation of liposomes didn't significantly change the particle size and surface morphology of the polymeric microspheres. In vitro, the liposomes were released at a nearly constant rate from the polymeric microspheres after an initial off-release period. It was found that decreasing the particle size of liposomes and increasing the pore size of the polymer matrix shortened the initial off-release period and increased the release rate. LIMs could integrate the advantages, avoid the disadvantages of the polymer-based and the lipid-based systems, and lead to new applications such as protecting biological activity of proteins and peptides, increasing the efficacy and decreasing the side effects of anticancer drugs, and facilitating the targeted gene delivery.



(A) Effect of the particle size of liposomes (no PEG was used)



(B) Effect of the pore size of polymer matrix (LUVs of particle size of 185.5 nm were used)

Figure 5.13 In vitro release of liposomes from LIMs

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The versatile applications of polymeric microspheres for controlled release have been demonstrated. Prepared by the technique of solvent extraction/evaporation, this system can be used to microencapsulate water-insoluble agents with an anticancer drug paclitaxel as the prototype, water-soluble agents with a protein HSA as the prototype, and other delivery devices with liposomes as the prototype. These agents/devices can be released in controlled manners.

The properties of polymeric microspheres can be engineered by material and process parameters. In the present thesis, we have mainly investigated the effects of materials of different hydrophobicity. These effects are dependent on the fabrication methods (single emulsion or double emulsion) and the material types (polymers, solvents, or additives). It has been found that the following novel materials could improve the quality of the polymeric microspheres: PLA-PEG-PLA as the polymer, ethyl acetate/acetone as the solvents, and Vitamin E TPGS as the additive.

The microsphere technique and the liposome technique have been combined in the present thesis to form a novel delivery device LIM, i.e. liposomes-in-microsphere. The liposomes remain intact after the microencapsulation and release. The in vitro release of liposomes from the polymer matrix could be controlled by the properties of liposomes and the

polymeric microspheres. LIMs could integrate the advantages and eliminate the disadvantages of the polymer based and the lipid based systems, and lead to new applications. Furthermore, the idea of combining different drug delivery systems could be applied to develop other new devices to meet various clinical requirements.

The successful preparation of LIM confirms that it is feasible to encapsulate supramolecular structures into microparticles. This fact might have implications in many areas such as nanotechnology and tissue engineering.

6.2 Recommendations

The clinical applications of the LIM system should be pursued. Most interesting development would be to examine its effects on the protection of protein activity. Preliminary studies in our laboratory have shown that interferon-β, a helical cytokine and currently the best drug to treat multiple sclerosis [Karpusas et al. 1998], lost practically all of its antiviral activity after being encapsulated into PLGA microspheres. Common protein stabilizers, such as trehalose, mannitol and Tween 20, showed no positive effect for interferon-β. In contrast to the above stabilizers, the liposomes in LIM could have protective effects for all proteins because its protective effects are based on physical cover. Since in the present thesis it has been shown that liposomes can be encapsulated into and released from microspheres in intact form, it should be reasonable to predict that LIM system could be very useful to protect the activity of interferon-β in its controlled delivery application.

It is suggested that the in vivo behavior of the drug delivery systems be examined. Only in the real biological environments can many questions be answered: where do the delivery devices go after they enter the animal/human body? What are the interactions between the delivery devices and tissues? Is the in vivo drug release kinetics the same as the in vitro one? How is the therapeutic effect of the drug after it is loaded into the delivery device?

Mathematical modeling could be applied in the research of controlled drug release. Mathematical modeling is powerful in rational design of drug delivery systems. It can help to identify the drug release mechanism and predict the drug release kinetics.

The feasibility to scale-up the fabrication process of the delivery devices should be investigated. This is another contribution that a chemical engineer can make in the area of controlled drug delivery. Many issues need to be addressed: are the techniques used in the present thesis suitable for large-scale production? How to control the quality of the products? etc.

REFERENCES

Amelinckx, S., Van Dyck, D., Van Landuyt, J., Van Tendeloo, G. (ed). Handbook of Microscopy: Applications in Materials Science, Solid-state Physics and Chemistry, Volume I. pp.33-53, Weinheim: VCH. 1997.

Anderson, J.M. and Shive, M.S. Biodegradation and Biocompatibility of PLA and PLGA Microspheres, Adv. Drug Deliver. Rev., *28*, pp.5-24. 1997.

Arshady, R. Preparation of Biodegradable Microspheres and Microcapsules: 2. Polylactides and Related Polyesters, J. Controlled Release, *17*, pp.1-21. 1991.

Bagwe, R.P., Kanicky, J.R., Palla, B.J., Patanjali, P.K., Shah, D.O. Improved Drug Delivery Using Microemulsions: Rationale, Recent Progress, and New Horizons, Crit. Rev. Ther. Drug., *18*, pp.77-140. 2001.

Baker, R. (ed). Biodegradable Drug Delivery Systems. pp.19-43, London: Academic press. 1992.

Barenholz, Yechezkel. Liposome Application: Problems and Prospects, Curr. Opin. Colloid In., *6*, pp.66-77. 2001.

Basu, S.C. and Basu, M. Liposomes Methods and Protocols. p.55, Totowa, N.J.: Humana press. 2002.

Becker, J.B., Robinson, T.E., Barton, P., Sintov, A., Siden, R., Levy, R.J. Sustained Behavioral Recovery from Unilateral Nigrostriatal Damage Produced by the Controlled Release of Dopamine from a Silicone Polymer Pellet Placed into the Denervated Striatum, Brain Res. *508*, pp.60-64. 1990.

Benita, S. and Levy, M.Y. Submicron Emulsions as Colloidal Drug Carriers for Intravenous Administration-comprehensive Physicochemical Characterization, J. Pharm. Sci., *82*, pp.1069-1079. 1993.

Bio-Rad company. Bio-Rad protein assay menu.

Bird, R.B., Stewart, W.E., Lightfoot, E.N. Transport Phenomena. pp.495-519, New York: John Wiley & Sons. 1960.

Black, F.E., Harshorne, M., Davies, M.C., Roberts, C.J., Tendler, S.J.B., Williams, P.M., Shakesheff, K.M. Surface Engineering and Surface Analysis of a Biodegradable Polymer with Biotinylated End Groups, Langmuir, *15*, pp.3157-3161. 1999.

Bodmeier, R. and McGinity, J.W. Solvent Selection in the Preparation of Poly(dl-lactide) Microspheres Prepared by the Solvent Evaporation Method, Int. J. Pharm., *43*, pp.179-186. 1988.

Boswell, G.A. (DuPont de Nemours and Co.) German patent 2051580, 1971.

Bradbury, S. and Bracegirdle, B. Introduction to Light Microscopy. p.23, New York: Springer. 1997.

Bradford, M.M. A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding, Anal. Biochem., *72*, pp.248-254. 1976.

Britannica Encyclopedia On-line: http://www.lib.nus.edu.sg/digital/.

Brown, M.E. (ed). Handbook of Thermal Analysis and Calorimetry, Vol. 1. pp.58-96, Amesterdam: Elsevier. 1998.

Brown, M.E. Introduction to Thermal Analysis: Techniques and Applications, 2nd edition. pp.77-98, Dordrecht & Boston: Kluwer Academic Publisher. 2001.

Bubert, H. and Jenett, H. Surface and Thin Film Analysis: Principles, Instrumentation, Applications. pp.106-132, Weinheim: Wiley-VCH. 2002.

Buckel, P. Recombinant Proteins for Therapy, Trends Pharmacol. Sci., *17*, pp.450-456. 1996.

Campbell, M.K. and Shawn, O.F. Biochemistry, 4th edition. pp.43-56, Singapore: Brooks/Cole. 2003.

Cannizzaro, S.M., Padera, R.F., Langer, R., Rogers, R.A., Black, F.E., Davies, M.C., Tendler, S.J.B., Shakesheff, K.M. A Novel Biotinylated Degradable Polymer for Cell-interactive Applications, Biotechnol. Bioeng., *58*, pp.529-535. 1998.

Capan, Y., Woo, B.H., Gebrekidan, S., Ahmed, S., DeLuca, P.P. Stability of Poly(L-lysine)-complexed Plasmid DNA during Mechanical Stress and Dnase I Treatment, Pharm. Dev. Technol., *4*, pp.491-498. 1999.

Castelli, F., Messina, C., Pignatello, R., Puglisi, G. Effect of pH on Diclofenac Release from Eudragit RS100® Microparticles, a Kinetic Study by DSC, Drug deliver., *8*, pp.173-177. 2001.

Ceh, B. Stealth® Liposomes: from Theory to Product, Adv. Drug Deliver. Rev., *24*, pp.165-177. 1997.

Chabner, B.A. and Longo, D.L. Cancer Chemotherapy and Biotherapy: Principles and Practice, 2nd edition. pp.97-122, Philadelphia: Lippincott-Raven Publishers. 1996.

Chastain, J. Handbook of X-ray Photoelectron Spectroscopy. pp.43-55, USA: Perkin-Elmer Corporation. 1992.

Chia, H.H., Yang, Y.Y., Chung, T.S., Ng, S., Heller, J. Auto-catalyzed Poly(ortho ester) Microspheres: a Study of Their Erosion and Drug Release Mechanism, J. Controlled Release, *75*, pp.11-25. 2001.

Chia, K.S. Trends in Cancer Incidence in Singapore 1968-1992. Report No 4, Singapore Cancer Registry. 1996.

Choi, Y.K., Bae, Y.H., Kim, S.W. Star-shaped Poly(ether-ester) Block Copolymers: Synthesis, Characterization and Their Physical Properties, Macromolecules, *31*, pp.8766-8774. 1998.

Cleland, J.L. and Jones, A.J.S. Stalbe Formulations of Recombinant Human Growth Hormone and Interferon-γ for Microencapsulation in Biodegradable Microspheres, Pharm. Res., *13*, pp.1464-1475. 1996.

Cleland, J.L. and Langer, R. Formulation and Delivery of Proteins and Peptides. pp.56-97, Washington DC: American Chemical Society. 1994.

Colin, M., Guenard, D., Guritte-Voegelein, F. Process for Preparing Derivatives of Baccatin III and of 10-Deacetyl Baccatin III, U.S. Patent 4924012, 1990.

Conway, B.R. and Alpar, H.O. Double Emulsion Microencapsulation of Proteins as Model Antigens Using Polylactide Polymers: Effect of Emulsifier on the Microsphere Characteristics and Release Kinetics, Eur. J. Pharm. Biopharm., *42*, pp.42-48. 1996.

Courvreur, P. Polycyanoacrylate Nanocapsules as Potential Lysosomotropic Carriers: Preparation, Morphology and Sorptive Properties, J. Pharm. Pharmaco., *31*, pp.331-332. 1979.

Crommelin, D.J.A., Daemen, T., Scherphof, G.L., Vingerhoeds, M.H., Heeremans, J.L.M., Kluft, C., Storm, G. Liposomes: Vehicles for the Targeted and Controlled Delivery of Peptides and Proteins, J. Controlled Release, *46*, pp. 165-175. 1997.

Dass, C.R. and Burton, M.A. Microsphere-mediated Targeted Gene Therapy of Solid Tumors, Drug Deliver., *6*, pp.243-252. 1999.

Dejaeger, N.C. and Tavernier, B.H. British Patent 1405108, 1971.

Deng, X.M., Li, X.H., Yuan, M.L., Xiong, C.D., Huang, Z.T., Jia, W.X., Zhang, Y.H. Optimization of Preparative Conditions for Poly-dl-lactide-polyethylene Glycol Microspheres with Entrapped Vibrio Cholera Antigens, J. Controlled Release, *58*, pp.123-131. 1999.

Deng, X.M., Xiong, C.D., Cheng, L.M., Xu, R.P. Synthesis and Characterization of Block Copolymers from d, l-Lactide and Poly(ethylene glycol) with Stannous Chloride, J. Polym. Sci. Polym. Lett., *28*, pp.411-416. 1990.

Dill, K.A. Strengthening Biomedicine's Roots, Nature, *400*, pp.309-310. 1999.

Dobias, B. Coagulation and Flocculation: Theory and Applications. pp.67-88, New York: Marcel Dekker. 1993.

Dordunoo, S.K., Jackson, J.K., Arsenault, L.A., Okataba, A.M.C., Hunter, W.L., Burt, H.M. Taxol Encapsulation in Poly(E-caprolactone) Microspheres, Cancer Chemother. Pharmacol., *36*, pp.279-282. 1995.

Dubernet, C. Thermoanalysis of Microspheres, Thermochim. Acta, *248*, pp.259-269. 1995.

Dumitriu, S. Polymeric Biomaterials, 2nd edition. pp.87-99, New York: Marcel Dekker. 2002.

Dvorak, J.A. The Application of Atomic Force Microscopy to the Study of Living Vertebrate Cells in Culture, Methods, *29*, pp.86-96. 2003.

Edlund, U. and Albertsson, A.C. Degradable Polymer Microspheres for Controlled Drug Delivery, Adv. Polym. Sci., *157*, pp.67-112. 2002.

Evora, C., Soriano, I., Rogers, R.A., Shakesheff, K.M., Hanes, J., Langer, R. Relating the Phagocytosis of Microparticles by Alveolar Macrophages to Surface Chemistry: the Effect of 1,2-Dipalmitoylphosphatidylcholine, J. Controlled Release, *51*, pp.143-152. 1998.

Fan, L.T. and Singh, S.K. Controlled Release: a Quantitative Treatment. pp.86-97, Berlin & New York: Springer-Verlag. 1989.

FASEB (Federation of American Societies for Experimental Biology). Molecular Medicine 2020: a Vision for the Future of Medical Research and Human Health. Public policy priority. 1998.

Feast, W.J., Munro, S., Richards, R.W. Polymer Surfaces and Interfaces. pp.120-145, New York: John Wiley & Sons. 1993.

Felt, O., Buri, P., Gurny, R. Chitosan: a Unique Polysaccharide for Drug Delivery, Drug Dev. Ind. Pharm., *24*, pp.979-993. 1998.

Feng, S.S. and Huang, G.F. Effects of Emulsifiers on the Controlled Release of Paclitaxel (taxol) from Nanospheres of Biodegradable Polymers, J. Controlled Release, *71*, pp.53-69. 2001.

Feng, S.S., Mu, L., Chen, B.H., Pack, D. Polymeric Nanospheres Fabricated with Natural Emulsifiers for Clinical Administration of an Anticancer Drug Paclitaxel, Mat.Sci. Eng. C-Bio S., *20 Sp. Iss.*, pp.85-92. 2002.

Fonseca, C., Simões, S., Gaspar, R. Paclitaxel-loaded PLGA Nanoparticles: Preparation, Physicochemical Characterization and In Vitro Anti-tumoral Activity, J. Controlled Release, *83*, pp.273-286. 2002.

Ford, J.L. and Timmins, P. Pharmaceutical Thermal Analysis: Techniques and Applications. pp.88-96, Chichester: E. Horwood; New York: Halsted press. 1989.

Freytag, T., Dashevsky, A., Tillman, L., Hardee G.E., Bodmeier, R. Improvement of the Encapsulation Efficiency of Oligonucleotide-containing Biodegradable Microspheres, J. Controlled Release, *69*, pp.197-207. 2000.

Fu, K., Klibanov, A.M., Langer, R. Protein Stability in Controlled Release System, Nat. Biotechnol., *18*, pp.24-25. 2000.

Gage, S.H. The Microscope, 17th edition. pp.45-66, Ithaca, N.Y.: Comstock Pub. Co. 1947.

Gillespie, A.M. Manual of Spectrofluorometric and Spectrophotometric Derivative Experiments. pp.32-56, Boca Raton: CRC press. 1994.

Gregoriadis, G. Liposome Technology, Volume I. pp.66-89, Boca Raton: CRC press. 1984a.

Gregoriadis, G. Liposome Technology, Volume III. pp.79-109, Boca Raton: CRC press. 1984b.

Guarino, R.A. New Drug Approval Process: the Global Challenge, 3rd edition. pp.82-95, New York: Marcel Dekker. 2000.

Guo, W.J., Gosselin, M.A., Lee, R.J. Characterization of a Novel Diolein-based LPDII Vector for Gene Delivery, J. Controlled Release, *83*, pp.121-132. 2002.

Gupta P.K. and Hung, C.T. Effect of Carrier Dose on the Multiple Tissue Disposition of Doxorubicin Hydrochloride Administered via Magnetic Albumin Microspheres in Rats, J. Pharm. Sci., *78*, pp.745-748. 1989.

Harrington, K.J. Liposomal Cancer Chemotherapy, Current Clinical Applications and Future Prospects, Expert Opin. Inv. Drug., *10*, pp.1045-1061. 2001.

Harper, E., Dang, W., Lapidus, R.G., Garver, R.I. Enhanced Efficacy of a Novel Controlled Release Paclitaxel Formulation (PACLIMER Delivery System) for Local-Regional Therapy of Lung Cancer Tumor Nodules in Mice, Clin. Cancer Res., *5*, pp.4242-4248. 1999.

Hatakeyama, T., Quinn, F.X. Thermal Analysis: Fundamentals and Applications to Polymer Science, 2nd edition. pp.83-99, New York: Wiley. 1999.

Heftmann, E. Chromatography, 2nd edition. pp.121-132, New York: Reinhold Pub. Corp. 1967.

Hemminger, W. Recommendations of the ICTAC Nomenclature Committee. ICTAC news. pp.106-122. December 1998.

Hollas, J.M. Modern Spectroscopy, 3rd edition. pp.38-66, Chichester and New York: John Wiley & Sons. 1996.

Holton, R., Somoza, C., Kim, H.B., Liang, F., Biediger, R.J., Boatman, P.D. First Total Synthesis of Taxol: 1. Functionalisation of B ring, J. Am. Chem. Soc., *116*, pp.1597-1598. 1994.

Honeywell-Nguyen, P.L., Frederik, P.M., Bomans, P.H.H., Junginger, H.E., Bouwstra, J.A. Transdermal Delivery of Pergolide from Surfactant-based Elastic and Rigid Vesicles: Characterization and In Vitro Transport Studies, Pharm. Res., *19*. pp.991-997. 2002.

Horwitz, S.B. Mechanism of Action of Taxol, Trends Pharmacol. Sci., *13*, pp.134-136. 1992.

Huang, X. and Braxel, C.S. On the Importance and Mechanisms of Burst Release in Matrix-controlled Drug Delivery Systems, J. Controlled Release, 73, pp. 121-136. 2001.

Huizing, M.T., Misser, V.H.S., Pieters, R.C., Huinink, W.W.T., Vencor, C.H.N., Vermorken, J.B., Pinedo, H.M., Beijnen, J.H. Taxanes - a New Class of Antitumor Agents, Cancer Invest., *13*, pp.381-404. 1995.

Igartua, M., Hernandez, R.M., Esquisabe, L.A., Gascon, A.R., Calve, M.B., Pedraz, J.L. Influence of Formulation Variables on the In Vitro Release of Albumin from Biodegradable Microparticulate Systems, J. Microencapsul., *14*, pp.349-356. 1997.

Jeong, B., Bae, Y.H., Lee, D.S., Kim, S.W. Biodegradable Block Copolymers as Injectable Drug-delivery Systems, Nature, *388*, pp.360-362. 1997.

Jones, C., Mulloy, B., Thomas, A.H. Microscopy, Optical Spectroscopy, and Macroscopic Techniques. pp.203-208, Totowa, N.J.: Humana press. 1994.

Karpusas, M., Whitty, A., Runkel, L., Hochman, P. Review: the Structure of Human Interferon-β: implications for activity, Cell Mol. Life Sci., *54*, pp.1203-1216. 1998.

Katooka, K., Harada, A., Nagasaki, Y. Block Copolymer Micelles for Drug Delivery: Design, Characterization and Biological Significance, Adv. Drug Deliver. Rev., *47*, pp.113-131. 2001.

Kim, J.H., Emoto, K., Lijima, M., Nagasaki, Y., Aoyagi, T., Okano, T., Sakurai, Y., Kataoka, K. Core-stabilized Polymeric Micelle as Potential Drug Carrier: Increased Solubilization of Taxol, Polym, Adv. Technol., *10*, pp.647-654. 1999.

Kim, S.C., Kim, D.W., Shim, Y.H., Bang, J.S., Oh, H.S., Kim, S.W., Seo, M.H. In Vivo Evaluation of Polymeric Micellar Paclitaxel Formulation: Toxicity and Efficacy, J. Controlled Release, *72*, pp.191-202. 2001.

Klausner, E.A., Lavy, E., Stepensky, D., Friedman, M., Hoffman, A. Novel Gastroretentive Dosage Forms: Evaluation of Gastroretentivity and Its Effect on Riboflavin Absorption in Dogs, Pham. Res., *19*, pp.1516-1523. 2002.

Kreuter, J. (ed). Colloidal Drug Delivery Systems. pp.78-99, New York: Marcel Dekker. 1994.

Krishnamurthy, R., Lumpkin, J.A., Sridhar, R. Inactivation of Lysozyme by Sonication under Conditions Relevant to Microencapsulation, Int. J. Pharm., *205*, pp.23-34. 2000.

Kumar, M.N.V.R. A Review of Chitin and Chitosan Applications, React. Funct. Polym., *46*, pp.1-27. 2000.

La, S.B., Okano, T., Kataoka, K. Preparation and characterization of micelle-forming polymeric drug indomethacin-incorporated poly(ethylene oxide)-poly(β-benzyl L-aspartate) block copolymer micelles, J. Pharm. Sci., 85, pp. 85-90. 1996.

Lakowicz, J.R. Principles of Fluorescence Spectroscopy. pp.98-113, New York: Plenum. 1983.

Lam, X.M., Duenas, E.T., Cleland, J.L. Encapsulation and Stabilization of Nerve Growth Factor into Poly(lactic-co-glycolic) Acid Microspheres, J. Pharm. Sci., *90*, pp.1356-1365. 2001.

Langer, R. Polymeric Delivery Systems for Controlled Drug Release, Chem. Eng. Commun., *6*, pp.1-48. 1980.

Langer, R. Drug Delivery and Targeting, Nature, *392*, pp.5-10. 1998.

Langer, R. Perspectives: Drug Delivery-Drugs on Target, Science, *293*, pp.58-59. 2001.

LaPorte, R.J. Hydrophilic Polymer Coatings for Medical Devices: Structure/properties, Development, Manufacture, and Applications. pp.113-128, Lancaster, PA: Technomic Publishing Company. 1997.

Lasic, D.D. Liposomes: from Physics to Applications. pp.122-134, Amsterdam & New York: Elsevier. 1993.

Lasic, D.D. and Martin, F. Stealth Liposomes. pp.113-130, Boca Raton: CRC press. 1995.

Li, X.H., Deng, X.M., Yuan, M.L., Xiong, C.D., Huang, Z.T., Zhang, Y.H., Jia, W.X. Investigation on Process Parameters Involved in Preparation of Poly-DL-lactide-poly(ethylene glycol) Microspheres Containing Leptospira Interrogans Antigens, Int. J. Pharm., *178*, pp.245-255. 1999.

Li, X.H., Deng, X.M., Yuan, M.L., Xiong, C.D., Huang, Z.T., Zhang, Y.H., Jia, W.X. In Vitro Degradation and Release Profiles of Poly-dl-lactide-poly(ethylene glycol) Microspheres with Entrapped Proteins, J. Appl. Polym. Sci., *78*, pp.140-148. 2000.

Li, Y., Kissel, T. Synthesis, Characterization and In Vitro Degradation of Star-block Copolymers Consisting of L-lactide, Glycolide and Branched Multi-arm Poly(ethylene Oxide), Polymer, *39*, pp.4421-4427. 1998.

Liebmann, J., Cook, J.A., Mitchell, J.B. Cremophor EL, Solvent for Paclitaxel, and Toxicity, Lancet, *342*, pp.1428-1428. 1993.

Liggins, R.T., D'Amours, S., Demetrick, J.S., Machan. L.S., Burt, H.M. Paclitaxel Loaded Poly(l-lactic acid) Microspheres for the Prevention of Intraperitoneal Carcinomatosis after a Surgical Repair and Tumor Cell Spill, Biomaterials, *21*, pp.1959-1969. 2000.

Liggins, R.T., Hunter, W.L., Burt, H.M. Solid-state Characterization of Paclitaxel, J. Pharm. Sci., *86*, pp.1458-1463. 1997.

Lopes, N.M., Adams, E.G., Pitts, T.W., Bhuyan, B.K. Cell Kill Kinetics and Cell Cycle Effects of Taxol on Human and Hamster Ovarian Cell Lines, Cancer Chemother. Pharmacol., *32*, pp.235-242. 1993.

Lundberg, B.B. A Submicron Lipid Emulsion Coated with Amphipathic Polyethylene Glycol for Parenteral Administration of Paclitaxel (Taxol), J. Pharm. Pharmacol., *49*, pp.16-21. 1997.

Magda, A.E., Piatt, D.M., Charpentier, B.A. (ed). Polymeric Delivery Systems: Properties and Applications. pp.104-116, Washington DC: American Chemical Society. 1993.

Mahato, R.J., Rolland, A., Tomlinson, E. Cationic Lipid-based Gene Delivery Systems: Pharmaceutical Perspectives, Pharm. Res., *14*, pp.853-859. 1997.

Mandal, T.K., Bostannian, L.A., Graves, R.A., Chapman, S.R. Poly(d,l-lactide-co-glycolide) Encapsulated Poly(vinyl alcohol) Hydrogel as a Drug Delivery System, Pharm. Res. *19*. pp.1713-1719. 2002.

Mathiowitz, E., Jacob, J.S., Jong, Y.S., Carino, G.P., Chickering, D.E., Chaturvedi, P., Santos, C.A., Vijayaraghavan, K., Montgomery, S., Bassett, M., Morrell, C. Biologically Erodable Microsphere as Potential Oral Drug Delivery System, Nature, *386*, pp.410-414. 1997.

Mayinger, F., Feldmann, O. Optical Measurements: Techniques and Applications, 2nd edition. pp.95-100, New York: Springer. 2001.

Mclean, J.R., Mortimer, A.J. A Cavitation and Free Radical Dosimeter for Ultrasound, Ultrasound Med. Biol., *14*, pp.59-64. 1988.

Meziani, M.J., Rollins, H.W., Allard, L.F., Sun, Y.P. Protein-protected Nanoparticles from Rapid Expansion of Supercritical Solution into Aqueous Solution, J. Phys. Chem. B, *106*, pp.11178-11182. 2002.

Mikulecky, D.C. and Caplan, S.R. The Choice of Reference Frame in the Treatment of Membrane Transport by Nonequilibrium Thermodynamics, J. Phys. Chem., *70*, pp.3049-3056. 1966.

Misik, V., Kirschenbaum, L.J., Riesz, P. Free Radical Production by Sonolysis of Aqueous Mixtures of N,N-Dimethylformamide: an EPR Spin Trapping Study, J. Phys. Chem., *99*, pp.5970-5976. 1995.

MIT (Massachusetts Institute of Technology) News Office. Langer Reviews Polymer Drug-delivery Work in Killian Lecture. MIT technology talk. March 18, 1998.

Morrison, I.D. and Ross, S. Colloidal Dispersions: Suspensions, Emulsions, and Foams. pp.134-138, New York: Wiley-Interscience. 2002.

Mu, L. and Feng, S.S. Fabrication, Characterization and In Vitro Release of Paclitaxel (Taxol) Loaded Poly(lactic-co-glycolic acid) Microspheres Prepared by Spray Drying Technique with Lipid/cholesterol Emulsifiers, J. Controlled Release, *76*, pp.239-254. 2001.

Mu, L. and Feng, S.S. Vitamin E TPGS Used as Emulsifier in the Solvent Evaporation/extraction Technique for Fabrication of Polymeric Nanospheres for Controlled Release of Paclitaxel (Taxol), J. Controlled Release, *80*, pp.129-144. 2002.

Mu, L., Feng, S.S., Kiang, T.W. Vitamin E TPGS Used in the Solvent Extraction/evaporation Technique for Fabrication of Polymeric Nanospheres of an Antineoplastic Drug Paclitaxel (taxol®) for Cancer Chemotherapy. In: Bioengineering Conference, American Society of Mechanical Engineers, 2001.

New, R.R.C. Liposomes: a Practical Approach. pp.122-131, Oxford & New York & Tokyo: IRL press. 1990.

Nicolaou, K.C., Guy, R.K., Pitsinos, E.N., Wrasidlo, W. A Water-soluble Prodrug of Taxol with Self-assembling Properties, Angew. Chem. Int. Edit., *33*, pp.1583-1587. 1994a.

Nicolaou, K.C., Yang, Z., Liu, J.J., Ueno, H., Biediger, R.J., Guy, R.K. Total Synthesis of Taxol, Nature, *367*, pp.630-634. 1994b.

Nihant, N., Schugens, C., Grandfils, C., Jerome, R., Teyssie, P. Polylactide Microparticles Prepared by Double Emulsion/evaporation Technique. I. Effect of Primary Emulsion Stability, Pharm. Res., *11*, pp.1479-1484. 1994.

O'Donnell, P.B., McGinity, J.W. Preparation of Microspheres by the Solvent Evaporation Technique, Adv. Drug Deliver. Rev., *28*, pp.25-42. 1997.

Olbrich, C., Bakowsky, U., Lehr, C.M., Muller, R.H., Kneuer, C. Cationic Solid-lipid Nanoparticles Can Effiencientyl Bind and Transfect Plasmid DNA, J. Controlled Release, *77*, pp.345-355. 2001.

Onyuksel, H., Ramakrishanan, S., Chai, H.B., Pezzuto, J.M. A Mixed Micellar Formulation Suitable for the Parenteral Administration of Taxol, Pharm. Res., *11*, pp.206-212. 1994.

Packer, L. and Fleischer, S. (ed). Biomembranes. pp.57-59, San Diego: Academic press. 1997.

Panchagnula, R. Pharmaceutical Aspects of Paclitaxel, Int. J. Pharm., *172*, pp.1-15. 1998.

Park, K. Controlled Drug Delivery: Challenges and Strategies. pp.132-138, Washington DC: American Chemical Society. 1997.

Pasch, H. and Trathnigg, B. HPLC of Polymers. pp.115-127, Berlin & New York: Springer. 1997.

Pean, J.M., Venier-Julienne, M.C., Filmon, R., Sergent, M., Phan-Tan-Luu, R., Benoit, J.P. Optimization of HSA and NGF Encapsulation Yields in PLGA Microparticles, Int. J. Pharm., *166*, pp.105-115. 1998.

Pekarek, K.J., Jacob, J.S., Mathiowitz, E. Double-walled Polymer Microspheres for Controlled Drug Release, Nature, *367*, pp.258-260. 1994.

Perum, T.J., Propst, C.L. Computer-aided Drug Design: Methods and Applications. pp.68-77, New York: Marcel Dekker. 1989.

Poole, C.F. and Poole, S.K. Chromatography Today. pp.1-3, Amsterdam & New York: Elsevier. 1991.

Rajeev, A.J. The Manufacturing Techniques of Various Drug Loaded Biodegradable Poly(lactide-co-glycolide) (PLGA) Devices, Biomaterials, *21*, pp.2475-2490. 2000.

Reich, G. Ultrasound-induced Degradation of PLA and PLGA during Microsphere Processing: Influence of Formulation Variables, Eur. J. Pharm. Biopharm., *45*, pp.165-171. 1998.

Richheimer, S.L., Tinnermeier, D.M., Timmons, D.W. High Performance Liquid Chromatographic Assay of Taxol, Anal. Chem., *64*, pp.2323-2326. 1992.

Rochow, T.G. and Tucker, P.A. Introduction to Microscopy by Means of Light, Electrons, X-rays, or Acoustics, 2nd edition. pp.265-327, New York and London: Plenum press. 1994.

Rosa, G.D., Iommelli, R., Rotonda, M.I.L., Miro, A., Quaglia, F. Influence of the Co-encapsulation of Different Non-ionic Surfactants on the Properties of PLGA Insulin-loaded Microspheres, J. Controlled Release, *69*, pp.283-295. 2000.

Ruan, G., Feng, S.S., Li, Q.T. Effects of Material Hydrophobicity on Physical Properties of Polymeric Microspheres Formed by Double Emulsion Process, J. Controlled Release, *84*, pp.151-160. 2002.

Sah, H.K., Smith, M.S., Chern, R.T. A novel Method of Preparing PLGA Microcapsules Utilizing Methylethyl Ketone, Pharm. Res., *13*, pp.360-367. 1996.

Sah, H.K., Toddywala, R., Chien, Y.W. The Influence of Biodegradable Microcapsule Formulations on the Controlled Release of a Protein, J. Controlled Release, *30*, pp.201-211. 1994.

Sah, H.K., Toddywala R., Chien Y.W. Biodegradable Microcapsules Prepared by a W/o/w Technique: Effects of Shear Force to Make a Primary W/o Emulsion on Their Morphology and Protein Release, J. Microencapsul., *12*, pp.59-69. 1995.

Saltzman, W.M. Drug Delivery: Engineering Principles for Drug Therapy. pp.97-105, New York: Oxford University press. 2001.

Sanders, L.M. and Hendren, R.W. Protein Delivery: Physical Systems. pp.128-134, New York: Plenum Press. 1997.

Schakenraad, J.M. Enzymatic Activity toward Poly(lactic acid) Implants, J. Biomed. Mater. Res., *24*, pp.529-545. 1990.

Sernelius, B.E. Surface Models in Physics. pp.109-115, Berlin: Wiley-VCH. 2001.

Shakesheff, K.M., Evora, C., Soriano, I., Langer, R. The Adsorption of Poly(vinyl alcohol) to Biodegradable Microparticles Studied by X-ray Photoelectron Spectroscopy (XPS), J. Controlled Release, *185*, pp.538-547. 1997.

Sharma, A., Straubinger, R.M. Novel Taxol Formulations: Preparation and Characterization of Taxol-containing Liposomes, Pharm. Res., *11*, pp.889-896. 1994.

Sharma, U., Balasubramanian, S.V., Straubinger, R.M. Pharmaceutical and Physical Properties of Paclitaxel (Taxol) Complexes with Cyclodextrins, J. Pharm. Sci., *84*, pp.1223-1230. 1995.

Sheppard, C. and Shotton, D. Confocal Laser Scanning Microscopy. pp.45-57, New York: Springer. 1998.

Smallwood, I.M. Handbook of Organic Solvent Properties. pp.38-45, London: Arnold. 1996.

Speyer, R.F. Thermal Analysis of Materials. pp.58-67, New York: Marcel Dekker. 1994.

Stallforthe, H. and Revell, P. (ed). Materials for Medical Engineering. pp.98-104, Weinheim: Wiley-VCH. 2000.

Stefanis, A.D. and Tomlinson, A.A.G. Scanning Proble Microscopes: from Surface Structure to Nano-scale Engineering. pp.145-156, Switzerland: Trans Tech Publications. 2001.

Stolnik, S., Illum, L., Davis, S.S. Long Circulating Microparticulate Drug Carriers, Adv. Drug Deliv. Rev., *16*, pp.195-214. 1995.

Sturesson, C. and Carlfors, J. Incorporation of Protein in PLG-microspheres with Retention of Bioactivity, J. Controlled Release, *67*, pp.171-178. 2000.

Sun, S.F. Physical Chemistry of Macromolecules: Basic Principles and Issues. pp.58-67, New York: John Wiley. 1994.

Swadesh, J. HPLC: Practical and Industrial Applications. pp.133-134, Boca Raton: CRC press. 1997.

Takeuchi, H., Yamamoto, H., Kawashima, Y. Mucoadhesive Nanoparticulate Systems for Peptide Drug Delivery, Adv. Drug Deliver. Rev., *47*, pp.39-54. 2001.

Takeuchi, H., Yamamoto, H., Niwa, T., Himo, T., Kawashima, Y. Enteral Absorption of Insulin in Rats from Mucoadhesive Chitosan-coated Liposomes, Pharm. Res., *13*, pp.896-901. 1996.

Tobio, M., Gref, R., Sanchez, A., Langer, R., Alonso, M.J. Stealth PLA-PEG Nanoparticles as Protein Carriers for Nasal Administration, Pharm. Res., *15*, pp.270-275. 1998.

Van Brunt, J. Perfecting Polymers to Release Drugs, Biotechnology, *4*, pp.756. 1986.

Vermette, P., Meagher, L., Gagnon, E., Griesser, H.J., Doillon, C.J. Immobilized Liposomes Layers for Drug Delivery Applications: Inhibition of Angiogenesis, J. Controlled Release, *80*, pp.179-195. 2002.

Vrancken, M.N. and Clays, D.A. US Patent 3526906, 1970.

Wang, F.J. and Wang, C.H. Sustained Release of Etanidazole from Spray Dried Microspheres Prepared by Non-halogenated Solvents, J. Controlled Release, *81*, pp.263-280. 2002.

Wang, Y.M., Sato, H., Adachi, I., Horikoshi, I. Preparation and Characterization of Poly(lactic-co-glycolic acid) Microspheres for Targeted Delivery of a Novel Anticancer Agent, Taxol, Chem. Pharm. Bull., *44* pp.1935-1940. 1996.

Wang, Y.M., Sato, H., Horikoshi, I. In Vitro and In Vivo Evaluation of Taxol Release from Poly(lactic-co-glycolic acid) Microspheres Containing Isopropyl Myristate and Degradation of the Microspheres, J. Controlled Release, *49*, pp.157-166. 1997.

Whiffen, D.H. Spectroscopy, 2nd edition. pp.67-76, London: Longman. 1972.

Willmott, N. and Daly, J. Microspheres and Regional Cancer Therapy. pp.77-87, Boca Raton: CRS press. 1994.

Wise, D.L. (ed). Encyclopedic Handbook of Biomaterials and Bioengineering. pp.95-106, New York: Marcel Dekker. 1995.

Yang, Y.Y., Chung, T.S., Ng, N.P. Morphology, Drug Distribution, and In Vitro Release Profiles of Biodegradable Polymeric Microspheres Containing Protein Fabricated by Double-emulsion Solvent Extraction/evaporation Method, Biomaterials, *22*, pp.231-241. 2001.

Yu, L., Bridgers, A., Polli, J., Vickers, A., Long, S., Roy, A., Winnike, R., Coffin, M. Vitamin E TPGS Increase Absorption Flux of an HIV Protease Inhibitor by Enhancing Its Solubility and Permeability, Pharm. Res., *16*, pp. 1812-1817. 1999.

LIST OF PUBLICATIONS

Journal Papers:

Ruan, G., Feng, S.S., Li, Q.T. Effects of Material Hydrophobicity on Physical Properties of Polymeric Microspheres Formed by Double Emulsion Process, J. Controlled Release, *84*, pp.151-160. 2002.

Ruan, G., Feng, S.S. Preparation and Characterizations of Poly(lactic acid)-Poly(ethylene glycol)-Poly(lactic acid) (PLA-PEG-PLA) Microspheres for Controlled Release of Paclitaxel, Biomaterials, 27, pp. 5037-5044. 2003.

Feng, S.S., **Ruan, G.**, Li, Q.T. Fabrication and Characterizations of a novel delivery device liposomes-in-microsphere (LIM), Biomaterials (in press).

Ruan, G., Feng, S.S., Ng, J.K. A Modification of Double Emulsion Process for Preparation of Polymeric Microspheres, J. Microencapsulation (accepted).

Conference Papers:

Ruan, G., Feng, S.S. Structural Properties and In Vitro Protein Release of PLGA and PELA Microspheres. In: Controlled Release Society Annual Meeting, 2002, Seoul, Korea.

Ruan, G., Feng, S.S. Development of a Novel Drug Delivery System Based on Liposome-encapsulated Microspheres (LEM). In: International Conference on Materials for Advanced Technologies, 2001, Singapore.

Feng, S.S., Mu, L., Huang, G.F., Zhou, W.Y., **Ruan, G.** Nanospheres of Biodegradable Materials Applied for Clinical Administration of Anticancer Drugs. In: International Conference on Materials for Advanced Technologies, 2001, Singapore.