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Nano-formulation and controlled delivery of low solubility anticancer drugs

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NANO-FORMULATION AND CONTROLLED

DELIVERY OF LOW SOLUBILITY

ANTICANCER DRUGS

By

Anshul Agarwal, M.D., M.S.

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

COLLEGE OF ENGINEERING AND SCIENCE
LOUISIANA TECH UNIVERSITY

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ABSTRACT

Optimal drug delivery and reduction of systemic adverse effects have been age old problems in chemotherapeutics in all types of human cancer. During chemotherapy, using water insoluble drugs like paclitaxel and tamoxifen, it has been realized that better formulations are needed for more specific and controlled drug delivery of these agents. In a novel approach to form high content stable nanocolloids of these drugs with controllable release rate, a sonicated layer-by-layer (LbL) polyelectrolyte coating technology is suggested. The desired features of pharmaceutical carriers for intravenous administration include their small size and biodegradability, good loading capacity for a given drug, high content of a drug in a final preparation, prolonged circulation in the blood, and ability to gradually concentrate in required areas (targeting) via passive accumulation. While these requirements are reasonably well met by a variety of drug carriers (liposomes, microcapsules, nanoparticles) developed for water-soluble drugs, the development of nanoparticulate drug carriers displaying all of these properties for the delivery of poorly soluble pharmaceuticals still represents a challenge.

Intravenous administration of these intrinsically hydrophobic agents is frequently associated with serious problems. One of these problems is that the diameter of blood capillaries is only a few micrometers or less, and intravenous administration of aggregates of undissolved material that form in an aqueous media would cause embolization before reaching and penetrating a target, such as tumor.

Additionally, the low solubility of hydrophobic drugs in combination with excretion and metabolic degradation often does not allow for achieving therapeutically significant systemic concentrations. As a result, many promising drug candidates never enter further development because of solubility problems.

Currently, the most popular approach to dissolve poorly soluble drugs and prepare their dosage forms with sufficiently high bioavailability is the use of micellar drug carriers, specifically polymeric micelles.

In this study LbL coating technology is proposed to make stable aqueous colloids of poorly soluble drugs with high stability, controllable release (faster or slower than bulk drug powders), and a very high content of the active drug. To achieve this, aqueous suspensions of poorly soluble drugs with micron range particles are subjected to a powerful ultrasonic treatment in order to decrease the size of individual drug particles to the nano level (between 100 and 200 nm), and while still keeping the obtained nanoparticles under sonication to prevent their fast agglomeration, they are stabilized in solution by applying the LbL technology (alternating addition of polycations and polyanions to the system) and assembling a thin polyelectrolyte coating on their surface. In the process of assembly, the highly charged polymeric layers are always present on the drug particle surface thus preventing particle aggregation after stopping the sonication. At the end of the process, stable nanocolloidal dispersions are formed. After the first polycation layer is deposited on the surface of a drug nanoparticle, it is stabilized by the addition of following oppositely charged polyanion. They form a stable electrostatic complex resulting in the appearance of a very thin but stable polymeric shell around each drug nanoparticle. This polyelectrolyte multilayer shell prevents particle aggregation, and
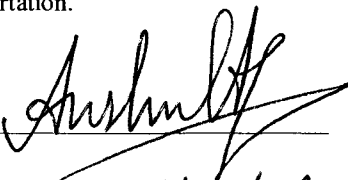
can be easily and reproducibly formed on the surface of any drug particle. By varying the charge density on each polymer or the number of coating cycles, particles with a different surface charge and different composition of the polymeric coating can be prepared. This provides a way to control drug release from such particles by designing the shell architecture at the nanometer level. The use of a polymer containing reactive groups (such as amino or carboxy-groups) for the last "outer" surface coating allow for the attachment of specific ligands, or reporter groups, or other moieties of interest to the nanoparticle surface.

By nanoencapsulation of such anticancer drugs as tamoxifen, paclitaxel, and camptothecin, we demonstrate the general applicability of this approach. The final content of the drug in the preparation as well as its release rate from the preparation can be controlled by the multilayer composition of the shell. The process key point is the deposition of the first polycation layer during the powerful sonication of the drug dispersion. In this process, dispersed by the ultrasound fine drug nanoparticles are immediately coated with a polyelectrolyte monolayer providing a high surface charge, which prevents the aggregation. Such drug nanocolloids remain stable in aqueous solutions after switching off the sonication.

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Date _____ 5/14/09

DEDICATION

To

My mother Veena Agarwal,
father Mr. Vishambhar Nath Agarwal,
wife Dr. Anagha Agarwal and
sister Shikha Agarwal.

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CHAPTER 1

INTRODUCTION

The objective of this research is to provide a universal and efficient platform for making stable colloids of various poorly water-soluble drugs with a high concentration of a drug in colloidal particles. Nanosized colloidal suspensions of poorly soluble drugs will dramatically increase drug solubility and bioavailability. Colloidal particles can also be easily made organ-specific (targeted) by modifying their surfaces with various specific ligands, such as monoclonal antibodies [1].

Layer by layer technology can be applied to poorly soluble drug nanoparticles to improve the solubilization and bioavailability by breaking them into stable colloids that will have a high content of the active ingredient. Nanoparticles will be generated by ultrasonication of the drug suspension. A polymeric coating, consisting of alternating layers of differently charged soluble polymers, will prevent coated drug particles from agglomeration. Drug content and release properties are to be controlled by the polymer type and number of polymeric layers.

Need for an Ideal Therapeutic Agent

Many potent drugs and drug candidates, especially anticancer drugs, are poorly soluble in water (e.g. tamoxifen, paclitaxel, camptothecin), which results in their low bioavailability and difficulties in preparing dosage forms [2]. Optimal drug delivery and

reduction of systemic adverse effects have been age old problems in chemotherapeutics of human cancer. With the advent of the use of water insoluble cancer drugs like paclitaxel and tamoxifen, it has been realized that better formulations are still needed for more specific and controlled delivery of these agents. The desired features of pharmaceutical carriers for parenteral (intravenous) administration include their small size, biodegradability, high content drug in final preparation, prolonged circulation in the blood, and, ideally, targeting required areas of interest passively (via enhanced permeability and retention (EPR) effect) or actively (via specific ligands, such as monoclonal antibodies) [1]. While these requirements are reasonably well met by a variety of drug carriers (liposomes, microcapsules, nanoparticles) developed for water-soluble drugs, the development of nanoparticulate drug carriers displaying all of these properties for the delivery of poorly soluble pharmaceuticals still represents a challenge [3, 4].

Mentioned below are some examples of insoluble chemotherapeutic agents:

1) Paclitaxel, a compound extracted from the bark of the Pacific yew tree, Taxus brevifolia is a drug used in the treatment of lung, ovarian, and breast cancers and advanced forms of Kaposi's sarcoma and acts by interfering with the normal function of microtubule growth [5].

2) Tamoxifen is an oral selective estrogen receptor modulator (SERM) which is used in breast cancer treatment, and is currently the world's largest selling breast cancer treatment. Tamoxifen was invented by ICI Pharmaceuticals [5].

3) Camptothecin, a plant alkaloid from Camptotheca acuminate, demonstrated strong antitumor activity against lung, ovarian, breast, pancreas, and stomach cancers by

targeting intracellular topoisomerase I, a nuclear enzyme that reduces the torsional stress of supercoiled DNA [6].

4) *Meso*-Tetraphenylporphine are porphyrins that are well known photosensitizing agents, which induce photodamage to malignant tumors by producing singlet O_2 via the energy transfer from the first triplet state of porphyrin to the ground triplet state of molecular oxygen [7].

Low solubility in water, however, tends to be an intrinsic property of many drugs, including the powerful anticancer agents mentioned above [2]. This is quite expected, since the membrane permeability and efficacy of various drugs increase with increasing hydrophobicity [2, 8, 9]. At the same time, intravenous administration of those intrinsically hydrophobic agents can be associated with serious problems. One of them is that the intravenous administration of relatively large aggregates/crystals of an insoluble drug that is formed in an aqueous media may embolize blood capillaries and cause unwanted effects, like tissue ischemia. Additionally, the low solubility of hydrophobic drugs in combination with excretion and metabolic degradation often does not allow for achieving therapeutically significant systemic concentrations. As a result, many promising drug candidates never enter further development and do not result in acceptable dosage forms because of solubility problems [3, 4].

Current Modalities of Developing an Ideal Therapeutic Agent

Various attempts to solve the problem of solubilization of insoluble drugs are associated with loading poorly soluble drugs (usually, hydrophobic molecules) into various nanosized pharmaceutical carriers, such as liposomes (drugs are loaded into the hydrophobic membrane of the liposome), micelles (drugs are loaded into the hydrophobic

core of the micelle), and oil-in-water emulsions [10, 11]. Although, many experimental data are available showing a dramatic increase in drug efficacy after loading into nanocarriers (forming stable colloidal systems), the general problems associated with these approaches include: relatively low loading efficacy of the drug into the nanocarrier (between 0.5 and 25% by weight, usually below 10% wt); the impossibility of using the same protocol for making solubilized forms of different drugs, since each drug requires its own specific conditions for solubilization; difficulties with the scaling up the technology; difficulties with controlling surface properties or surface composition of such nanosystems; insufficient storage stability of such systems and their instability in the body [11].

Currently, the most popular approach to solubilize poorly soluble drugs and prepare their dosage forms with sufficiently high bioavailability is the use of micellar drug carriers that is various polymeric micelles [12-16]. Micelles are colloidal particles, usually spherical, with size in the nanometer range into which many amphiphilic molecules self-assemble spontaneously. In an aqueous environment, hydrophobic fragments of amphiphilic molecules form the core of a micelle, which is segregated from the environment by hydrophilic parts of the molecules forming the micelle corona. The hydrophobic core of micelles may be used as a cargo space for encapsulation of a variety of sparingly soluble therapeutic and diagnostic agents. Such encapsulation substantially increases the bioavailability of pharmaceuticals, protects them from destructive factors upon parenteral administration, and beneficially modifies their pharmacokinetics and biodistribution including the target accumulation via the EPR effect [17, 18].

However, there exists a set of serious problems associated with micellar carriers, which include low loading efficacy of the drug into the micelles (usually well below 5% wt); the impossibility to use the same protocol for making solubilized forms of different drugs, since each drug requires its own specific conditions for solubilization; problems with controlling the release rate of the drug from micelles; difficulties with the scaling up the technology; insufficient storage stability of micelles and their frequent instability in the body [11].

Our Approach Towards Developing an Ideal Chemotherapeutic Agent

There exists an interesting approach to assemble polyelectrolyte multilayer shells having wall thickness of few nanometers on various particles, including very small ones, through a LbL assembly [19-21]. A LbL assembly is a nanoassembly technique based on alternate adsorption of oppositely charged polyelectrolytes, nanoparticles and proteins [22-24]. These polycation/polyanion multilayers may have required composition with a component location precision of one nanometer. An application of LbL assembly to tiny solid cores allows for the formation of microcapsules. Among many other applications, the formation of LbL polyelectrolyte capsules was used to prepare sustained release formulation of some water-soluble drugs, such as ibuprofen, furosemide, nifedipine, and insulin [25, 26]. These resulted in drug carrying microparticles, where coating with 4-5 polycation/polyanion bilayers allowed for reaching 3-4 hrs release of the drugs normally completely dissolving in 1-2 minutes.

During the last several years, it was clearly demonstrated, that the passive accumulation of drug carriers in the areas with leaky vasculature, such as tumors, depends on the "physiological" properties of each particular cancer, tumor vasculature

cut-off size being the most important parameter controlling penetration of drugs and drug carriers through the endothelium and into the interstitium. In particular, it was shown that different tumors possess different vascular permeability and, in certain cases, this permeability can be rather low (small cut-off size of 200 nm or less), which prevents many drug carriers from accumulation in tumors, such as Lewis lung carcinoma [18]. One should expect that LbL nanocolloids of poorly soluble drugs, being sufficiently small in size, can also provide an efficient way of drug delivery in such areas. Here, we suggest a novel application for the LbL coating technology to make stable aqueous colloids of poorly soluble drugs with high stability, controllable release rate, and a very high content of the active drug.

Layer by Layer Assembly

The assembly of organized macromolecular and nanoparticle has become increasingly important. The methods used for the assembly of ultrathin films with various degrees of molecular order and stability include: spin coating and solution casting, thermal deposition, polyion LbL assembly, chemical self-assembly, Langmuir-Blodgett technique and free-standing films (in this paper we will not discuss the methods of inorganic film growth). The optimal combination of molecular order and stability of films determines the practical usefulness of these technologies [27].

The most ordered macromolecular films are free-standing liquid crystalline films, but they are very unstable. Langmuir-Blodgett (LB) method allows one to construct amphiphile multilayers with a thickness ranging from 5 to 500 nm, however it does not have any industrial applications, because only small, flat substrates can be covered by an LB-film, and it produces intrinsic defects at the lipid grain borders [28-30]. Another

method that can be applied to surface modification (especially in biomaterials) is monolayer self-assembly, based on thiol or silane compounds. By this method, one can achieve self-assembly of 2-5 nm thick monolayers on silicon or gold surfaces, but there is no simple means for constructing thicker films using this approach [31-33]. Other widely used methods for the industrial manufacture of thin films are spin coating and thermal deposition of macromolecules onto a substrate. Unfortunately, unlike the methods considered above, these methods do not allow one to control the molecular order in the films.

Finally, there is yet a newer method for film self-assembly that makes use of the alternate adsorption of oppositely charged macromolecules (polymers, nanoparticles and proteins) [21, 34-37]. The assembly of alternating layers of oppositely charged linear or branched polyions and nanoparticles is simple and provides the means to form 5 –500 nm thick films with monolayers of various substances growing in a pre-set sequence on any substrate at a growth step of about 1 nm. These films have a lower molecular order than LB or free-standing films but they have the advantage of high strength and easy preparation. T. Mallouk [30] has called this technique "molecular beaker epitaxy," meaning that, with simple instruments and thus exploiting the materials self-assembly tendency, one can produce molecularly organized films similar to the ones obtained for metals and semiconductors using highly sophisticated and expensive molecular beam epitaxy technology.

General Procedure

A cleaned substrate of any shape and dimension is immersed into a dilute solution of a cationic polyelectrolyte, for a time optimized for the adsorption of a single

monolayer (ca 1 nm thick), and then it is rinsed and dried. The next step is the immersion of the polycation-covered substrate into a dilute dispersion of polyanions or negatively charged nanoparticles (or any other nanosize charged species) also for a time optimized for the adsorption of a monolayer, then rinsed and dried. These operations complete the self-assembly of a polyelectrolyte monolayer and monoparticulate layer sandwich unit onto the substrate (Figure 1.1). Subsequent sandwich units are self-assembled analogously. Linear polycation / polyanion multilayers can be assembled by similar means. Different nanoparticles, enzymes and polyions may be assembled in a pre-planned order in a single film [22].
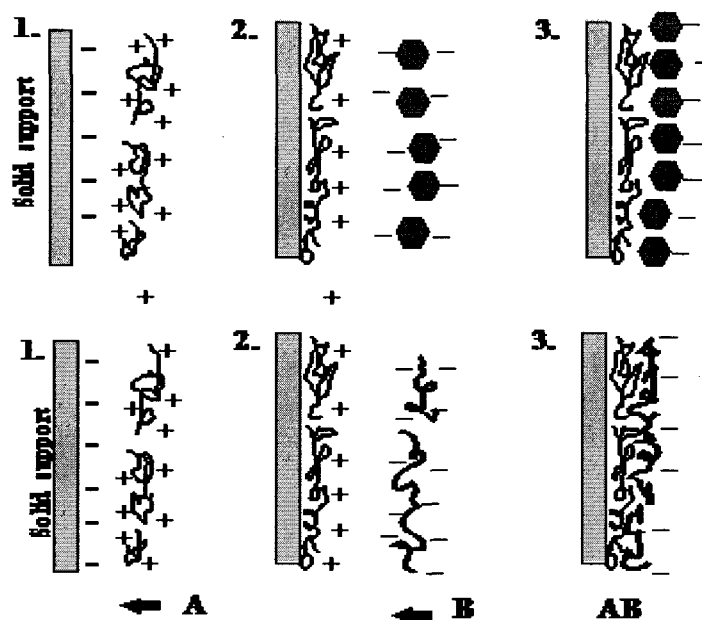
Figure 1.1 Schematic showing the concept of electrostatic LbL assembly.

Standard Assembly Procedure

As a standard approach to film preparation we employ the following steps: 1) Take aqueous solutions of polyion, nanoparticles or protein at a concentration of 0.1 - 1

mg/mL and adjust the pH in such a way that the components are oppositely charged 2) Take a substrate carrying a surface charge (e.g., plates or polymer films covered by a layer of cationic poly(ethylenimine) which may be readily attached to many surfaces) 3) Carry out alternate immersion of the substrate in the component's solutions for 10 minute with 1 minute intermediate water rinsing. To rinse a sample use a solution with pH that keeps the polyions ionized 4) Dry the sample using a stream of nitrogen (note: drying may hinder the assembly process, and it is not necessary for the procedure) [22].

Polyions predominately used in the assembly (Figure 1.2) are as follows: polycations - poly(ethylenimine) (PEI), poly(dimethyldiallylammonium chloride) (PDDA), poly(allylamine) (PAH), polylysine, chitosan; polyanions - poly(styrenesulfonate) (PSS), poly(vinylsulfate), poly(acrylic acid), dextran sulfate, sodium alginate, heparin, DNA . One can grow polymer nanocomposite films by means of the sequential adsorption of different material monolayers that employ hundreds of commercially available polyions. The only requirement is that there be a proper (positive / negative) alternation of the component charges [23].

We suggest using the LBL coating technology to make stable colloids of poorly soluble drugs. For this purpose, the aqueous suspensions of poorly soluble drugs with particle size of the order of microns are subjected to physical treatment, such as an ultrasonic treatment, to decrease the size of individual particles to the nanolevel (between 50 and 1000 nm, preferably, between 100 and 200 nm), and then are stabilized in solution by the formation of the thin polymeric layer on their surface. This polymeric layer prevents particle agglomeration after stopping the physical treatment, which results in the formation of stable colloidal dispersions with high drug content in each colloidal particle

(more than 50% wt and up to 90% wt). The polymeric coating is formed based on the polyelectrolyte complexing process, when, for example drug nanosuspensions formed by ultrasonications are incubated in the presence of a watersoluble biocompatible polymer (polycation or polyanion) to allow for its deposition on their surfaces. After that, the first polymeric layer is stabilized by the addition of another, oppositely charged polyelectrolyte, which forms a firm electrostatic complex with the first one resulting in the appearance of a very thin but extremely stable polymeric shell around each drug nanoparticle. This shell prevents particle agglomeration, and can be easily and reproducibly formed on the surface of any drug particle. By varying the charge density on each polymer, or the number of coating cycles, drug particles can be prepared with a different surface charge and different thickness of the polymeric coat. This, in turn, provides an easy way to control drug release from such particles. In addition, used polymers can also incorporate reactive groups allowing for further attachment of various specific ligands or reporter groups or other moieties to their surface. These moieties can also be incorporated into the polymeric shell during its formation of the drug nanoparticle.

Polycations (MW 50 000-70 000)

NH₃⁺ Cl⁻

PAH

PEI (protonated)

H₃C   CH₃   Cl⁻
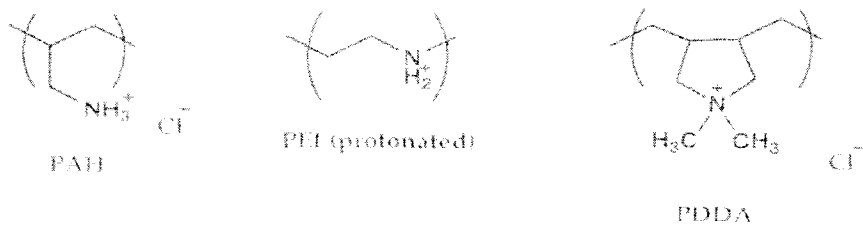
PDDA

Polyanions (MW 50 000-70 000

SO₃⁻ K⁺

PVS

SO₃⁻ Na⁺

PSS
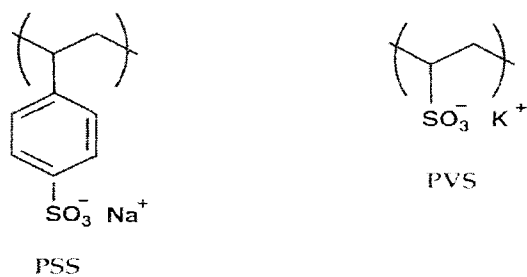
PDDA   **PSS**   **PAH**
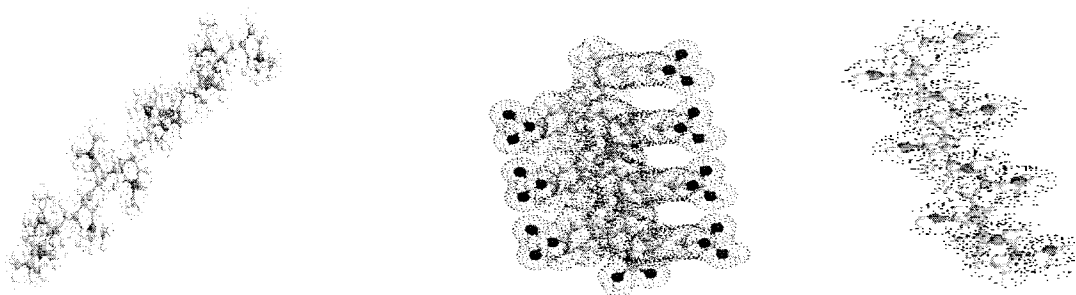
Figure 1.2 Polyion's structural formulas.

CHAPTER 2

INSTRUMENTS AND METHODS

Ultra Sonicator

A MISONOX S 3000 power sonicator was used for all the nanoparticle suspension formation experiments prior to LbL. The drug was subjected to very high sonication energy, up to 18 W via the sonicator probe, as shown in Figure 2.1. For all sterile sample preparations for animal studies, the sonicator tip could be detached and autoclaved.
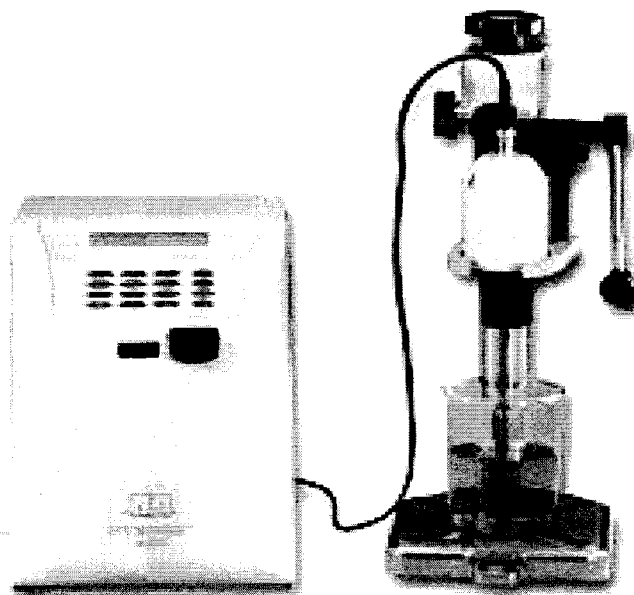
Figure 2.1 MISONOX S 3000 power sonicator.

Ultracentrifugation Machine

An Eppendorf Centrifuge 5804 R machine, Figure 2.2, was used to carry out all the Layer by Layer assembly nanoparticle separation and polyelectrolyte washing steps. This machine was able to provide centrifugation speeds upto 15000 rpm and the enviournment temperature could be controlled down to zero degrees centigrade, although for our experiments usually speeds up to 11000 rpm for 7-10 minutes were sufficient to separate nanoparticles from the colloidal suspension.

Figure 2.2 Eppendorf centrifuge 5804 R machine.

Zeta Potential Meter

A ZetaPlus Brookhaven microelectrophorectic instrument, Figure 2.3, was used to measure the surface charge on nanoparticles and the hydrodynamic diameter. Either buffer or water was used as a medium for the colloidal suspension of drug nanoparticles.

Figure 2.3 ZetaPlus Brookhaven microelectrophorectic instrument.

Confocal Microscopy

Lieca TCS SP2 laser scanning confocal microscope, Figure 2.4 was used to visualize the morphology of colloidal drug particles and for the proof that the polyelectrolyte layer is being assembled over the drug particles by direct visualization of the FITC labeled PAH. Although only particle sizes >= 1 micron in size could be viasualized in detailed morphology, nanoparticles upto 100 nano meters were also visualized as tiny dots without much detailed morphology. These results were needed before proceeding with further experiments and Electron Microscopy Imaging.
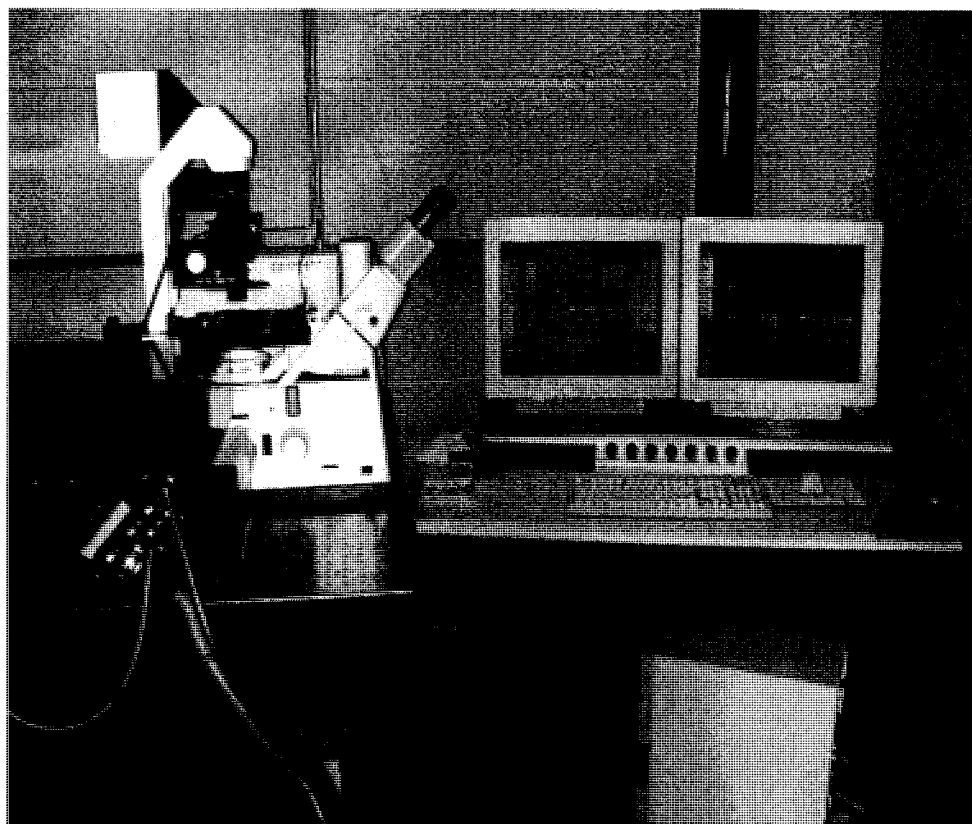
Figure 2.4 Lieca laser scanning microscope.

Scanning Electron Microscopy

A Hitachi S 4800 Scanning Electron Microscope, Figure 2.5, was used for the most detailed morphology and characterization of the formed drug nanoparticles. Drug particles as small as 100 nanometers or less were very conveniently visualized and measured with this microscope. Bare silicon substrates were used to make diluted samples of the drug nanoparticles into templates for visualization. The samples were not heat dried but allowed to dry overnight at room temperature.
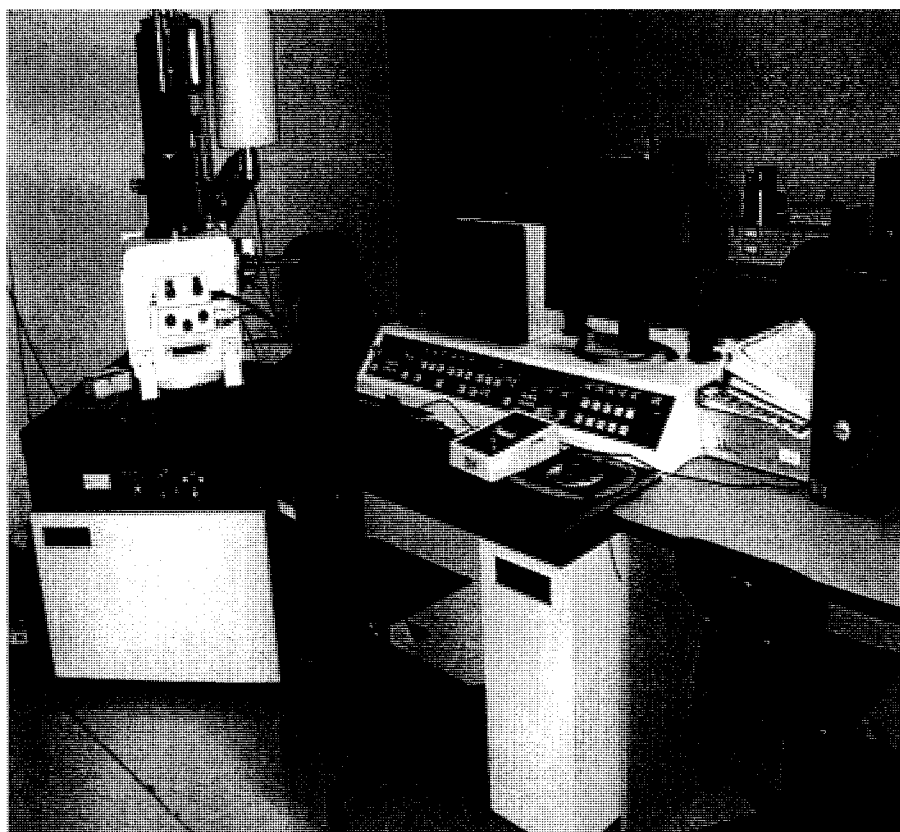
Figure 2.5 Hitachi S 4800 Scanning Electron Microscope.

Ultra Violet Spectrophotometry

An Aligent 8453 UV- Vis Spectrophotometer, Figure 2.6, was used to calculate

standard curves for all the drug release pharmacodynamic diffusion experiments through

a 200 nanometer nitrocellulose membrane in a horizontal diffusion chamber. The peak

was observed at 240 nm for both Paclitaxel and Tamoxifen.

Figure 2.6 Aligent 8453 UV- Vis Spectrophotometer.

CHAPTER 3

STABLE NANOCOLLOIDAL DRUG FORMULATION
USING SYNTHETIC POLYELECTROLYTES

Stable nanocolloids of insoluble drugs with very high drug content (up to 90% wt) can be easily and reproducibly prepared through the application of the LbL technology, the alternate adsorption of oppositely charged polyelectrolytes on the surface of drug nanoparticles produced by ultrasonication of larger drug crystals. Such polymeric coating prevents drug nanoparticle aggregation and creates a firm polymeric shell on their surfaces [21]. Drug release rate from such nanocolloidal particles can be easily controlled by assembling multilayer shells with variable shell density and thickness. Various additional functions, such as specific targeting ligands, can be easily attached to the surface on nanocolloidal particles of poorly soluble drugs by using a polymer with free reactive groups for the "outer" coating. This may represent a novel approach to preparing convenient dosage forms of poorly soluble drugs [38].

Here, we demonstrate a novel application for the LbL coating technology to make stable aqueous nanocolloids of poorly soluble drugs with a very high content of the active drug and a controllable drug release rate [39]. To achieve this, aqueous suspensions of these drugs with micron range particles are subjected to the ultrasonic treatment in order to decrease the size of individual drug particles to the nano level (between 100 and 200 nm) and, while still keeping the nanoparticles formed under the sonication to

prevent their fast agglomeration, stabilize them in solution by applying the LbL coating

method (alternating addition of polycations and polyanions to the system) and assembling

a thin polyelectrolyte shells on their surfaces (Figure 3.1). In the process of the assembly,

the highly charged polymeric layer is formed on the drug particle surface already after the

first polymer application, and this layer prevents drug particle aggregation after

terminating the sonication. At the end of the process, stable coated nanocolloidal drug

dispersions are formed with high drug content in each particle (more than 50% wt and up

to 90% wt).
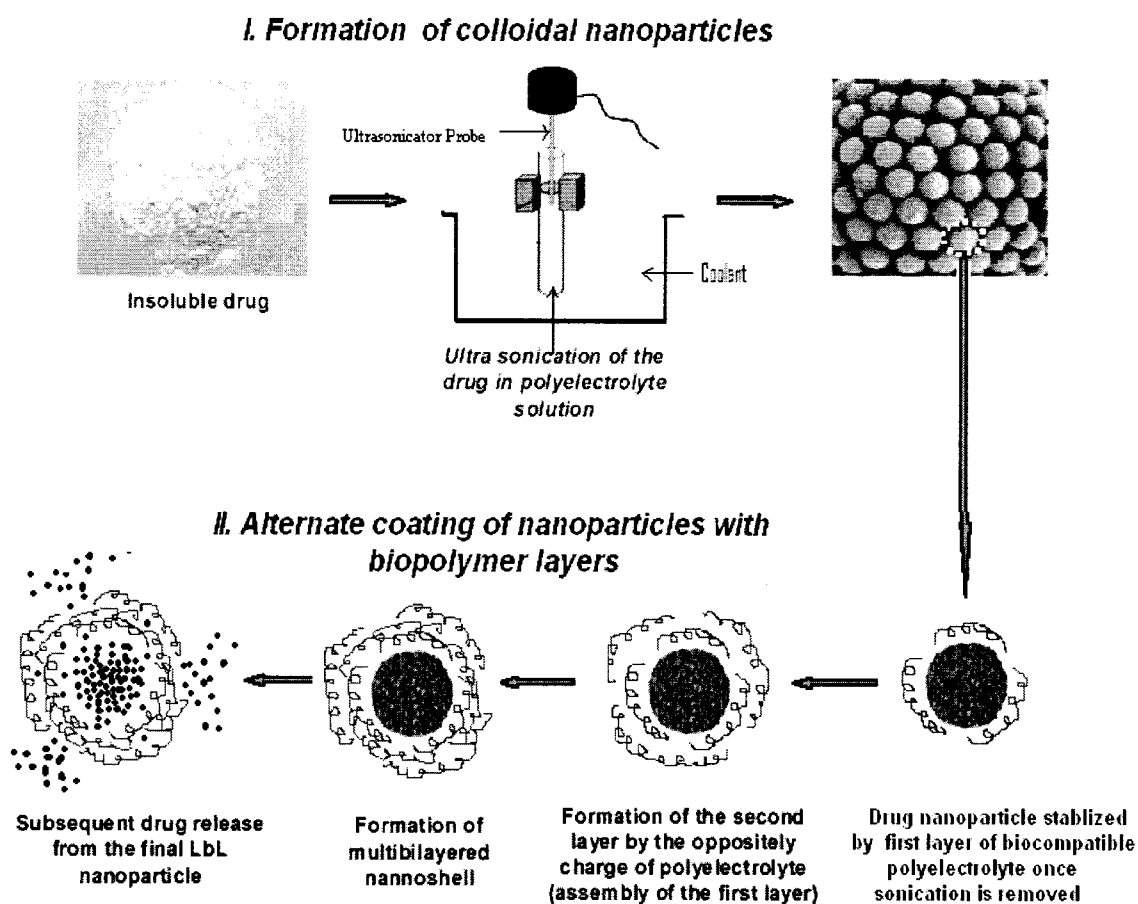
Figure 3.1 Principal scheme of nanocolloidal particles formation using LbL from low solubility drugs.

LbL assembly can utilize a broad variety of polyiones, including many biocompatible ones. Polycations can be represented by poly(ethyleneimine) (PEI), poly(allylamine) (PAA), polylysine, chitosan, gelatin B, amino-dextran, protamine sulfate; polyanions – by poly(styrene sulfonate) (PSS), poly(acrylic acid), dextran sulfate, carboxymethyl cellulose, sodium alginate, hyaluronic acid, gelatin A, chondroitin and heparin. After, for example, the first polycation layer is deposited on the surface of the drug nanoparticle; it is followed by the addition of an oppositely charged polyanion and results in formation of a stable interpolyelectrolyte complex shell (3-30 nm) around each drug nanoparticle. This polyelectrolyte multilayer shell can be easily and reproducibly formed on the surface of any drug particle. By varying the charge density on each polymer or the number of coating cycles, particles with a different surface charge and different composition of the polymeric coat can be prepared. This provides a way to control drug release from such particles by designing the shell architecture at the nanometer level. The use of a polymer containing reactive group (such as an amino or carboxylic group) for the last "outer" surface layer will allow for the attachment of specific ligands, or reporter groups, and other moieties of interest to drug nanoparticles (see example in Figure 3.2) [40].
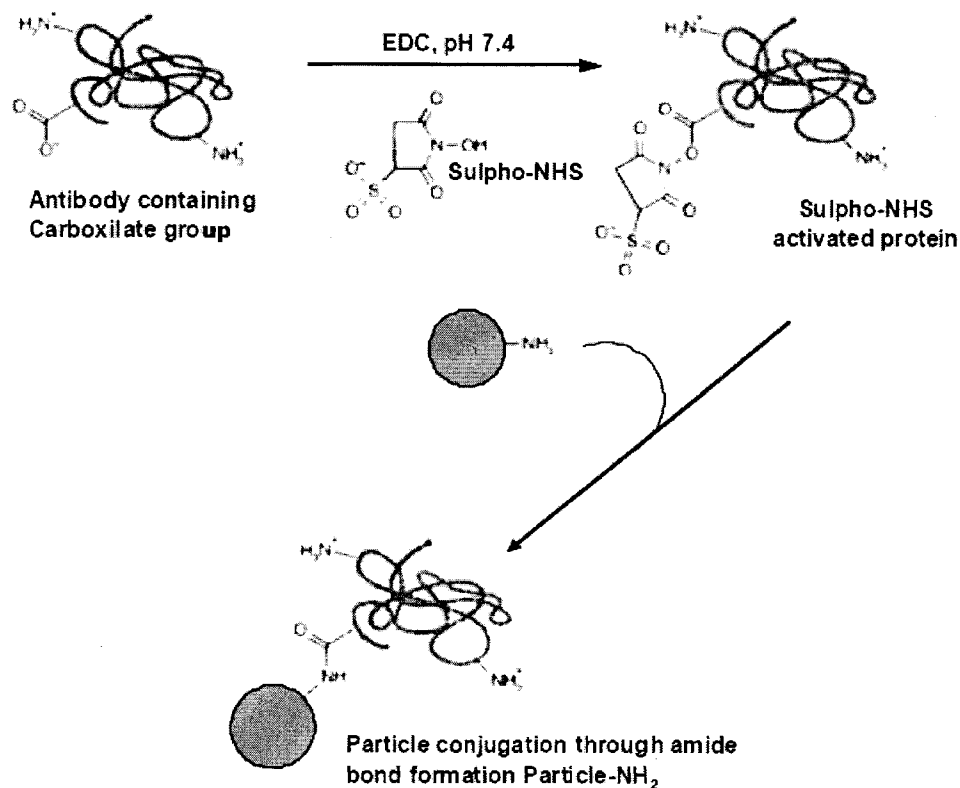
Figure 3.2 Schematics of the conjugation of an antibody (or any other ligand) to the LbL paclitaxel nanoparticles via free amino-groups of the "outer" polymeric layer.

We present here the results of our studies on using the LbL assembly for the preparation of stable nanocolloids of poorly soluble drugs with very high drug content. By nanoencapsulation of such anticancer drugs as tamoxifen and paclitaxel, we demonstrate the general applicability of this approach to prepare non-targeted and targeted drug nanoparticles with very high drug content and controlled drug release rate.

Materials and Methods

Poorly soluble and potent anti-cancer drugs tamoxifen (TMF) and paclitaxel (PCT) have been used for our experiments (solubility below 1 μg/mL). The polyelectrolytes used for the LbL assembly included positively charged poly(allylamine

hydrochloride) (PAH), FITC-labeled PAH, and poly(dimethyldiallylamide ammonium chloride) (PDDA); all at the concentration of 2 mg/mL; and negatively charged sodium poly(styrene sulphonate) (PSS); also at the concentration of 2 mg/mL. Deionized water and PBS, pH 7.4, were used as the solvents. Ultra Sonicator 3000 (Misonix Inc, Farmingdale NY) was used for drug crystals disintegration at the power of 3 - 18 W and sonication time of 10-30 minute. To prevent sample overheating during the sonication and to keep the temperature in the range of 20-30°C, liquid nitrogen was used to cool the sample tubes liquid nitrogen was used. A quartz Crystal Microbalance (9 MHz QCM, USI-System, Japan) was used to measure the thickness of the polyelectrolyte multilayer. Surface potential (zeta potential) and particle size measurements were performed using the ZetaPlus Microelectrophoresis (Brookhaven Instruments). Field Emission Scanning Electron Microscope (Hitachi, S-4800) was used for particle imaging. Laser Scanning Confocal Microscope, Leica TCS SP2 (Leica Microsystems Inc) was also used to control the shell formation and to follow the colloid stability.

LbL Assembly and Some Properties of Nanocolloids

Initially, for disintegration, all drug samples were ultra sonicated with cooling at 18 W for up to 30 minutes in 1 mL volume before any polyelectrolyte was added. The size of the drug particles formed was periodically measured. Prior to the addition of the first layer of polyelectrolyte, the zeta potential reading was also taken. Polycations were used to form the first surface layer, since drug nanoparticles of both drugs were found to bear the intrinsic negative charge. Drug samples were then centrifuged at 14,000 rpm for 7 minute, washed and re-suspended in water or PBS to remove the excess polyelectrolyte before further zeta potential readings were taken. Then, the coating process was repeated

without the ultrasound applied and with the use of the oppositely charged polymer (polyanion, in this case). Zeta potential measurements were taken after each next layer addition. The images of colloidal particles formed were normally taken immediately and 48 hrs after the LbL assembly was completed to check for the stability of the colloids formed. Dry samples were prepared for SEM imaging using 5-10 µL of the colloidal suspension obtained. Sample droplets on bare silicon wafers were dried by heating them at 50°C for 1 hr or, by keeping overnight at room temperature. Drug colloids were kept in a low volume of saturated solution to prevent drug release.

Drug Release from Colloidal Particles at Sink Conditions

To study the release rate of different drugs from the colloidal particles prepared using the LbL assembly, the samples prepared using a different number of coating cycles were placed in 1 mL horizontal diffusion chambers made of cellulose acetate membrane and stirred in a large volume of PBS, pH 7.2, to mimic sink conditions expected *in vivo*. The concentration of the released drugs was measured by the HPLC.

Attachment of Ligand Moieties to the LbL Nanocolloids of Poorly Soluble Drugs

To prepare nanocolloids with the "reactive" surface suitable for the covalent attachment of various ligands to their surfaces, PAH containing free amino groups was used to form the outer layer on the drug particles. Paclitaxel was used in this series of experiments. To conjugate the monoclonal nucleosome-specific 2C5 antibody (mAb 2C5) for recognizing a broad variety of cancer cells via the cancer cell surface-bound nucleosomes [41, 42], the reaction was carried out in two steps (Figure 3.2). In the first step, the carboxylate groups on the mAb 2C5 were activated using 1-ethyl-3-

carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) to make

it amine-reactive. In the second step, the activated antibody was added to LbL paclitaxel

nanoparticles coated with polyamino-containing PAH polymer. All reactions were carried

out in HBS, pH 7.4 at 4°C with continuous stirring in presence of argon gas. The

modified particles were centrifuged at 12 rpm for 10 minute and re-suspended twice

using PBS to remove the unconjugated antibody.

The amount of paclitaxel in all the nanoparticle preparations was measured by the

reversed phase-HPLC. The particles were dissolved in the mobile phase prior to injecting

into the HPLC column. A D-7000 HPLC system equipped with a diode array (Hitachi,

Japan) and Spherisorb ODS2 column, 4.6mm×250mm (Waters, Milford, MA, USA) was

used. The column was eluted with acetonitrile/water (65:35%, v/v) at 1.0 mL/min.

Paclitaxel peak was detected at 227 nm. Injection volume was 50µL; all samples were

analyzed in triplicate.

Antibody Activity Preservation on the Surface of LbL Drug Nanocolloid

To verify the preservation of mAb 2C5 specific activity after the conjugation with

LbL-paclitaxel nanoparticles, a standard ELISA was performed. Briefly, ELISA plates

pretreated with 40 µg/mL polylysine solution in TBS, pH 7.4, were coated with 50 µL of

40µg/mL nucleosomes (the water-soluble fraction of calf thymus nucleohistone,

Worthington Biochemical, Lakewood, NJ) and incubated for 1 h at room temperature.

The plates were rinsed with 0.2% casein, 0.05% Tween 20 in TBS (casein/ TBS), pH 7.4.

To these plates, serial dilutions of mAb 2C5-containing samples were added and

incubated for 1 hr at room temperature. The plates were extensively washed with

casein/TBS and coated with horseradish peroxidase goat antimouse IgG conjugate (ICN

Biomedical, Aurora, OH) diluted according to the manufacturer's recommendation. After 1 h incubation at room temperature, the plates were washed with casein/TBS. Bound peroxidase was quantified by the degradation of its substrate, diaminobenzidine supplied as a ready-for-use solution, Enhanced K-Blue TMB substrate (Neogen, Lexington, KY). The intensity of the color developed was analyzed using an ELISA reader at the wavelength of 492 nm, Labsystems Multiscan MCC/340 (Labsystems and Life Sciences International, UK).

Cytotoxicty of Targeted Paclitaxel LbL Nanocolloid

The cytotoxicity of various concentrations of LbL-paclitaxel nanoparticles against and MCF-7 and BT-20 cells was studied using an MTT test. A ready-for-use CellTiter 96® Aqueous One solution of MTT (Promega, Madison, WI) was used according to a protocol suggested by the manufacturer. Formulations with paclitaxel concentration of up to 200 ng/mL dispersed in Hanks' buffer were added to cells grown in 96-well plates to about 40% confluence. After 48 hr or 72 hr of incubation at 37°C, 5% CO_2, plates were washed three times with Hanks' buffer followed by the addition of 100 μL of media and 20 μL of CellTiter 96® Aqueous One solution. After 1 hr of incubation at 37°C, 5% CO_2, the cell survival rate was estimated by measuring the color intensity of the MTT degradation product at 492 nm using an ELISA plate reader. Untreated cells were considered as 100% growth.

Results

LbL-Stabilized Drug Nanocolloids and Surface Zeta Potential

To find the optimal sonication condition, we have performed our initial experiments with tamoxifen crystals at a drug concentration in the suspension of 2

mg/mL. Figure 3.3 clearly shows that particle size expectedly and strongly depends on the sonication time. After 30 minutes of sonication at 18 W, particles with the size of about 100 nm were obtained (with the addition of polycationic PDDA prior to the size measurement to prevent particle re-aggregation). When similar sonication conditions were applied to paclitaxel, particle sizes of about 100 nm were also obtained. Further increase in the sonication time did not result in a significant decrease in drug particle size.
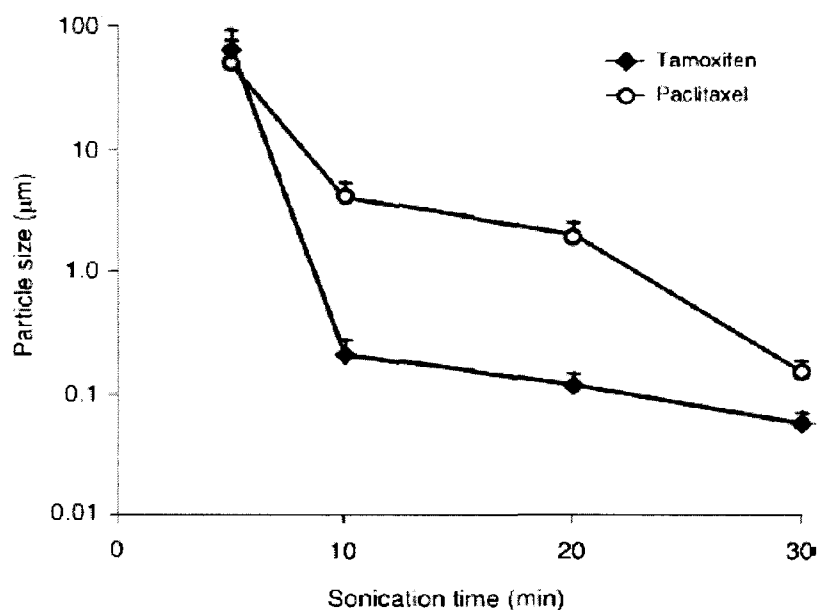
Figure 3.3 The dependence of drug particle size on the duration of sonication.

In experiments with tamoxifen and paclitaxel nanocrystals (2 mg/mL), PAH was present in solution for the first polymeric monolayer on the particle surface and prevents particle aggregation after the removal of ultrasound. Figure 3.4 gives values of the zeta potential during the process of sequential polycation/polyanion adsorption on tamoxifen cores. After the addition of the PDDA polycation to the initially negatively charged tamoxifen particles under sonication, drug nanoparticles are recharged to the positive

potential of ca. +45 mV and form stable colloidal solution when the sonication is terminated. Then, we proceeded with the LbL assembly on tamoxifen nanoparticles through the PSS polyanion adsorption adding one more monolayer to the shell, and again reversed the surface potential to the negative value of -17 mV. By adding the PDDA polycation again, we again made tamoxifen particles positively charged at ca. +80 mV. The fourth polymer layer (negative PSS this time) made the tamoxifen particles negative again, and so on. At the end, we obtained tamoxifen nanoparticles coated with an organized multilayer shell with the composition (PDDA/PSS)$_3$.
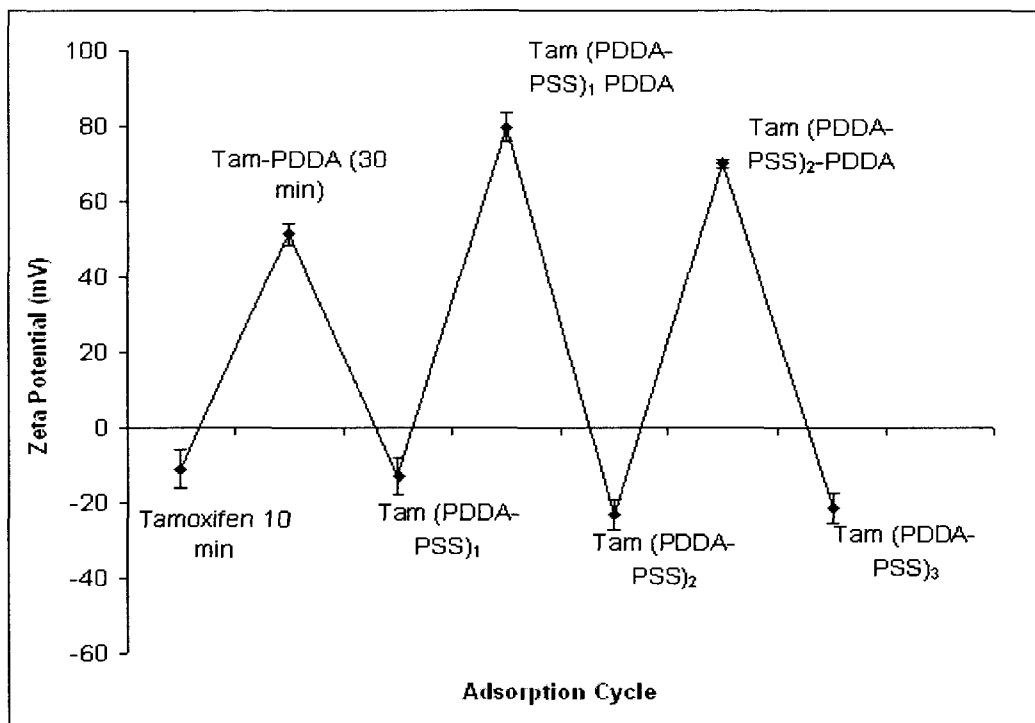
Figure 3.4 Changes in drug particle zeta potential during the process of the LbL assembly. LbL assembly of tamoxifen (2 mg/mL) resulting in the product coated with three bilayers composed of PDDA and PSS.

In Figure 3.5, similar changes in zeta potential values are demonstrated during the LbL shell assembly process for paclitaxel. Again, we have initially negatively charged

bare drug nanoparticles which, after the sonication with the addition of another polycation (PAH), became positive and formed stable colloids. Further assembly with the corresponding changes in zeta potential values allowed for the formation of the final shell with (PAH/PSS)$_2$ composition.
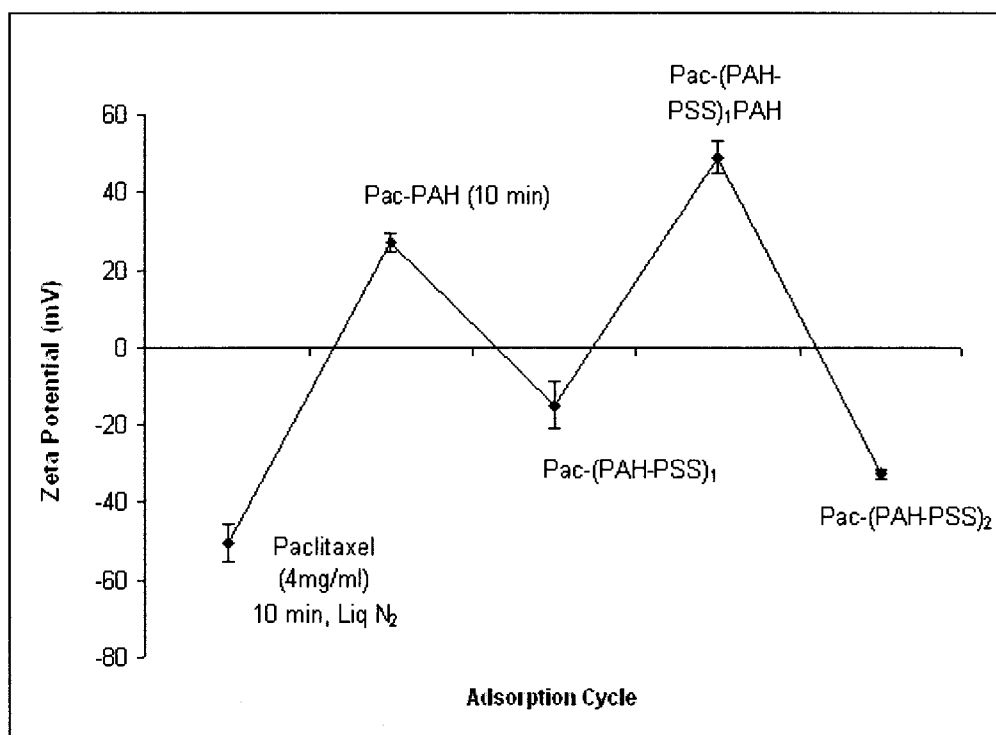
Figure 3.5 LbL assembly of paclitaxel (4 mg/mL) resulting in the product coated with two bilayers composed of PAH and PSS.

In separate experiments with quartz Crystal Microbalance (QCM) monitoring of the PDDA/PSS or PAH/PSS assembly on a quartz resonator, we found that a single polycation/polyanion bilayer has a thickness of 1.5 nm in the dry state. Polyelectrolyte multilayer thickness doubles in water [39]. Therefore, one could estimate our (PDDA/PSS)$_3$ shell thickness as ca. 4.5 nm in the dry state and 9 nm in an aqueous solution, and (PAH/PSS)$_2$ shell thickness as ca. 3 nm in the dry state and 6 nm in water.

Nanoparticle Imaging and Some Properties

The particle size of all the drug samples formulated by the LbL technology into nanocolloidal state was confirmed by scanning electron microscopy and confocal fluorescent microscopy. Figure 3.6 shows SEM images of prepared colloidal tamoxifen particles. Particles of tamoxifen have mainly a spherical shape and have a diameter of 120 ± 30 nm. LbL-coated paclitaxel has the elongated rod-like shape with approximate measurements of 50 x 120 nm.

The samples were rinsed with deionized water to get rid of the extra polyelectrolyte and 1 µL of the sample that was dried on a bare silicon wafer at room temperature for 12 hrs was used for SEM imaging. The samples were not sputtered prior to the imaging. The silicon wafer with the samples was mounted on a metal stub with an adhesive. During the drying process the nanoparticles become partially aggregated, as can be seen in the Figure 3.7a. To prove that this aggregation is the result of SEM sample preparation and does not proceed in aqueous suspension, we made fluorescence confocal images of our samples. With this in mind, we have used FITC-labeled PAH to prepare LbL-coated drug particles following same protocol as for the samples shown on Figure 3.6 a-b. Figure 3.8, showing the fluorescence image of LbL-coated tamoxifen particles in suspension, does not reveal any aggregation. One can see multiple individual fluorescent green dots (the color is due to FITC labeling), although the confocal microscope resolution of ca. 100 nm does not allow for seeing the detailed structure of individual nanocapsules.

Taking into account that the thickness of a single polymeric layer is approx. 1.5 nm in the dry state, we can easily calculate that the drug content in stable nanocolloidal particles of poorly soluble drugs is from 85 % wt (in case of tamoxifen particles with a triple bilayer coating) to approx. 90% wt (in case of paclitaxel particles with a double

layer coating), which is dramatically higher than that for any other solubilization method. Colloidal suspensions of both drugs are completely stable during the two weeks of observation.

Figure 3.6   SEM images: (a-c) tamoxifen nanoparticles of ca. 120 nm after 30 minute sonication in the presence of 2 mg/mL PAH.

a)   b)
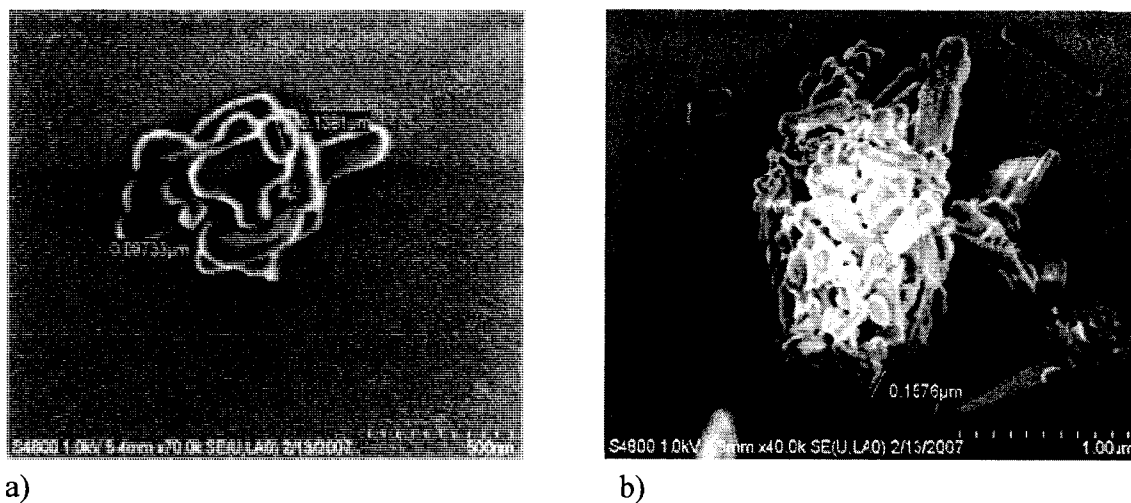
Figure 3.7 SEM images: (a-b) paclitaxel nanoparticles after 30 minute sonication and assembly of the LbL coating with (PAH-PSS)$_2$ composition.
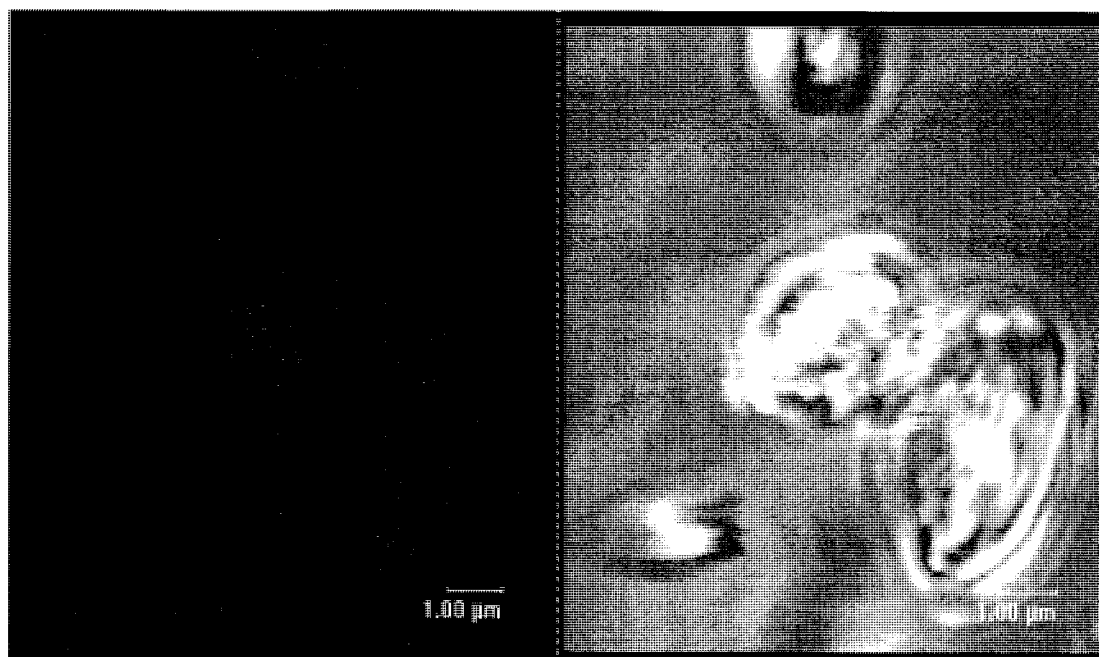
Figure 3.8 Confocal fluorescent image of an aqueous dispersion of LbL tamoxifen nanocolloid coated with FITC-labeled PAH.

Drug Release from LbL Nanocolloids

LbL technology allows for the easy control of the drug release rate from polymer-stabilized colloidal nanoparticles by simple changes of coating thickness or composition. Figure 3.9 presents the release curves for tamoxifen with different coating thickness in standard sink conditions at the same drug concentration of 2 mg/mL using the Peppas model (exponential approximation) [43]. As one could expect, we observe a slower release rate as the number of polyelectrolyte layers in the shell increases. At sink conditions, non-coated tamoxifen crystals (both without and with sonication) solubilize within approx. 2 hr, while LbL coating allows for easily extending this time to approx. 10 hr (extrapolation). Similar results were also obtained for paclitaxel (data not shown). Clearly, slower release rates are also possible depending on the architecture of the LbL coating.
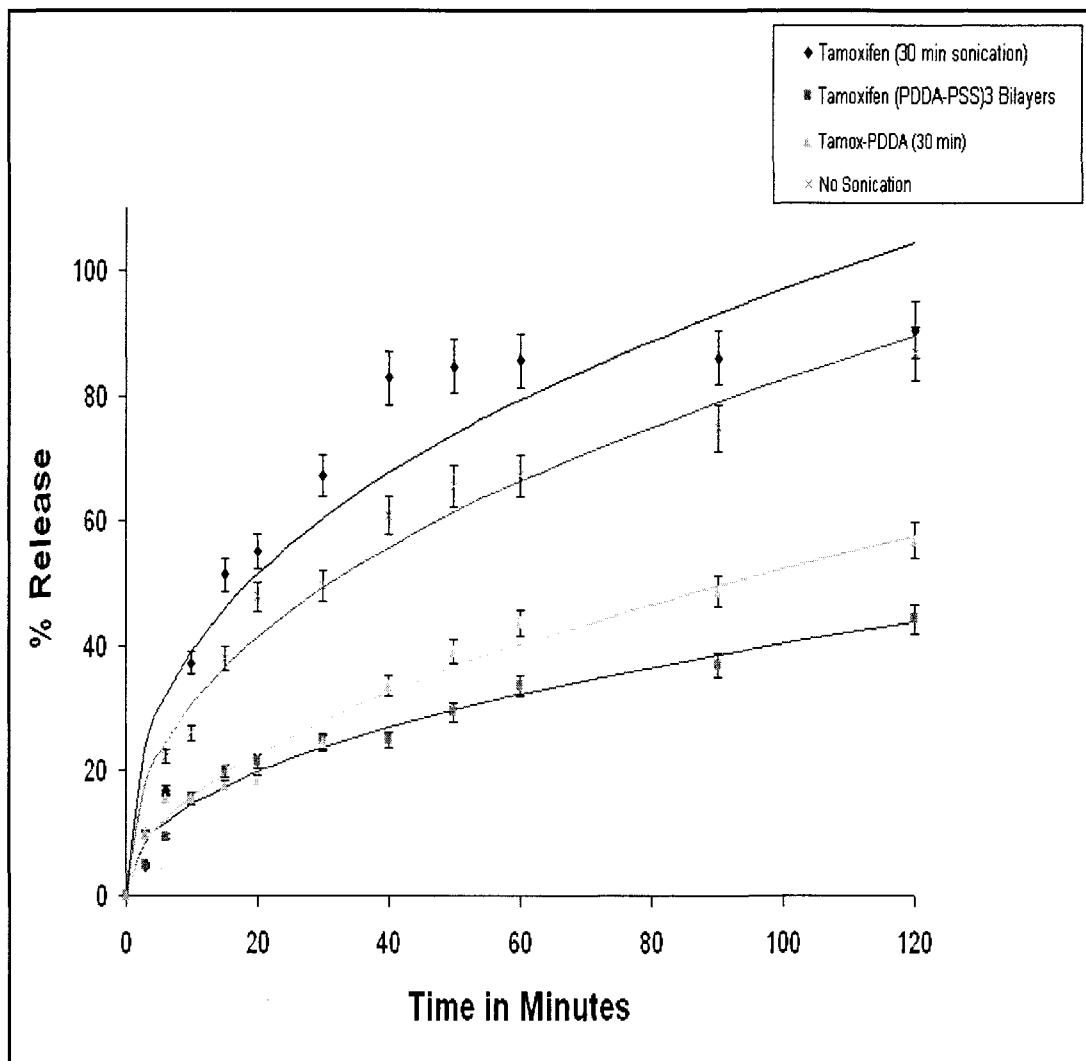
Figure 3.9 Controlled release of tamoxifen from the LbL nanocolloidal partricles.

Shown in Figure 3.9 are dissolution rates of free tamoxifen as drug crystals without sonication – 1, and nanoparticles of sonicated non-coated drug - 2 and tamoxifen release form the ca. 125 nm LbL nanocolloidal particles with different coating composition: PDDA coating – 3, and (PDDA/PSS)₃ coating – 4, at sink conditions in PBS buffer at pH 7.4.

Surface Modification of LbL Coated Drug Nanoparticles

To confirm that LbL-coated drug nanoparticles can be easily derivatized on the surface in order to impart them various additional properties including targetability, we have attached tumor-specific mAb 2C5 to the paclitaxel nanoparticles via free amino groups belonging to the surface layer of PAH.

ELISA with the nucleosome monolayer (specific antigen for mAb 2C5), the results of which are presented in Figure 3.10, clearly confirms that 2C5-modified LbL-coated paclitaxel nanoparticles acquire the ability to specifically recognize the target antigen, i.e. become targeted.
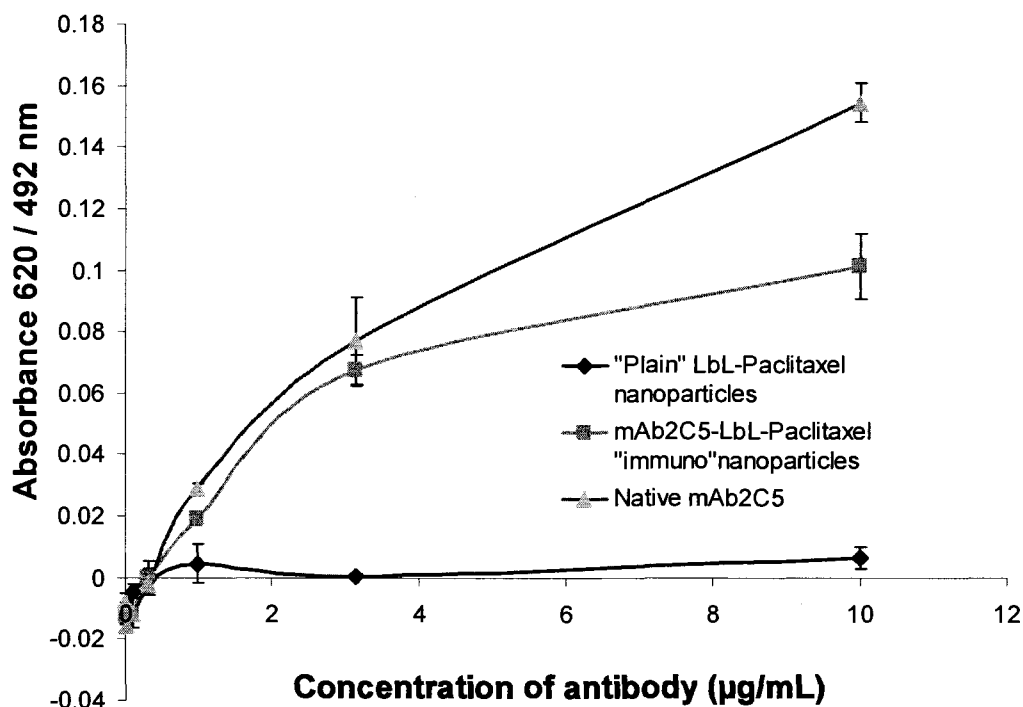
Figure 3.10 ELISA for 2C5-LbL paclitaxel immuno-nanoparticles (the values are average ± SD).

CHAPTER 4

PACLITAXEL AND TAMOXIFEN NANOPARTICLE LbL WITH BIOCOMPATIBLE COATINGS

Drug nanoparticle stabilization by LbL procedure using synthetic polyelectrolyte was first established [39], and then it was transferred to biocompatible polymers, such as cationic: polylysine, protamine sulphate and anionic: dextran sulfate, and bovine serum albumin (Figure 4.1) [44]. This was needed for in-vivo use of the drug nanoparticles to prevent any anaphylactic reactions in amimals [45]. Just as in previous experiments, the first polycation layer is deposited on the surface of the drug nanoparticle, and is then followed by the addition of an oppositely charged polyanion which results in the formation of a stable inter-polyelectrolyte complex shell around each drug nanoparticle. By varying the charge density of each polymer or the number of coating cycles, particles with a different surface charge and different composition can be prepared. The use of a polymer containing reactive groups (such as amino groups) for the last "outer" surface layer allows for the attachment of specific ligands and other moieties of interest to drug nanoparticles. We present here a proprietary method (Nemucore Medical Innovations Inc., Boston) of sonicated LbL assembly for the preparation of stable nanocolloids of paclitaxel and tamoxifen with very high drug content.

Negative

Positive

polyaspartic acid   polyglutamic acid

poly(L-lysine) hydrochloride

polyarginine hydrochloride

polyaminoacids

Gelatin B

Protamine sulfate, Gelatin A

Alginic acid   Chondroitin sulfate   Chitosan

polysaccharides

dextran sulfate

CMC -Carboxymethyl Cellulose

R = H or

$-CH_2-\overset{O}{\overset{\|}{C}}-ONa$

Figure 4.1 Biocompatible polyelectrolytes for *in-vivo* application.

Materials and Methods

Poorly soluble and potent anti-cancer drugs paclitaxel and tamoxifen have been used. The polyelectrolyte used included positively charged polylysine, poly (dimethyldiallyl ammonium chloride) (PDDA), protamine sulphate (PS); and negatively charged sodium poly (styrene sulphonate) (PSS) and bovine serum albumin (BSA). Ultrasonicator 3000 (Misonix Inc) was used for drug crystals disintegration at a power of

18 W. To prevent sample overheating during the sonication and to keep the temperature in the range of 20-25°C, liquid nitrogen was used to cool the sample tubes.

For disintegration, all drug samples were ultra sonicated with cooling for 10 minutes before polyelectrolyte was added. After the polyelectrolyte was added, the samples were sonicated for another 20 minutes. Polycations were used to form the first surface layer, since drug nanoparticles were found to bear an intrinsic negative charge. Drug samples were then centrifuged washed and re-suspended in PBS buffer to get rid of the excessive polycation before further zeta potential readings were taken. Then, the coating process was repeated with polyanion, followed by polycations, and so on. The resulting high concentration drug colloids were kept in a low volume of saturated solution to prevent drug release. To study the release rate of drugs from the colloidal particles, the samples were placed in horizontal diffusion chambers made of cellulose acetate membrane and stirred in a large volume of PBS, pH 7.2, to mimic the sink conditions expected *in-vivo*.

Attachment of Ligand Moieties to the LbL Nanocolloids of Poorly Soluble Drugs

To prepare nanocolloids with the "reactive" surface suitable for the covalent attachment of various ligands to their surface, polycation containing free amino groups was used to form the outer layer on the drug nanoparticles. To conjugate the monoclonal nucleosome-specific 2C5 antibody (mAb 2C5) for recognizing a broad variety of cancer cells [41, 42], the reaction was carried out in 2 steps. In the first step, the carboxylate groups on the mAb 2C5 were activated using 1-ethyl-3-carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) to make it amine-reactive. In the second step, the activated antibody was added to LbL paclitaxel nanoparticles coated

with polyamino-containing polymer. To verify the preservation of mAb 2C5 specific

activity after the conjugation with LbL-paclitaxel nanoparticles, a standard ELISA was

performed. The cytotoxicity of various concentrations of LbL-paclitaxel nanoparticles

against MCF-7 and BT-20 cells was studied using an MTT test.

Results and Discussion

After 30 minutes of LbL assisted sonication, paclitaxel particle sizes of about 50 x

50 x 120 nm were obtained (Figure 4.2). Further increase in the sonication time did not

result in a significant decrease in drug particle size. Initially we have initially negatively

charged bare drug nanoparticles, which after the sonication with the addition of cationic

polylysine or protamine sulphate became positive and formed stable colloids. Further

assembly with the corresponding changes in zeta potential values allowed formation of

the two bilayer shell with biocompatible protamine sulfate and albumin: $(PS/BSA)_2$.

(a) (b) (c)
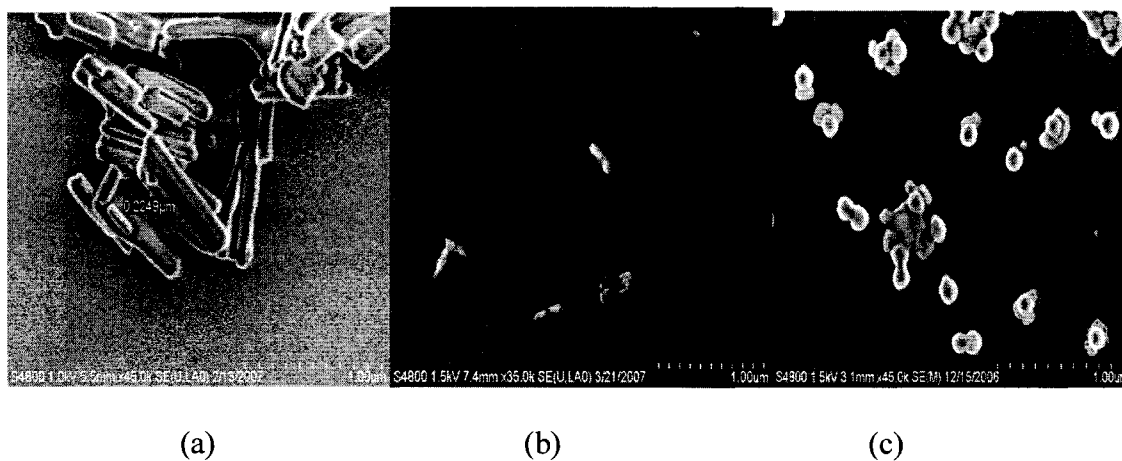
Figure 4.2 SEM images (Hitachi-S4800) of LbL encapsulated drug nanoparticles.
Paclitaxel: (a) 20 minute sonication + LbL coating, (b) smaller concentration sample
with 30 minute sonication + LbL coating with protamine sulfate / albumin, size 50 x 50 x
120 nm, (c) 120 nm diameter tamoxifen particles prepared via sonication in the presence
of polylysine.

Figure 4.3 gives values of the surface zeta potential during the process of sequential four step protamine sulfate/ albumin adsorption on paclitaxel nanocores. The thickness of this shell was estimated with a Quartz Crystal Microbalance as ca. 6 nm in dry state and 12 nm in water. Similar zeta potential alternation was observed during shell assembly on tamoxifen nanoparticles.
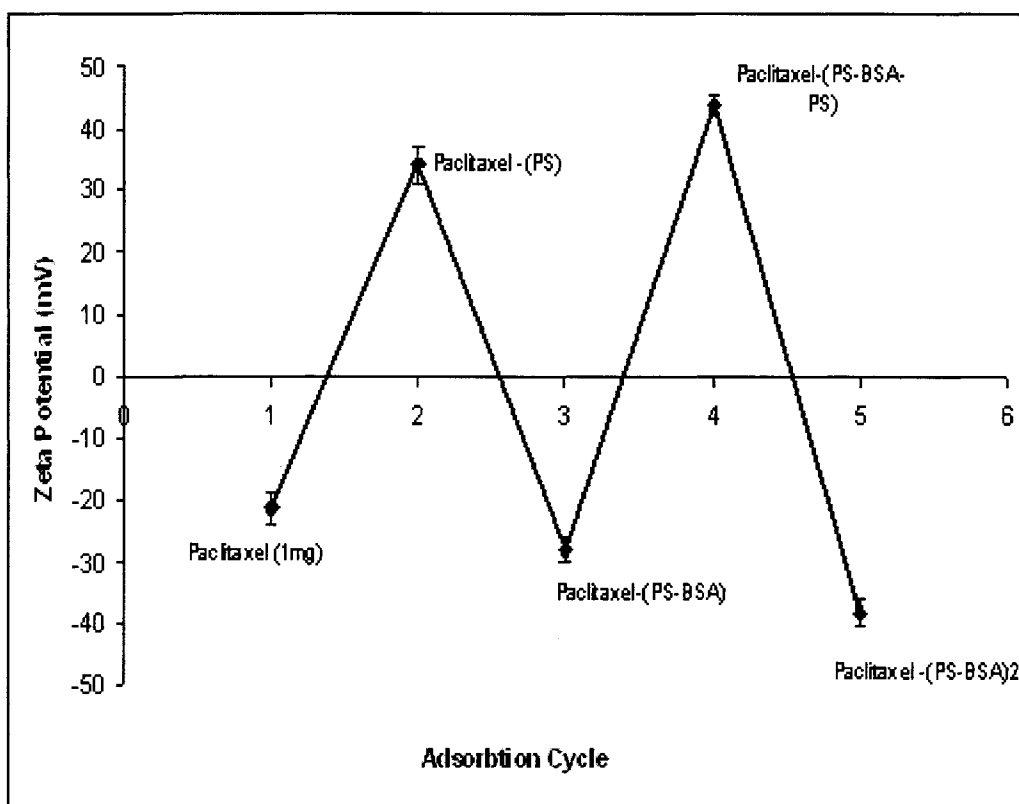
Figure 4.3 Changes on paclitaxel particle zeta potential in the process of the LbL coating with two bilayers of protamine sulphate (PS) and bovine serum albumin (BSA).

Nanoparticle Imaging and Drug Release

The particle size of all the drug samples formulated by the LbL technology into nanocolloidal state was confirmed by scanning electron microscopy and confocal fluorescent microscopy (Figure 4.2). At 20 minute sonication, we obtained submicron

particles, and further sonication with polycations and liquid nitrogen cooling resulted in nanosized particles. LbL-coated paclitaxel has the elongated rod-like shape of approximately 50 x 50 x 120 nm. Particles of tamoxifen demonstrate mainly spherical shape and have a diameter of 120 ± 30 nm. The spherical shape of tamoxifen is probably due to the melting and then solidifying of the drug nanoparticle during powerful sonication (instantaneous local temperature during collapsing cavitations' microbubbles reaches hundreds degrees). This was confirmed from the amorphous form of the resulting Tamoxifen nanoparticles. The SEM images were obtained after drying the samples, and during this process the nanoparticles become partially aggregated. This aggregation does not proceed in aqueous suspension, as it was demonstrated with fluorescence confocal images of our samples [39].

Taking into account that the thickness of a single polymeric layer is approx. 3 nm in dry state, we can calculate that the drug content in stable nanocolloidal drug particles is from 90 % wt (in case of paclitaxel particles with a double layer coating) to 85 % wt (in case of tamoxifen particles with a triple bilayer coating) which is dramatically higher than that for the case of other solubilization methods. Colloidal suspensions of both drugs were stable during a month of observation.
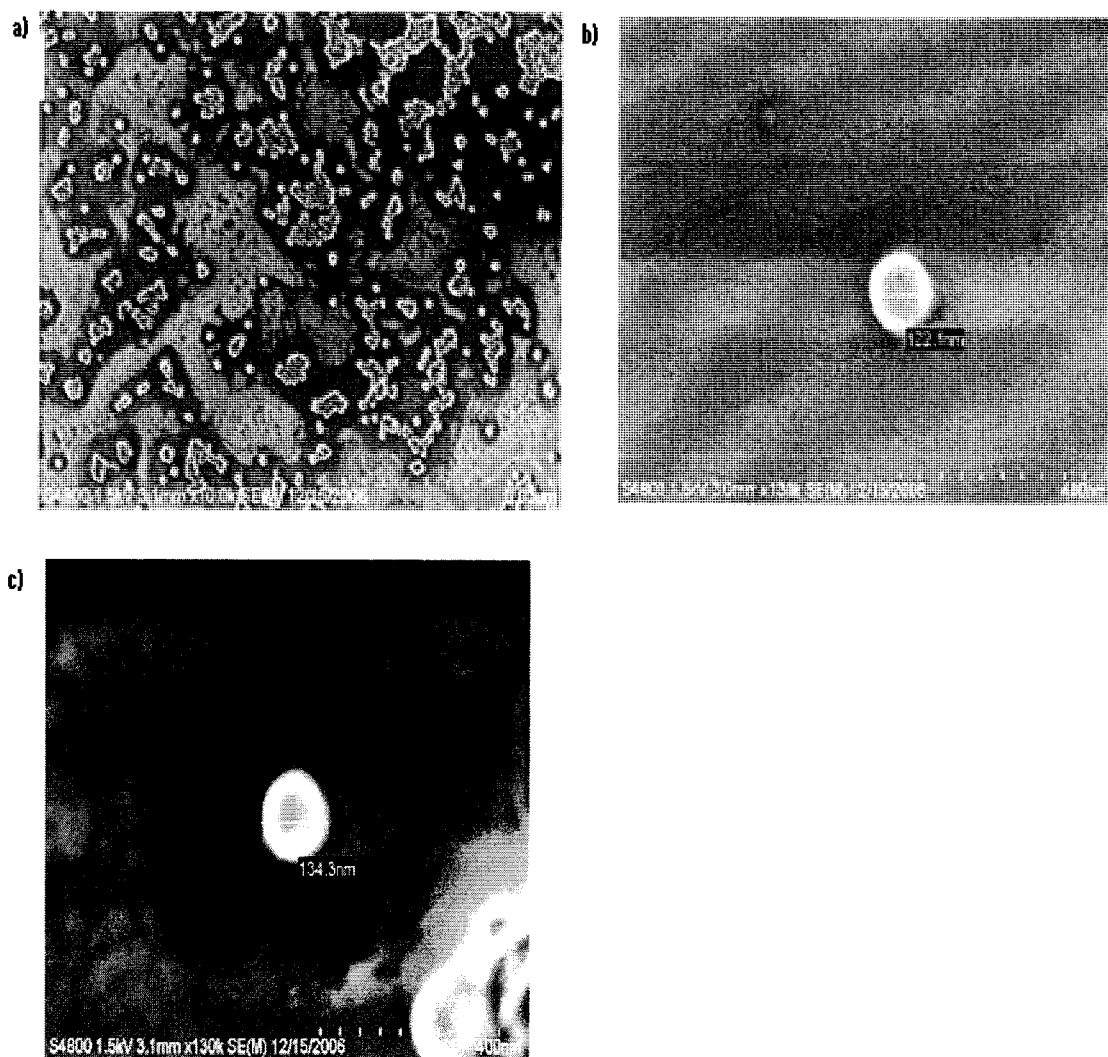
LbL technology allows control of the drug release rate from polymer-stabilized colloidal nanoparticles by simple changes of coating thickness or composition. As one could expect, we observed a slower release rate as the number of polyelectrolyte layers in the shell increases. At sink conditions, non-coated tamoxifen particles (after sonication) dissolve within approx. 2 hr, while LbL coating allows the extension of this time to 10 hrs. Figure 4.4 gives the release curves for 2 mg/mL paclitaxel in PBS buffer at pH 7.2

through a 0.2 micrometer pore nitrocellulose membrane. The upper most curves represent

the bare, uncoated micronized drug, and the two lower curves show the release from 125-

nm diameter nanoparticles coated with one PDDA monolayer and a thicker shell of three

bilayers of (PDD/PSS)₃. Similar results were obtained also for tamoxifen. These are

initial release experiments done with synthetic polymers. Experiments with

biocompatible shells of (protomine sulfate / albumin)₃ gave similar release rates.
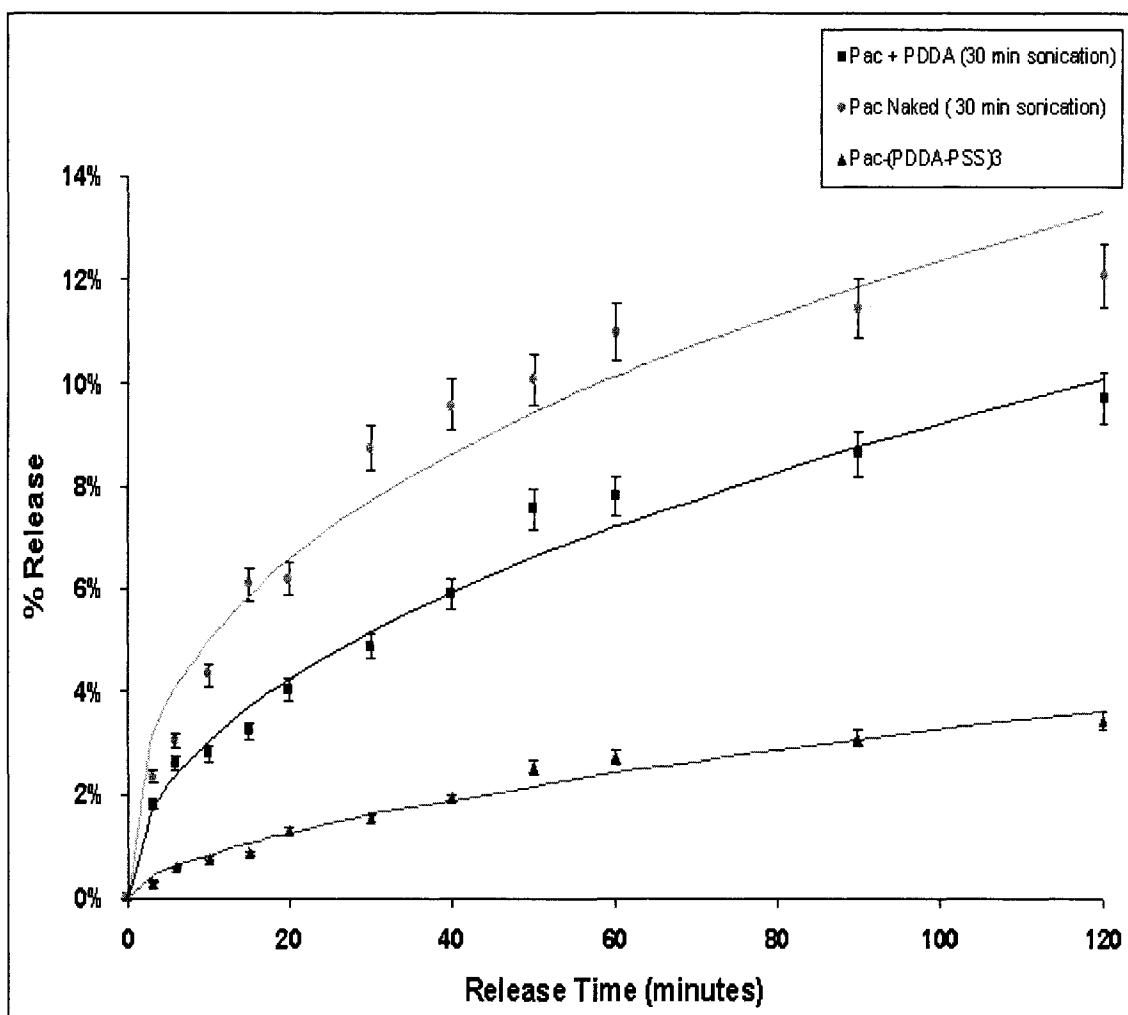
Figure 4.4 Release of paclitaxel nanoparticles at sink condition in PBS buffer at pH 7.2
after LbL-coating with PDDA and (PDDA-PSS)₃ (lower two curves as compared to
uncoated drug nanoparticles in the upper curve).

Surface Modification of LbL-Coated Drug Nanoparticles

To confirm that LbL-coated drug nanoparticles can be easily derivatized on the surface in order to impart to them various additional properties, including targeted delivery, we have attached tumor-specific mAb 2C5 to the paclitaxel nanoparticles via free amino groups belonging to the outermost surface layer of PAH.

Increased Cytotoxicity of Tumor Cell-Targeted LbL Drug Nanocolloids *in vitro*

Our preliminary *in vitro* experiments with MCF-7 and BT-20 cancer cell lines clearly confirmed that targeted drug nanoparticles demonstrate higher cytotoxicity than their non-targeted counterparts. At a paclitaxel concentration of 100 ng/mL for the MCF-7 cells (Figure 4.5) and 30 ng/mL for the BT-20 cells (Figure 4.6), when virtually no cytotoxic effect can be observed with non-targeted LbL-coated paclitaxel nanoparticles (around 95% of cancer cells remains alive after incubation for 48 or 72 hrs), 2C5-targeted LbL-coated paclitaxel particles kill at least 30% of the cancer cells (Figure 4.7). These results confirms that LbL technology allows for decorating the surface of stable colloidal drug particles with a very high drug content with various additional functions as needed.
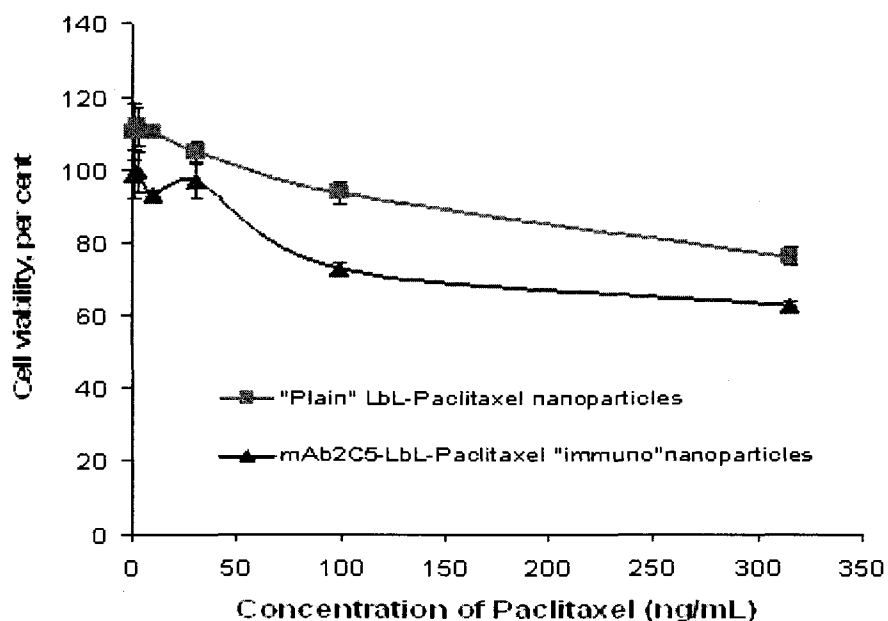
Figure 4.5 Cytotoxic effects of different concentrations of paclitaxel in "Plain" LbL-Paclitaxel nanoparticles and mAb2C5-LbL-Paclitaxel "immuno" nanoparticles on MCF-7 cells after 48 hrs of incubation with the formulations.
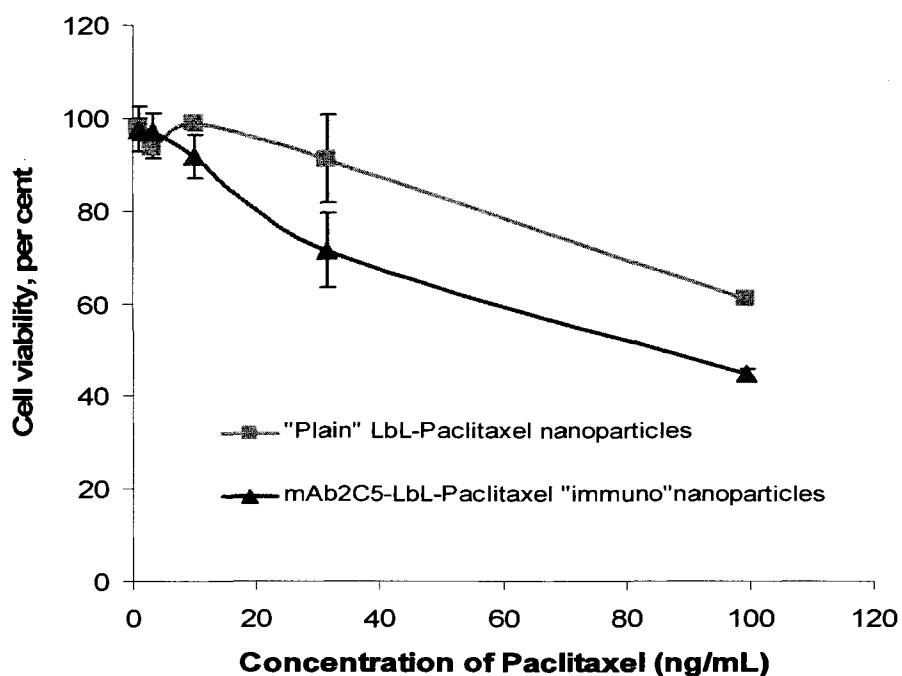
Figure 4.6 Cytotoxic effects of different concentrations of paclitaxel in "Plain" LbL-Paclitaxel nanoparticles and mAb2C5-LbL-Paclitaxel "immuno" nanoparticles on BT-20 cells after 72 hrs of incubation with the formulations.

a)

b)
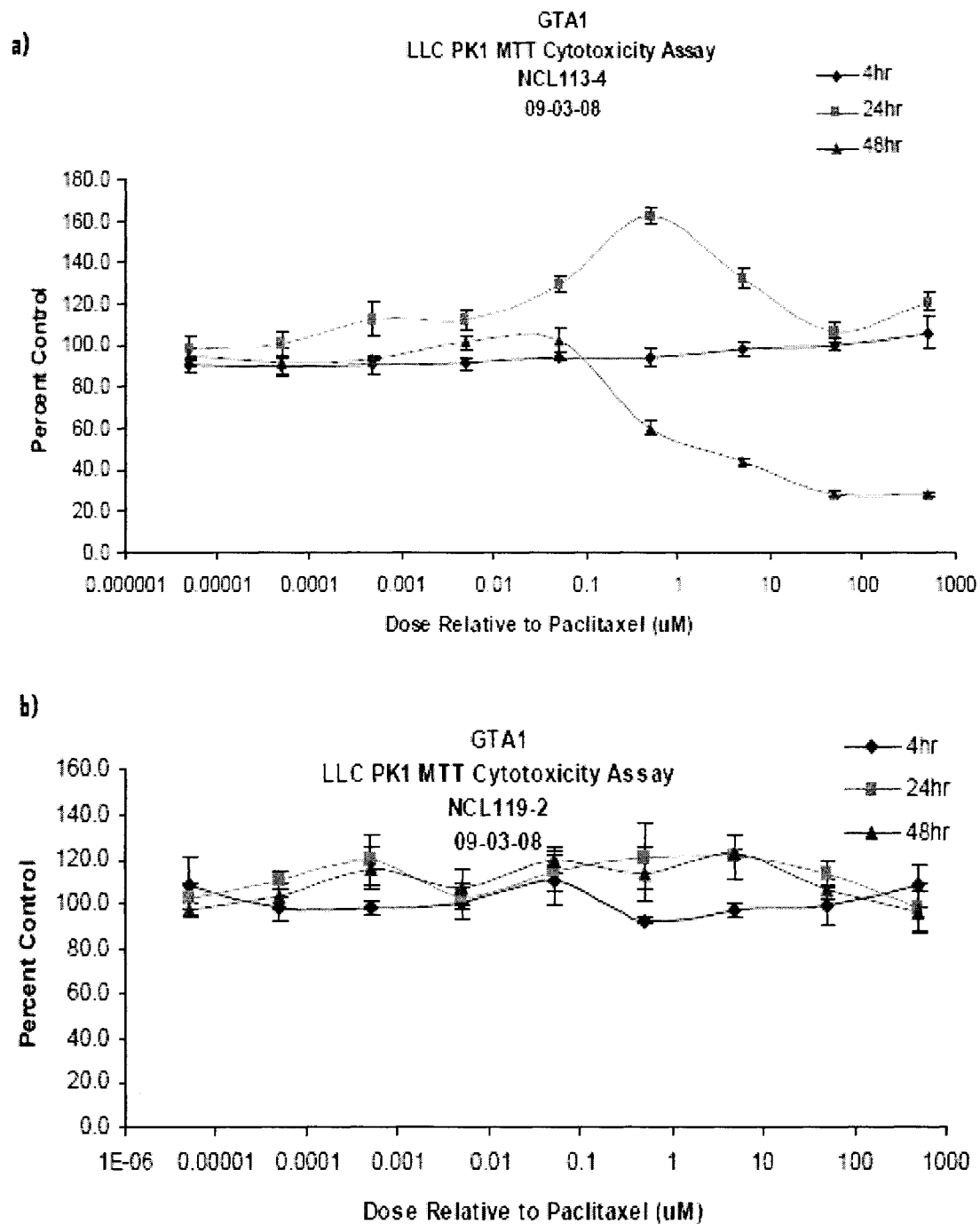
Figure 4.7 MTT cytotoxic assay of (a) paclitaxel (PS-BSA)$_2$ nanoparticles as compared to (b) negative control Gelatin (PS-BSA)$_2$ nanoparticles.

CHAPTER 5

STABLE NANOPARTICLES OF GADOLINIUM
FOR MOLECUALR IMAGING

Gadopentate Dimeglumine

Many potent radio diagnostic drugs, like Gadolinium based MRI contrast agent Gadopentate Dimeglumine, are associated with high incidence of toxicity and difficulties in preparing dosage forms [46]. Making these substances to have better bioavailability can simplify their administration, result in easier-to-make dosage forms, and minimize their non-specific toxicity and side-effects upon their administration. It can also allow for converting many known powerful but insoluble substances into clinically acceptable drugs. In recent years, serious problems have arisen involving the use of gadolinium for help with MRI's and MRA's in patients who have kidney problems or renal disease [47]. When gadolinium is injected into an otherwise-healthy patient, it is generally is eliminated by the kidneys after the examination, and does not cause problems. However, those with kidney problems are generally unable to expel the substance from their systems, which results in prolonged exposure to the agent within their bodies. Prolonged exposure to gadolinium can result in the development of Nephrogenic Systemic Fibrosis (NSF), which is also known as Nephrogrenic Fibrosing Dermopathy (NFD) [47]. Only a few studies involving nanometer sized gadolinium particles have been reported, which is

mainly due to the challenges associated with fabricating a stable colloidal suspension of gadolinium in an aqueous media [48, 49].

We elaborate on the use of the LbL polyelectrolyte coating technology to fabricate and characterize new particulate contrast media, gadopentate dimeglumine nanoparticles with biocompatible coating as prototype multimodal imaging and therapeutic agents. For this purpose, the aqueous suspensions of Gadopentate dimeglumine with particle size of the order of microns are subjected to unltrasonic treatment to decrease the size of individual particles to the nanolevel (between 50 and 1000 nm, preferably, with size distribution maximum between 100 and 200 nm), and then, with sonication treatment on, are stabilized in solution by the formation of the thin polyelectrolyte layer on their surface. This charged polymeric layer, with zeta potential of ca +15 mV, prevents particle agglomeration after stopping the physical treatment, which results in the formation of stable colloidal dispersions with high drug content in each colloidal particle (more than 50% wt and up to 90% wt). Further building of the shell through additional alternate adsorption of polyanion (such as sodium polystyrene sulfonate) and polycations (such as polyallylamine) allows formation of a thicker shell with higher zeta potential of ca 30-40 mV.

Application of LbL Assembly on Gadolinium

The aqueous suspensions of Gadopentate dimeglumine with particle size of the order of microns is subjected to physical treatment, such as unltrasonic treatment, to decrease the size of individual particles to the nanolevel (between 50 and 1000 nm, preferably, between 100 and 200 nm), and then are stabilized in solution by the formation of a thin polymeric layer on their surface. This polymeric layer prevents particle

agglomeration after stopping the physical treatment, which results in the formation of stable colloidal dispersions with high contrast content in each colloidal particle (more than 50% wt and up to 90% wt). The polymeric coating is formed based on the polyelectrolyte complexing process, when gadolinium nanosuspensions formed by ultrasonications are incubated in the presence of a watersoluble biocompatible polymer (polycation or polyanion) to allow for its deposition on their surface. After that, the first polymeric layer is stabilized by the addition of another, oppositely charged polyelectrolyte, which forms a firm electrostatic complex with the first one resulting in the appearance of a very thin but extremely stable polymeric shell around each contrast nanoparticle. This shell prevents particle agglomeration, and can be easily and reproducibly formed on the surface of any drug particle. By varying the charge density on each polymer, or the number of coating cycles, gadolinium particles can be prepared with a different surface charge and different thicknesses of the polymeric coat. This, in turn, provides an easy way to control drug release from such particles. In addition, the polymers used can also incorporate reactive groups allowing for further attachment of various specific ligands or reporter groups or other moieties to their surfaces. These moieties can also be incorporated into the polymeric shell during the formation of the drug nanoparticle.

Fabrication of Gd-DTPA- Dimeglumine Nanoparticles into a Stable Aqueous Solution

Reagents

1) Gd-DTPA-Dimeglumine- 469 mg/ml diluted in 10X Deionized H_2O (from *ViewGam*).

2) Aqueous Solvent – DI water at pH 7.0

3) PAH- poly(allylamine hydrochloride)

4) FITC attached to PAH for fluorescent imaging.

Instruments

1) Zeta Plus- zeta potential Instrumet by Brookhaven Corporation.

2) Scanning Electron Microscope (HITACHI S-4800)

3) Ultra Sonicator- MISONIX 3000 at 18 W power.

4) Leica TCS SP2 Laser Scanning Confocal Microscope.

Substrate used for SEM analysis was bare silicon plate with out plasma sputtered Au coating.

Methods

A total 46.9 mg/ml of Gd-DTPA-Dimeglumine was taken in 1 ml buffer volume and sonicated in the MISONIX SONICATOR -3000 for 7 minute. Zeta Readings are taken after sonication and before mixing the PAH solution. The solution was then mixed with 2mg/ml of PAH for 20 minute, and then centrifuged, washed two times with DI water, Zeta measurements, taken again.

Powerful sonication in the presence of polycation PAH resulted in the Gd-DTPA-Dimeglumine sub-micronizing with simultaneous coating, which prevented drug re-aggregation.

Coating architecture: Gd-PAH provided stable colloids with diameter of 105 nm ±10% and enhanced surface charge (negative or positive will depend on the polyelectrolyte at the outermost layer).

Results

Zeta Potential Measurements

A reversal of surface charge was seen as shown in Figure 5.1. Again an inverse

relation of particle size and sonication time was observed (Figure 5.2).
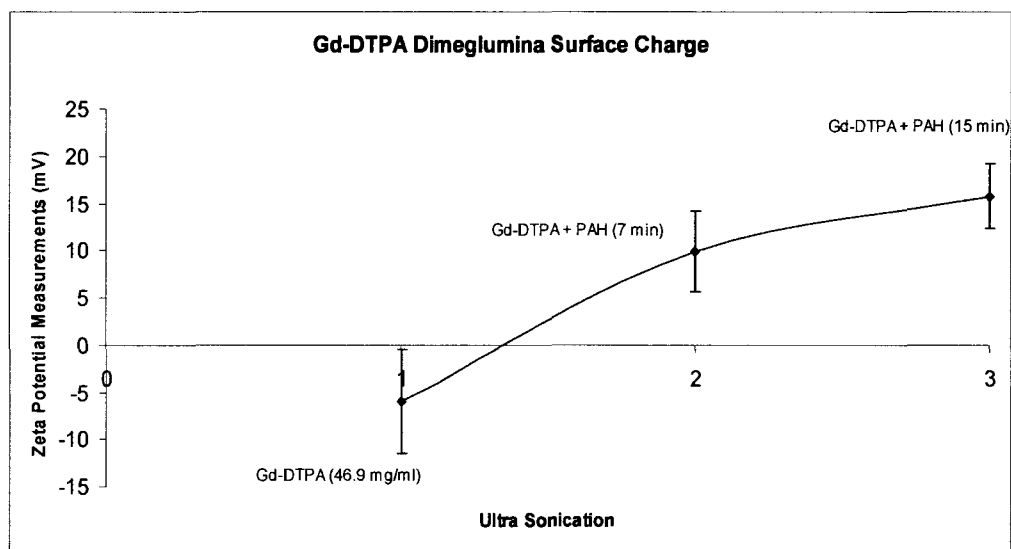
Figure 5.1 Surface charge measurements of Gd-DTPA Dimeglumine before and after nanofabrication. An average of seven readings were taken.
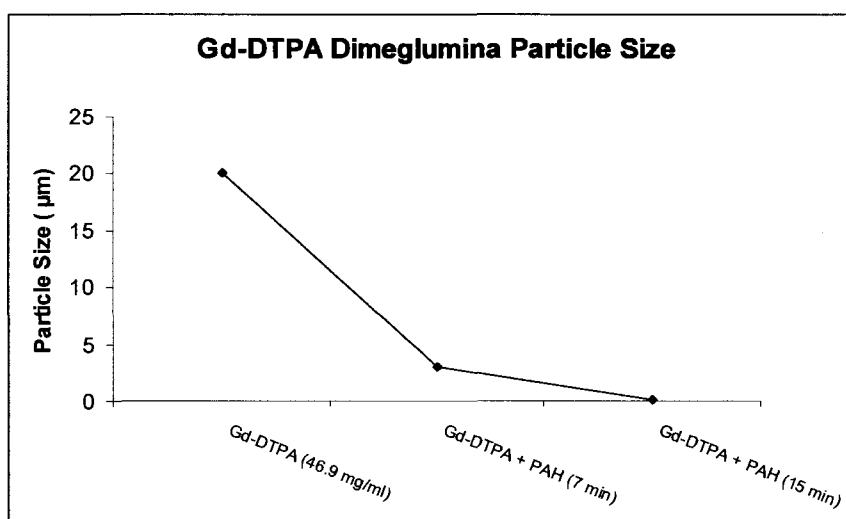
Figure 5.2 Inverse relationship of ultrasonication time with particle size.

Scanning Electron Microscopy Imaging

Untampered Gd-DTPA particles (Figure 5.3) were much larger than the

nanofabricated ones as shown in Figure 5.4 and Figure 5.5.

Gd-DTPA Dimeglumine Without Fabrication or Sonication.
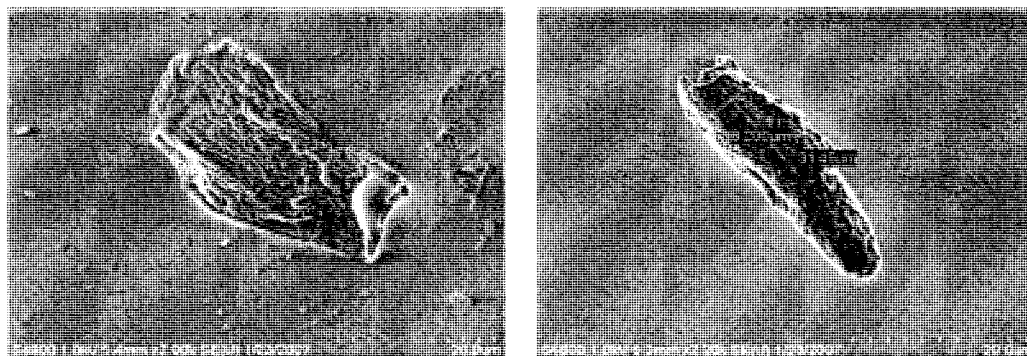
a)   b)

Figure 5.3 Scanning electron microscopy images of Gd-DTPA Dimeglumine without any sonication or coating. Particle Size ranged from 8 - 20 μm (a) & (b).

Nano-fabricated Gd-DTPA Dimeglumine
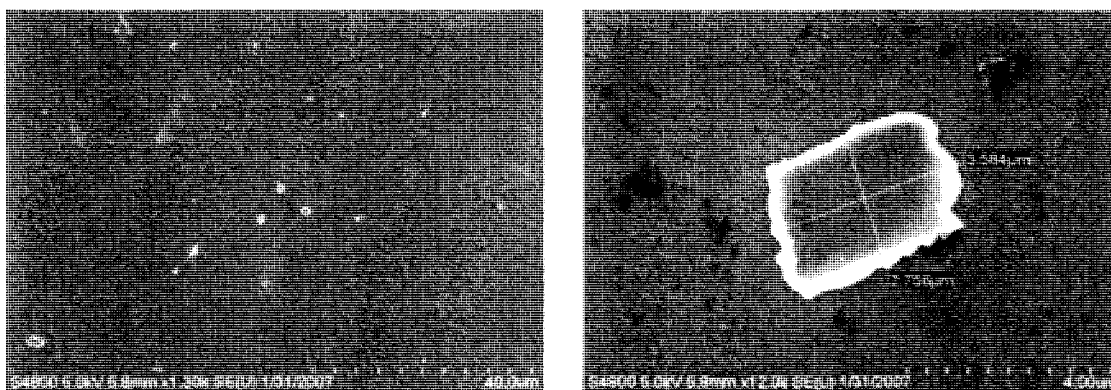
a)   b)

Figure 5.4 Scanning electron microscopy images of Gd-DTPA Dimeglumine (a) after utra sonication at 18 W, for 7 minutes, in the presence of 2 mg/ml PAH, (b) particle size ranges from 2-3 μm as seen on higher magnification.
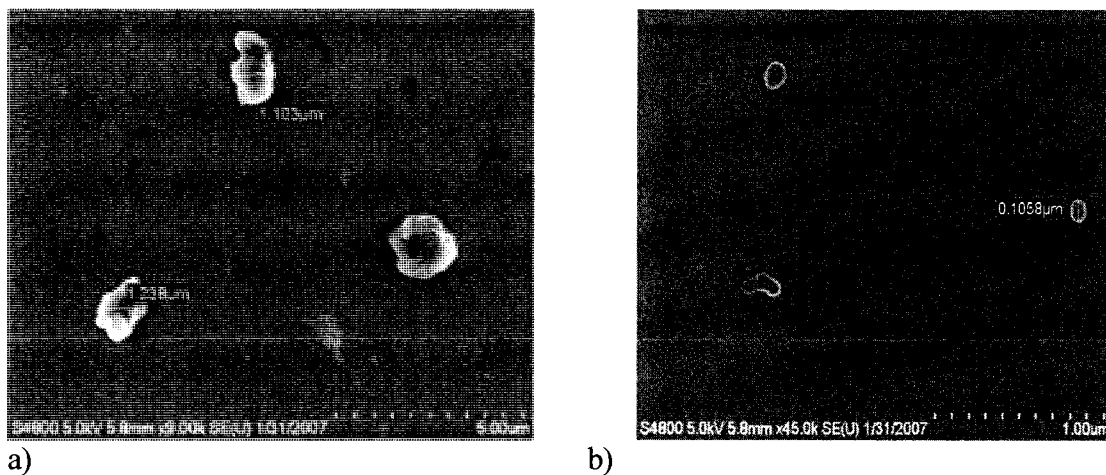
Figure 5.5 Scanning electron microscopy images of Gd-DTPA Dimeglumine (a) after utra sonication at 18 W, for 15 minutes, in the presence of 2 mg/ml PAH, (b) smallest particle size attained was 105 nanometers as seen at higher magnification.

Confocal Imaging

Proof of LbL layer was demonstrated by confocal imaging (Figure 5.6).
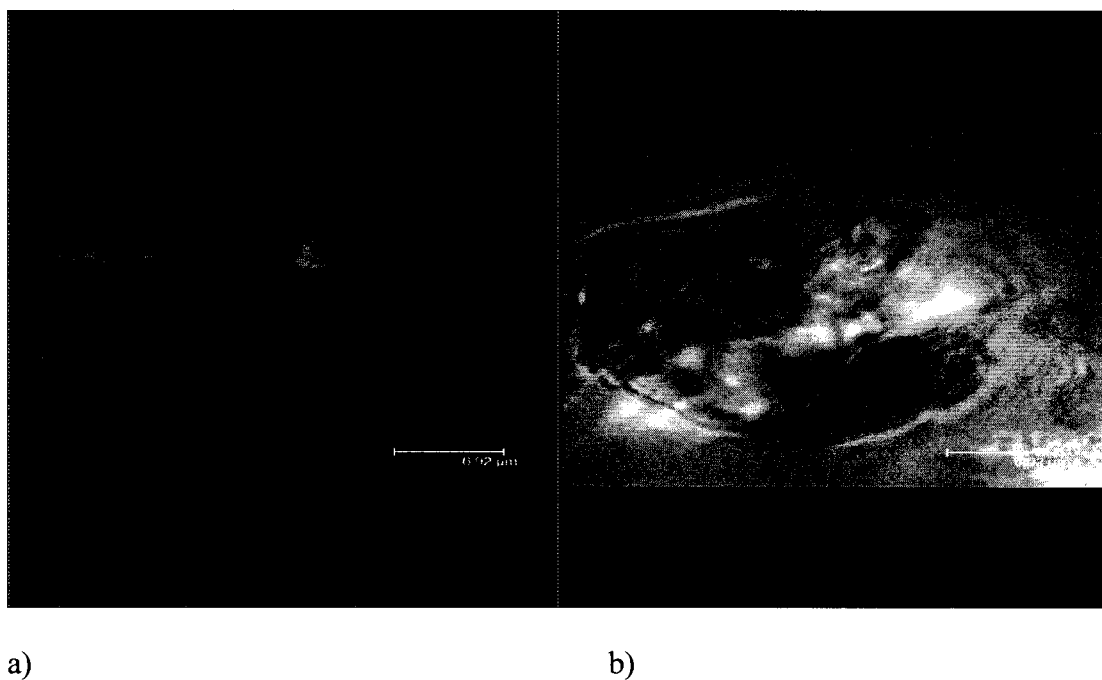
Figure 5.6 (a) Confocal and (b) Transmission images of Gd-DTPA Dimeglumine after utra sonication at 18 W, for 7 minutes, in the presence of 2 mg/ml PAH- FITC.

Novel Features of Our Technology

1. Stable nanocolloids of various poorly bioavailable drugs can be prepared following the same procedure, i.e. the procedure is applicable to a broad variety of toxic and poorly soluble pharmaceutical agents

2. The properties of the polymeric coat (i.e. surface properties of drug particles) can be easily controlled though LbL polycation / polyanion assembly, forming a shell of the needed molecular composition and increased surface charge.

3. The composition and the architecture of the particle surface (of the polymeric shell) can be easily controlled, resulting in easy control over its biological properties and making complexes of two different agents.

The purpose of this research is to provide a universal and efficient platform for making stable colloids of gadopentate dimeglumine with a high concentration of the agent in the colloidal particles. Nanosized colloidal suspensions of gadolinium will dramatically increase its solubility, bioavailability, and golmerular filteration through the kidneys, thus reducing its half life and adverse effects on the patients even with compromised renal function. Colloidal particles can also be easily made organ-specific (targeted) by modifying their surfaces with various specific ligands, such as monoclonal antibodies, etc., to increase their diagnostic/therapeutic potential.

CHAPTER 6

CONCLUSIONS AND DISCUSSION

In all the work explained above, we have clearly concluded the following:

1) Stable nanocolloids of insoluble drugs with very high drug content can be easily and reproducibly prepared through the application of LbL technology, i.e. combination of sonication and alternate adsorption of oppositely charged polyelectrolytes, resulting in nanoparticles with the content of the drug far exceeding other known systems

2) Drug release rate from such nanocolloidal particles can be easily controlled by assembling organized multilayer shells with required wall composition, density and thickness;

3) Various additional functions, such as specific targeted ligands, can be easily attached to the surface on nanocolloidal particles of poorly soluble drugs by using a polymer with free reactive groups for the "outer" coating and preserve their specific properties upon the attachment;

4) Since drugs are not modified in any way in the process of solubilization and release as free drug molecules, there is no concern regarding any possible changes in drug activity *in vivo*. However, to deliver a desired dose of a poorly soluble drug in the body, a very small quantity of a polymer (polymeric carrier) is required compared with any other protocols currently used for administration of poorly soluble pharmaceuticals, thus

simplifying the administration procedure and decreasing the possibility of carrier-related side-reactions. This may represent a promising approach to obtaining convenient dosage forms of poorly soluble drugs.

To summarize, the following formulation technique was demonstrated:

1) All drugs used were formulated into stable colloids by powerful sonication with polycation coating with resultant particle sizes ranging from 130 nanometers to 400 nanometers.

2) Effective LbL assembly was possible, at least up to two bilayers, providing the needed shell multilayer architecture for adjustable release and for binding of antibodies to amine groups in the outermost layer (i.e. polylysine) for targeted delivery.

3) A negative surface potential of -15, -25 mV was found for all four drugs after powerful 10 minute to 30 minute sonication.

4) As the sonication time and power (to 18 W per mL) were increased, the drug particle size was reduced showing a time dependence of size with sonication. In one embodiment, polycation coating was applied to the samples simultaneously with sonication to prevent re-aggregation.

5) Zeta potential readings show stable polycation coating with drug surface re-charging to + 20 mV. This positive surface charge keeps drugs in a stable colloid form and prevents re-aggregation. After the drug was coated, the surface potential remained the same even 48 hrs later, which demonstrates stable coating.

6) Negatively charged polyelectrolyte PSS addition failed to form stable colloids of these drugs. Drug nanoparticle re-aggregated to larger size. Therefore, coating drug nanoparticles with oppositely charged polyelectrolyte is a condition of the process.

7) Sonication at lower temperatures of 5-10° C (with surrounding ice or liquid nitrogen) showed even smaller particle sizes were attained.

SEM and confocal images taken 2-7 days after the sample formation demonstrated the stability of the drug aqueous nanocolloids.

8) Two-twelve hrs release at pH 7.2 can be observed for the drug nanocolloids depending on the polyelectrolyte shell composition. A longer release is achievable with tighter capsule walls. For long storage of the colloids without dissolution, certain pH conditions with low solubility rate may be useful.

Commercial Applications and Future Work

We have presented here a proprietary method (Nemucore Medical Innovations Inc., Boston) of sonicated LbL assembly for the preparation of stable nanocolloids of paclitaxel and tamoxifen with very high drug content.

Since many currently used "good" drugs have difficult to deal with dosage forms and many potentially good drug candidates never enter further development because of their low solubility and thus bioavailability and impossibility to make an acceptable dosage form. The use of the suggested approach can eliminate these problems.

We await Phase I clinical trials of our nanoformulation, specially the cancer drugs paclitaxel and tamoxifen once the pre-clinical, *in vivo* studies are completed.

APPENDIX

COMMERCIAL VIABILITY AND
SUMMARY OF RESULTS

Cross-Reference to Related Applications

This application claims the benefit of priority to U.S. Provisional Patent Application No. 60/959,728, filed July 16, 2007, in collaboration with Northeastern University in Boston, the contents of which are incorporated by reference herein in their entirety.

Field of the Invention

The invention is in the field of therapeutic nanoparticles for medical screening and treatment.

Background of the Invention

Many potent drugs and drug candidates, especially anticancer drugs, are poorly soluble in water (e.g., tamoxifen, paclitaxel, and camptothecin). Their poor solubility results in their low bioavailability and difficulties in preparing dosage forms.

Current attempts to solve this problem are associated with loading poorly soluble drugs (usually hydrophobic molecules) into various nanosized pharmaceutical carriers, such as liposomes (drugs are loaded into the hydrophobic membrane of the liposome), micelles (drugs are loaded into the hydrophobic core of the micelle), and oil-in-water

emulsions. However, many general problems are associated with these approaches. For example, the nanocarriers exhibit relatively low loading efficacy of the drug into the nanocarrier (between 0.5% and 25% by weight, and often below 10% by weight); the protocols cannot be standardized, since each drug requires its own specific conditions for solubilization; scaling up the technology is difficult; controlling surface properties or surface composition of such nanosystems is difficult; and the nanocarriers have insufficient storage stability and demonstrate instability in the body.

Summary of the Invention

The invention is based, at least in part, on the discovery of a universal platform for making stable nanocolloids containing high concentration of water insoluble drugs. This discovery was exploited to develop the invention, which, in one aspect, features a nanoparticle comprising a compound; a first defined solid polymeric layer comprising a first polymer, the first layer surrounding the compound; and a second defined solid polymeric layer comprising a second polymer, the second layer surrounding the first layer, the first polymer and the second polymer having opposite charges, and the nanoparticle having a diameter of between about 100 nm and about 500 nm. In certain embodiments, the nanoparticle has a diameter of between about 100 nm and about 450 nm, between about 100 nm and about 400 nm, between about 100 nm and about 300 nm, between about 100 nm and about 250 nm, between about 100 nm and about 200 nm, between about 100 nm and about 150 nm, or about 100 nm.

In some embodiments, the compound is present in the nanoparticle between about 5% by weight and about 95% by weight, between about 20% by weight and about 90% by weight, between about 40% by weight and about 85% by weight, between about 60%

by weight and about 85% by weight, between about 75% by weight to about 90% by weight, and between about 80% by weight and about 90% by weight.

In other embodiments, the first polymeric layer and the second polymeric layer have a combined thickness of between about 5 nm and about 30 nm, between about 5 nm and about 25 nm, between about 5 nm and about 20 nm, between about 5 nm and about 15 nm, and between about 5 nm and about 10 nm.

In certain embodiments, the first polymer is positively charged and the second polymer is negatively charged. In other embodiments, the first polymer is negatively charged and the second polymer is positively charged.

In some embodiments, the compound is a therapeutic compound described herein. In one embodiment, the compound is a cancer therapeutic described herein. In particular embodiments, the compound is tamoxifen or paclitaxel. In other embodiments, the compound is a low soluble anticancer drugs, camptothecin, topotecan, irinotecan, KRN 5500 (KRN), meso-tetraphenylporphine, dexamethasone, a benzodiazepine, allopurinol, acetohexamide, benzthiazide, chlorpromazine, chlordiazepoxide, haloperidol, indomethacine, lorazepam, methoxsalen, methylprednisone, nifedipine, oxazepam, oxyphenbutazone, prednisone, prednisolone, pyrimethamine, phenindione, sulfisoxazole, sulfadiazine, temazepam, sulfamerazine, ellipticin, porphine derivatives for photo-dynamic therapy, and/or trioxsalen. In some embodiments, the nanoparticle contains more than one type of compound.

In yet other embodiments, the first polymer is poly (dimethyldiallylamide ammonium chloride) (PDDA), poly (allylamine hydrochloride) (PAH), or protamine sulfate (PS). In certain embodiments, the first polymer is poly (allylamine), poly

(dimethyldiallyammonim chloride) polylysine, poly (ethylenimine), poly (allylamine), dextran amine, polyarginine, chitosan, gelatine A, or protamine sulfate. In some embodiments, the second polymer is sodium poly (styrene sulphonate) (PSS) or human serum albumin (HSA). In particular embodiments, the second polymer is polyglutamic or alginic acids, poly(acrylic acid), poly(aspartic acid), poly(glutaric acid), dextran sulfate, carboxymethyl cellulose, hyaluronic acid, sodium alginate, gelatine B, chondroitin sulfate, and/or heparin.

In certain embodiments, the first polymer is a biocompatible and/or biodegradable polymer. In other embodiments, the second polymer is a biocompatible and/or biodegradable polymer. In other embodiments, both the first and the second polymer are biocompatible and/or biodegradable.

In yet other embodiments, the nanoparticle further comprises a third polymeric layer surrounding the second polymeric layer. In particular embodiments, the third polymeric layer comprise a third polymer having an opposite charge from the second polymer. In some embodiments, the third polymeric layer comprises PDDA. In certain embodiments, the first polymer and the third polymer are the same.

In other embodiments, the compound is poorly soluble in water. In particular embodiments, the compound has a solubility in aqueous medium of less than about 10 mg/ml, of less than about 5 mg/ml, of less than about 2.5 mg/ml, of less than about 1 mg/ml, or of less than about 0.5 mg/ml. In some embodiments, outermost polymeric layer is modified with a targeting agent. In certain embodiments, the targeting agent is an antibody. In particular embodiments, the antibody is an antibody against IL2 receptor a, complement system protein C5, CD11a, CD20, TNF-alpha, T cell CD3 receptor, T cell

VLA4 receptor, F protein of RSV, epidermal growth factor receptor, vascular endothelial growth factor, glycoprotein IIb/IIIa, CD52, or epidermal growth factor receptor. In other embodiments, the antibody is a monoclonal 2C5 antibody.

In some embodiments, the nanoparticle does not contain a detergent, surfactant, or oil. In other embodiments, the compound is released from the nanoparticle at a slower rate. In another aspect, the invention features a nanoparticle comprising a compound; and a polymeric coating comprising alternating polymeric layers of oppositely charged polymers; the nanoparticle having a diameter of about 100 nm to about 500 nm. In certain embodiments, the nanoparticle comprises two, three, four, five, or six polymeric layers of oppositely charged polymers.

In certain embodiments, the nanoparticle has a diameter of between about 100 nm and about 450 nm, between about 100 nm and about 400 nm, between about 100 nm and about 300 nm, between about 100 nm and about 250 nm, between about 100 nm and about 200 nm, between about 100 nm and about 150 nm, or about 100 nm.

In some embodiments, the polymers are polymers described herein. In particular embodiments, the nanoparticle comprises a first polymeric layer comprising poly(dimethyldiallylamide ammonium chloride) (PDDA), poly(allylamine hydrochloride) (PAH), or protamine sulfate (PS). In other embodiments, the nanoparticle comprises a second polymeric layer comprising sodium poly (styrene sulphonate) (PSS) or human serum albumin (HSA). In yet other embodiments, the nanoparticle comprises a third polymeric layer comprising poly (dimethyldiallylamide ammonium chloride) (PDDA), poly (allylamine hydrochloride) (PAH), or protamine sulfate (PS). In still other embodiments, the nanoparticle comprises a fourth polymeric layer comprising sodium

poly (styrene sulphonate) (PSS) or human serum albumin (HSA). And in still other embodiments, the nanoparticle comprises a fifth polymeric layer comprising poly (dimethyldiallylamide ammonium chloride) (PDDA), poly (allylamine hydrochloride) (PAH), or protamine sulfate (PS). In yet another embodiment, the nanoparticle comprises a sixth polymeric layer comprising sodium poly (styrene sulphonate) (PSS) or human serum albumin (HSA).

In other embodiments, the compound is poorly soluble in water. In particular embodiments, the compound has a solubility in aqueous medium of less than about 10 mg/ml, of less than about 5 mg/ml, of less than about 2.5 mg/ml, of less than about 1 mg/ml, or of less than about 0.5 mg/ml. In certain embodiments, the compound is a therapeutic compound described herein. In some embodiments, the compound is tamoxifen or paclitaxel, and the compound is present between about 5% by weight and about 95% by weight, between about 20% by weight and about 90% by weight, between about 40% by weight and about 85% by weight, between about 60% by weight and about 85% by weight, between about 75% by weight to about 90% by weight, and between about 80% by weight and about 90% by weight. In some embodiments, the nanoparticle is a nanoparticle described herein.

In another aspect, the invention features a method of making a stable nanoparticle, the method comprising subjecting a water-insoluble compound to ultrasonication; and adding a first polymer to the compound in the presence of ultrasonication, the polymer added at a concentration sufficient to form a stable first polymeric layer around the compound.

In some embodiments, after ultrasonication, the water-insoluble compound has a negative charge in the absence of the polymer. In other embodiments, the polymer added to the compound has a positive charge.

In particular embodiments, the ultrasonication is performed at about 20 °C to about 30 °C. In certain embodiments, the ultrasonication is performed at between about 10 °C and about 40 °C, between about 15 °C and about 35 °C, or between about 10 °C and about 25 °C.

In certain embodiments, the nanoparticle has a diameter of between about 100 nm and about 450 nm, between about 100 nm and about 400 nm, between about 100 nm and about 300 nm, between about 100 nm and about 250 nm, between about 100 nm and about 200 nm, between about 100 nm and about 150 nm, or about 100 nm.

In other embodiments, the compound is poorly soluble in water. In particular embodiments, the compound has a solubility in aqueous medium of less than about 10 mg/ml, of less than about 5 mg/ml, of less than about 2.5 mg/ml, of less than about 1 mg/ml, or of less than about 0.5 mg/ml.

In certain embodiments, the compound is a therapeutic compound described herein. In some embodiments, the compound is tamoxifen or paclitaxel, and the compound is present between about 5% by weight and about 95% by weight, between about 20% by weight and about 90% by weight, between about 40% by weight and about 85% by weight, between about 60% by weight and about 85% by weight, between about 75% by weight to about 90% by weight, and between about 80% by weight and about 90% by weight. In some embodiments, the nanoparticle is a nanoparticle described herein.

In other embodiments, the first polymer is poly (dimethyldiallylamide ammonium chloride) (PDDA), poly (allylamine hydrochloride) (PAH), or protamine sulfate (PS). In particular embodiments, the method further comprising adding a second polymer to the nanoparticle after the first polymeric layer is formed. In some embodiments, the second polymer is sodium poly (styrene sulphonate) (PSS) or human serum albumin (HSA).

In yet another aspect, the invention features a method of treating a subject having a tumor, the method comprising administering to the subject a nanoparticle in an amount sufficient to reduce tumor size or number of tumor cells, wherein the nanoparticle comprises a compound; a first defined solid polymeric layer comprising a first polymer, the first layer surrounding the compound; and a second defined solid polymeric layer comprising a second polymer, the second layer surrounding the first layer, the first polymer and the second polymer having opposite charges, and the nanoparticle having a diameter of about 100 nm to about 500 nm.

In certain embodiments, the nanoparticle has a diameter of between about 100 nm and about 450 nm, between about 100 nm and about 400 nm, between about 100 nm and about 300 nm, between about 100 nm and about 250 nm, between about 100 nm and about 200 nm, between about 100 nm and about 150 nm, or about 100 nm.

In certain embodiments, the compound is a therapeutic compound described herein. In some embodiments, the compound is tamoxifen or paclitaxel, and the compound is present between about 5% by weight and about 95% by weight, between about 20% by weight and about 90% by weight, between about 40% by weight and about 85% by weight, between about 60% by weight and about 85% by weight, between about 75% by weight to about 90% by weight, and between about 80% by weight and about

90% by weight. In some embodiments, the nanoparticle is a nanoparticle described herein.

In some embodiments, the subject is a vertebrate. In certain embodiments, the subject is a mammal. In particular embodiments, the subject is a human.

Detailed Description of the Invention

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein, including GenBank database sequences, are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Definitions

A "subject" is a mammal, e.g., a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, or non-human primate, such as a monkey, chimpanzee, baboon or rhesus.

The term "biodegradable" refers to a substance that is decomposed (e.g., chemically or enzymatically) or broken down in component molecules by natural biological processes (e.g., in vertebrate animals such as humans).

The term "biocompatible" refers to a substance that has no unintended toxic or injurious effects on biological functions in a target organism.

The term "targeting agent" refers to a ligand or molecule capable of specifically or selectively (i.e., non-randomly) binding or hybridizing to, or otherwise interacting with, a desired target molecule. Examples of targeting agents include, but are not limited to, nucleic acid molecules (e.g., RNA and DNA, including ligand-binding RNA molecules such as aptamers, antisense, or ribozymes), polypeptides (e.g., antigen binding proteins, receptor ligands, signal peptides, and hydrophobic membrane spanning domains), antibodies (and portions thereof), organic molecules (e.g., biotin, carbohydrates, and glycoproteins), and inorganic molecules (e.g., vitamins). A nanoparticle described herein can have affixed thereto one or more of a variety of such targeting agents.

The term "nanoparticle" refers to a particle having a diameter in the range of about 50 nm to about 1000 nm. Nanoparticles include particles capable of containing a therapeutic or diagnostic agent that can be released within a subject. The terms "nanoparticle" and "nanocolloids" are used interchangeably herein.

The term, "about" means a numeric value having a range of ± 10% around the cited value.

The term, "treat", "treating" or "treatment" refers to administering a therapy in an amount, manner (e.g., schedule of administration), and/or mode (e.g., route of administration), effective to improve a disorder (e.g., a disorder described herein) or a symptom thereof, or to prevent or slow the progression of a disorder (e.g., a disorder described herein) or a symptom thereof. This can be evidenced by, e.g., an improvement in a parameter associated with a disorder or a symptom thereof, e.g., to a statistically

significant degree or to a degree detectable to one skilled in the art. An effective amount, manner, or mode can vary depending on the subject and may be tailored to the subject. By preventing or slowing progression of a disorder or a symptom thereof, a treatment can prevent or slow deterioration resulting from a disorder or a symptom thereof in an affected or diagnosed subject.

A "solid" layer refers to a defined firm border between a compound within a nanoparticle and the environment external to the compound. For example, nanoparticles described herein can have one or more solid polymeric layers that reduce or restrict the access of external molecules to the compound at the core of the nanoparticle.

The term "polymer", as used herein, refers to a molecule composed of repeated subunits. Such molecules include, but are not limited to, polypeptides, polynucleotides, polysaccharides or polyalkylene glycols. Polymers can also be biodegradable and/or biocompatible.

The terms "polypeptide", "peptide", and "protein" are used interchangeably herein and refer to a polymer of amino acid residues. The terms apply to naturally occurring amino acid polymers as well as amino acid polymers in which one or more amino acid residues are non-natural amino acids. Additionally, such polypeptides, peptides, and proteins include amino acid chains of any length, including full length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

The term "stable" means that, for a period of at least six months after the nanoparticles are made, a majority of the nanoparticles remain intact at RT. As used herein, a compound that is "poorly soluble", when referring to a compound, means a

compound that has a solubility in aqueous medium of less than about 10 mg/ml, such as less than about 1 mg/ml.

The term "drug" refers to any substance used in the prevention, diagnosis, alleviation, treatment, or cure of a disease or condition.

The term "zeta potential" means the electric potential across an ion layer, e.g., a charged polymeric layer, around a charged colloidal nanoparticle.

The methods described in part, a layer-by-layer (LBL) coating technology to make stable colloids of poorly soluble drugs. For this purpose, aqueous suspensions of poorly soluble drugs with a particle size of the order of microns are subjected physical treatment, such as ultrasonic treatment or ball milling (crushing), to decrease the size of individual particles to the nanolevel (e.g., between about 25 nm and about 1000 nm, between about 100 nm and about 500 nm, or between about 100 nm and about 200 nm), which are then stabilized in solution by the formation of a thin polymeric layer on their surface. This polymeric layer prevents particle agglomeration after stopping the physical treatment, which results in the formation of stable colloidal dispersions with high drug content in each colloidal particle (e.g., more than about 50% by weight and up to about 90% by weight). The polymeric coating is formed based on a polyelectrolyte complexing process, when drug nanosuspensions formed by, for example, ultrasonication, are incubated in the presence of a water soluble, polymer (polycation or polyanion) to allow for its deposition on their surface. The first polymeric layer can then be stabilized by the addition of another, oppositely-charged polyelectrolyte, which forms a firm electrostatic complex with the first layer. This results in the appearance of a very thin, but stable, polymeric layer or shell around each nanoparticle of a compound. This shell can prevent

particle agglomeration, and can be easily and reproducibly formed on the surface of any compound particle. By varying the charge density on each polymer, or the number of coating cycles, drug particles can be prepared with a different surface charge and different thickness of the polymeric coat. This, in turn, provides a way to control drug release from such particles.

LbL self-assembly of molecularly organized films was first developed with linear polyions, nanoparticles, dyes and proteins for coating of large surfaces. The formation of alternate outermost layers of the opposite charge at every adsorption cycle is an important part of the procedure. An alternate assembly of linear polyanions and polycations typically provides 1-2 nm growth step for a single bilayer, and a number of bilayers, which can be built up, can vary from one to few hundreds.

Compounds

A nanoparticle as described herein can contain many types of compounds, such as therapeutic agents. Such therapeutic agents can be, but are not limited to, steroids, analgesics, local anesthetics, antibiotic agents, chemotherapeutic agents, immunosuppressive agents, anti-inflammatory agents, antiproliferative agents, antimitotic agents, angiogenic agents, antipsychotic agents, central nervous system (CNS) agents; anticoagulants, fibrinolytic agents, growth factors, antibodies, ocular drugs, and metabolites, analogs, derivatives, fragments, and purified, isolated, recombinant and chemically synthesized versions of these species, and combinations thereof.

Representative useful therapeutic agents include, but are not limited to, tamoxifen, paclitaxel, low soluble anticancer drugs, camptothecin and its derivatives, e.g., topotecan and irinotecan, KRN 5500 (KRN), meso-tetraphenylporphine, dexamethasone,

benzodiazepines, allopurinol, acetohexamide, benzthiazide, chlorpromazine, chlordiazepoxide, haloperidol, indomethacine, lorazepam, methoxsalen, methylprednisone, nifedipine, oxazepam, oxyphenbutazone, prednisone, prednisolone, pyrimethamine, phenindione, sulfisoxazole, sulfadiazine, temazepam, sulfamerazine, ellipticin, porphine derivatives for photo-dynamic therapy, and/or trioxsalen. These agents are commercially available from, e.g., Merck & Co., Barr Laboratories, Avalon Pharma, and Sun Pharma, among others. Nanosized colloidal suspensions of poorly soluble drugs can increase drug solubility and bioavailability.

Polymers

The nanoparticles described herein can be produced by encapsulating a compound described herein within one or more layers of polymers, creating a defined polymeric layer. In some instances, polycation polymers are used. Such polycation polymers include, without limitation, poly (allylamine), poly (dimethyldiallyammonim chloride) polylysine, poly (ethylenimine), poly (allylamine), and natural polycations such as dextran amine, polyarginine, chitosan, gelatine A, and/or protamine sulfate. In other instances, polyanion polymers are used, including, without limitation, poly(styrenesulfonate), polyglutamic or alginic acids, poly(acrylic acid), poly(aspartic acid), poly(glutaric acid), and natural polyelectrolytes with similar ionized groups such as dextran sulfate, carboxymethyl cellulose, hyaluronic acid, sodium alginate, gelatine B, chondroitin sulfate, and/or heparin. These polymers can be synthesized, isolated, or commercially obtained.

In certain instances, biodegradable and/or biocompatible polymers are used. These include, without limitation, substantially pure carbon lattices (e.g., graphite),

dextran, polysaccharides, polypeptides, polynucleotides, acrylate gels, polyanhydride, poly(lactide-co-glycolide), polytetraflouroethylene, polyhydroxyalkonates, cross-linked alginates, gelatin, collagen, cross-linked collagen, collagen derivatives (such as succinylated collagen or methylated collagen), cross-linked hyaluronic acid, chitosan, chitosan derivatives (such as methylpyrrolidone-chitosan), cellulose and cellulose derivatives (such as cellulose acetate or carboxymethyl cellulose), dextran derivatives (such carboxymethyl dextran), starch and derivatives of starch (such as hydroxyethyl starch), other glycosaminoglycans and their derivatives, other polyanionic polysaccharides or their derivatives, polylactic acid (PLA), polyglycolic acid (PGA), a copolymer of a polylactic acid and a polyglycolic acid (PLGA), lactides, glycolides, and other polyesters, polyglycolide homoploymers, polyoxanones and polyoxalates, copolymer of poly(bis(p-carboxyphenoxy)propane)anhydride (PCPP) and sebacic acid, poly(1-glutamic acid), poly(d-glutamic acid), polyacrylic acid, poly(dl-glutamic acid), poly(1-aspartic acid), poly(d-aspartic acid), poly(dl-aspartic acid), polyethylene glycol, copolymers of the above listed polyamino acids with polyethylene glycol, polypeptides, such as, collagen-like, silk-like, and silk-elastin-like proteins, polycaprolactone, poly(alkylene succinates), poly(hydroxy butyrate) (PHB), poly(butylene diglycolate), nylon-2/nylon-6-copolyamides, polydihydropyrans, polyphosphazenes, poly(ortho ester), poly(cyano acrylates), polyvinylpyrrolidone, polyvinylalcohol, poly casein, keratin, myosin, and fibrin, silicone rubbers, or polyurethanes, and the like. Other biodegradable materials that can be used include naturally derived polymers, such as acacia, gelatin, dextrans, albumins, alginates/starch, and the like; or synthetic polymers, whether

hydrophilic or hydrophobic. The materials can be synthesized, isolated, and are commercially available.

Targeting Agents

In some instances, a nanoparticle described herein includes a targeting agent that is attached, fixed, or conjugated to, the nanoparticle via the outermost layer of the nanoparticle. In certain situations, the targeting agent specifically binds to a particular biological target. Nonlimiting examples of biological targets include tumor cells, bacteria, viruses, cell surface proteins, cell surface receptors, cell surface polysaccharides, extracellular matrix proteins, intracellular proteins and intracellular nucleic acids. The targeting agents can be, for example, various specific ligands, such as antibodies, monoclonal antibodies and their fragments, folate, mannose, galactose and other mono-, di-, and oligosaccharides, and RGD peptide.

The nanoparticles and methods described herein are not limited to any particular targeting agent, and a variety of targeting agents can be used. Examples of such targeting agents include, but are not limited to, nucleic acids (e.g., RNA and DNA), polypeptides (e.g., receptor ligands, signal peptides, avidin, Protein A, and antigen binding proteins), polysaccharides, biotin, hydrophobic groups, hydrophilic groups, drugs, and any organic molecules that bind to receptors. In some instances, a nanoparticle described herein can be conjugated to one, two, or more of a variety of targeting agents. For example, when two or more targeting agents are used, the targeting agents can be similar or dissimilar. Utilization of more than one targeting agent in a particular nanoparticle can allow the targeting of multiple biological targets or can increase the affinity for a particular target.

The targeting agents can be associated with the nanoparticles in a number of ways. For example, the targeting agents can be associated (e.g., covalently or noncovalently bound) to other subcomponents/elements of the nanoparticle with either short (e.g., direct coupling), medium (e.g., using small-molecule bifunctional linkers such as SPDP (Pierce Biotechnology, Inc., Rockford, IL)), or long (e.g., PEG bifunctional linkers (Nektar Therapeutics, Inc., San Carlos, CA)) linkages. Alternatively, such agents can be directly conjugated to the outermost polymeric layer.

In addition, polymers used to produce the nanoparticles described herein can also incorporate reactive groups (e.g., amine groups such as polylysine, dextranemine, profamine sulfate, and/or chitosan). The reactive group can allow for further attachment of various specific ligands or reporter groups (e.g., ^{125}I, ^{131}I, I, Br, various chelating groups such as DTPA, which can be loaded with reporter heavy metals such as ^{111}In, 99m-Tc, GD, Mn, fluorescent groups such as FITC, rhodamine, Alexa, and quantum dots), and/or other moieties (e.g., ligands, antibodies, and/or portions thereof). These moieties can also be incorporated into the polymeric shell during its formation of a nanoparticle described herein.

Antibodies as Targeting Agents

In some instances, the targeting agents are antigen binding proteins or antibodies or binding portions thereof. Antibodies can be generated to allow for the specific targeting of antigens or immunogens (e.g., tumor, tissue, or pathogen specific antigens) on various biological targets (e.g., pathogens, tumor cells, and normal tissue). Such antibodies include, but are not limited to, polyclonal antibodies; monoclonal antibodies or antigen binding fragments thereof; modified antibodies such as chimeric antibodies, reshaped antibodies, humanized antibodies, or fragments thereof (e.g., Fv, Fab', Fab,

F(ab')₂); or biosynthetic antibodies, e.g., single chain antibodies, single domain antibodies (DAB), Fvs, or single chain Fvs (scFv).

Methods of making and using polyclonal and monoclonal antibodies are well known in the art [50]. Methods for making modified antibodies and antibody fragments (e.g., chimeric antibodies, reshaped antibodies, humanized antibodies, or fragments thereof, e.g., Fab', Fab, F(ab')₂ fragments); or biosynthetic antibodies (e.g., single chain antibodies, single domain antibodies (DABs), Fv, single chain Fv (scFv), and the like), are known in the art and can be found [51] .

In some instances, the antibodies recognize tumor specific epitopes (e.g., TAG-72 U.S. 5,892,020; 5,892,019; and 5,512,443) [52]; human carcinoma antigen (U.S. 5,693,763; 5,545,530; and 5,808,005); TP1 and TP3 antigens from osteocarcinoma cells (U.S. 5,855,866); Thomsen-Friedenreich (TF) antigen from adenocarcinoma cells (U.S. 5,110,911); "KC-4 antigen" from human prostrate adenocarcinoma (U.S. 4,708,930 and 4,743,543); a human colorectal cancer antigen (U.S. 4,921,789); CA125 antigen from cystadenocarcinoma (U.S. 4,921,790); DF3 antigen from human breast carcinoma (U.S. 4,963,484 and 5,053,489); a human breast tumor antigen (U.S. 4,939,240); p97 antigen of human melanoma (U.S. 4,918,164); carcinoma or orosomucoid-related antigen (CORA) (U.S. 4,914,021); a human pulmonary carcinoma antigen that reacts with human squamous cell lung carcinoma but not with human small cell lung carcinoma (U.S. 4,892,935); T and Tn haptens in glycoproteins of human breast carcinoma [53], MSA breast carcinoma glycoprotein [54]; MFGM breast carcinoma antigen [55]; DU-PAN-2 pancreatic carcinoma antigen [56]; CA125 ovarian carcinoma antigen [57]; and YH206 lung carcinoma antigen [58] .

For example, to target breast cancer cells, the nanoparticles can be modified with folic acid, EGF, FGF, and antibodies (or antibody fragments) to the tumor-associated antigens MUC 1, cMet receptor and CD56 (NCAM).

Other antibodies that can be used recognize specific pathogens (e.g., *Legionella peomophilia, Mycobacterium tuberculosis, Clostridium tetani, Hemophilus influenzae, Neisseria gonorrhoeae, Treponema pallidum, Bacillus anthracis, Vibrio cholerae, Borrelia burgdorferi, Cornebacterium diphtheria, Staphylococcus aureus,* human papilloma virus, human immunodeficiency virus, rubella virus, and polio virus).

Antibodies or ligands that can be attached to the nanoparticles described herein include, without limitation, antibodies to IL2 receptor a, complement system protein C5, CD11a, CD20, TNF-alpha, T cell CD3 receptor, T cell VLA4 receptor, F protein of RSV, epidermal growth factor receptor, vascular endothelial growth factor, glycoprotein IIb/IIIa, CD52, and epidermal growth factor receptor.

Antibody attachment to nanoparticles can be performed through standard covalent binding to free amine groups in the outermost polycation layer of polylysine or amine dextran [59-61].

For example, during formation of a polycation /polyanion multilayer shell, at every stage of the assembly, about 50% of pending ionized groups reacts with a previous layer, and another about 50% is free at the outermost shell providing a surface charge indicated by a given surface potential. Therefore, the number of amine or acidic reactive groups at the outermost shell may correspond to half of the pending groups in the polymer, e.g., 3,000 pending amine groups for poly (lysine) or poly (allylamine) in the outermost layer

of a 100 nm diameter nanoshell. Standard methods of protein covalent binding are known, such as covalent binding through amine groups [62].

To activate the polymer coat of the particle, a polymer can be used for the last layer of the particle which has free amino, carboxy, SH-, epoxy-, and/or other groups that can react with ligand molecules directly or after preliminary activation with, e.g., carbodiimides, SPDP, SMCC, and/or other mono- and bifunctional reagents.

Signal Peptides as Targeting Agents

In some instances, the targeting agents include a signal peptide. These peptides can be chemically synthesized or cloned, expressed and purified using known techniques. Signal peptides can be used to target the nanoparticles described herein to a discreet region within a cell. In some situations, specific amino acid sequences are responsible for targeting the nanoparticles into cellular organelles and compartments. For example, the signal peptides can direct a nanoparticle described herein into mitochondria. In other examples, a nuclear localization signal is used.

Nucleic Acids as Targeting Agents

In other instances, the targeting agent is a nucleic acid (e.g., RNA or DNA). In some examples, the nucleic acid targeting agents are designed to hybridize by base pairing to a particular nucleic acid (e.g., chromosomal DNA, mRNA, or ribosomal RNA). In other situations, the nucleic acids bind a ligand or biological target. For example, the nucleic acid can bind reverse transcriptase, Rev or Tat proteins of HIV [63]; human nerve growth factor [64]; or vascular endothelial growth factor [65]. Nucleic acids that bind ligands can be identified by known methods, such as the SELEX procedure (see, e.g., U.S. 5,475,096; 5,270,163; and 5,475,096; and WO 97/38134; WO 98/33941; and WO 99/07724). The targeting agents can also be aptamers that bind to particular sequences.

Other Targeting Agents

The targeting agents can recognize a variety of epitopes on preselected biological targets (e.g., pathogens, tumor cells, or normal cells). For example, in some instances, the targeting agent can be sialic acid to target HIV [66], influenza [67], Chlamydia [68] , Neisseria meningitidis, Streptococcus suis, Salmonella, mumps, newcastle, reovirus, Sendai virus, and myxovirus; and 9-OAC sialic acid to target coronavirus, encephalomyelitis virus, and rotavirus; non-sialic acid glycoproteins to target cytomegalovirus [69] and measles virus [70]; CD4 [71], vasoactive intestinal peptide [72], and peptide T [73] to target HIV; epidermal growth factor to target vaccinia [74]; acetylcholine receptor to target rabies [75]; Cd3 complement receptor to target Epstein-Barr virus [76]; .beta.-adrenergic receptor to target reovirus [77]; ICAM-1 [78], N-CAM, and myelin-associated glycoprotein MAb [79] to target rhinovirus; polio virus receptor to target polio virus [80]; fibroblast growth factor receptor to target herpes virus [81]; oligomannose to target Escherichia coli; and ganglioside G_{M1} to target Neisseria meningitides.

In other instances, the targeting agent targets nanoparticles according to the disclosure to factors expressed by oncogenes. These can include, but are not limited to, tyrosine kinases (membrane-associated and cytoplasmic forms), such as members of the Src family; serine/threonine kinases, such as Mos; growth factor and receptors, such as platelet derived growth factor (PDDG), SMALL GTPases (G proteins), including the ras family, cyclin-dependent protein kinases (cdk), members of the myc family members, including c-myc, N-myc, and L-myc, and bcl-2 family members.

In addition, vitamins (both fat soluble and non-fat soluble vitamins) can be used as targeting agents to target biological targets (e.g., cells) that have receptors for, or

otherwise take up, vitamins. For example, fat soluble vitamins (such as vitamin D and its analogs, vitamin E, Vitamin A), and water soluble vitamins (such as Vitamin C) can be used as targeting agents.

Therapeutic Administration

The nanoparticles described herein can be used to treat (e.g., mediate the translocation of drugs into) diseased cells and tissues. In this regard, various diseases are amenable to treatment using the nanoparticles and methods described herein. An exemplary, nonlimiting list of diseases that can be treated with the subject nanoparticles includes breast cancer; prostate cancer; lung cancer; lymphomas; skin cancer; pancreatic cancer; colon cancer; melanoma; ovarian cancer; brain cancer; head and neck cancer; liver cancer; bladder cancer; non-small lung cancer; cervical carcinoma; leukemia; neuroblastoma and glioblastoma; T and B cell mediated autoimmune diseases; inflammatory diseases; infections; hyperproliferative diseases; AIDS; degenerative conditions, vascular diseases, and the like. In some cases, the treated cancer cells are metastatic. The route and/or mode of administration of a nanoparticle described herein can vary depending upon the desired results. Dosage regimens can be adjusted to provide the desired response, e.g., a therapeutic response.

Methods of administration include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intracerebral, intravaginal, transdermal, rectal, by inhalation, or topical, particularly to the ears, nose, eyes, or skin. The mode of administration is left to the discretion of the practitioner.

In some instances, a nanoparticle described herein is administered locally. This is achieved, for example, by local infusion during surgery, topical application (e.g., in a cream or lotion), by injection, by means of a catheter, by means of a suppository or enema, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In some situations, a nanoparticle described herein is introduced into the central nervous system, circulatory system or gastrointestinal tract by any suitable route, including intraventricular, intrathecal injection, paraspinal injection, epidural injection, enema, and by injection adjacent to the peripheral nerve. Intraventricular injection can be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

This disclosure also features a device for administering a nanoparticle described herein. The device can include, e.g., one or more housings for storing pharmaceutical compositions, and can be configured to deliver unit doses of a nanoparticle described herein.

Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent, or via perfusion in a fluorocarbon or synthetic pulmonary surfactant.

In some instances, a nanoparticle described herein can be delivered in a vesicle, in particular, a liposome [82, 83].

In yet other situations, a nanoparticle described herein can be delivered in a controlled-release system or sustained-release system [84]. Other controlled or

sustained-release systems discussed in the review by Langer, Science 249:1527-1533 (1990) can be used. In one case, a pump can be used [82, 85-87].

In yet other situations, a controlled- or sustained-release system can be placed in proximity of a target of nanoparticle described herein, reducing the dose to a fraction of the systemic dose.

A nanoparticle described herein is formulated as a pharmaceutical composition that includes a suitable amount of a physiologically acceptable excipient [88]. Such physiologically acceptable excipients can be, e.g., liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The physiologically acceptable excipients can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea and the like. In addition, auxiliary, stabilizing, thickening, lubricating, and coloring agents can be used. In one situation, the physiologically acceptable excipients are sterile when administered to an animal. The physiologically acceptable excipient should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms. Water is a particularly useful excipient when a nanoparticle described herein is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid excipients, particularly for injectable solutions. Suitable physiologically acceptable excipients also include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Other examples of suitable physiologically acceptable excipients are described in reference [88]. The pharmaceutical compositions,

if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

Liquid carriers can be used in preparing solutions, suspensions, emulsions, syrups, and elixirs. A nanoparticle described herein can be suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both, or pharmaceutically acceptable oils or fat. The liquid carrier can contain other suitable pharmaceutical additives including solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers, or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (particular containing additives described herein, e.g., cellulose derivatives, including sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g., glycols) and their derivatives, and oils (e.g., fractionated coconut oil and arachis oil). For parenteral administration the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. The liquid carriers can be in sterile liquid form for administration. The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellant.

In other instances, a nanoparticle described herein is formulated for intravenous administration. Compositions for intravenous administration can comprise a sterile isotonic aqueous buffer. The compositions can also include a solubilizing agent. Compositions for intravenous administration can optionally include a local anesthetic such as lignocaine to lessen pain at the site of the injection. The ingredients can be supplied either separately or mixed together in unit dosage form, for example, as a dry

lyophilized powder or water-free concentrate in a hermetically sealed container such as an ampule or sachette indicating the quantity of active agent. Where a nanoparticle described herein is administered by infusion, it can be dispensed, for example, with an infusion bottle containing sterile pharmaceutical grade water or saline. Where a nanoparticle described herein is administered by injection, an ampule of sterile water for injection or saline can be provided so that the ingredients can be mixed prior to administration.

In other circumstances, a nanoparticle described herein can be administered across the surface of the body and the inner linings of the bodily passages, including epithelial and mucosal tissues. Such administrations can be carried out using a nanoparticle described herein in lotions, creams, foams, patches, suspensions, solutions, and suppositories (e.g., rectal or vaginal). In some instances, a transdermal patch can be used that contains a nanoparticle described herein and a carrier that is inert to the nanoparticle described herein, is non-toxic to the skin, and that allows delivery of the agent for systemic absorption into the blood stream via the skin. The carrier can take any number of forms such as creams or ointments, pastes, gels, or occlusive devices. The creams or ointments can be viscous liquid or semisolid emulsions of either the oil in water or water in oil type. Pastes of absorptive powders dispersed in petroleum or hydrophilic petroleum containing a nanoparticle described herein can also be used. A variety of occlusive devices can be used to release a nanoparticle described herein into the blood stream, such as a semi-permeable membrane covering a reservoir containing the nanoparticle described herein with or without a carrier, or a matrix containing the nanoparticle described herein.

A nanoparticle described herein can be administered rectally or vaginally in the form of a conventional suppository. Suppository formulations can be made using methods known to those in the art from traditional materials, including cocoa butter, with or without the addition of waxes to alter the suppository's melting point, and glycerin. Water-soluble suppository bases, such as polyethylene glycols of various molecular weights, can also be used.

The amount of a nanoparticle described herein that is effective for treating disorder or disease is determined using standard clinical techniques known to those with skill in the art. In addition, in vitro or in vivo assays can optionally be employed to help identify optimal dosage ranges. The precise dose to be employed can also depend on the route of administration, the condition, the seriousness of the condition being treated, as well as various physical factors related to the individual being treated, and can be decided according to the judgment of a health-care practitioner. For example, the dose of a nanoparticle described herein can each range from about 0.001 mg/kg to about 250 mg/kg of body weight per day, from about 1 mg/kg to about 250 mg/kg body weight per day, from about 1 mg/kg to about 50 mg/kg body weight per day, or from about 1 mg/kg to about 20 mg/kg of body weight per day. Equivalent dosages can be administered over various time periods including, but not limited to, about every 2 hr, about every 6 hr, about every 8 hr, about every 12 hr, about every 24 hr, about every 36 hr, about every 48 hr, about every 72 hr, about every week, about every two weeks, about every three weeks, about every month, and about every two months. The number and frequency of dosages corresponding to a completed course of therapy can be determined according to the judgment of a health-care practitioner.

In some instances, a pharmaceutical composition described herein is in unit dosage form, e.g., as a tablet, capsule, powder, solution, suspension, emulsion, granule, or suppository. In such form, the pharmaceutical composition can be sub-divided into unit doses containing appropriate quantities of a nanoparticle described herein. The unit dosage form can be a packaged pharmaceutical composition, for example, packeted powders, vials, ampoules, pre-filled syringes or sachets containing liquids. The unit dosage form can be, for example, a capsule or tablet itself, or it can be the appropriate number of any such compositions in package form. Such unit dosage form can contain from about 1 mg/kg to about 250 mg/kg, and can be given in a single dose or in two or more divided doses.

Nanoparticle Drug Kits

A nanoparticle described herein can be provided in a kit. In some instances, the kit includes (a) a container that contains a nanoparticle and, optionally (b) informational material. The informational material can be descriptive, instructional, marketing or other material that relates to the methods described herein and/or the use of the nanoparticles, e.g., for therapeutic benefit.

The informational material of the kits is not limited in its form. In some instances, the informational material can include information about production of the nanoparticle, molecular weight of the nanoparticle, concentration, date of expiration, batch or production site information, and so forth. In other situations, the informational material relates to methods of administering the nanoparticles, e.g., in a suitable amount, manner, or mode of administration (e.g., a dose, dosage form, or mode of administration described herein). The method can be a method of treating a subject having a disorder.

In some cases, the informational material, e.g., instructions, is provided in printed matter, e.g., a printed text, drawing, and/or photograph, e.g., a label or printed sheet. The informational material can also be provided in other formats, such as Braille, computer readable material, video recording, or audio recording. In other instances, the informational material of the kit is contact information, e.g., a physical address, email address, website, or telephone number, where a user of the kit can obtain substantive information about the nanoparticles therein and/or their use in the methods described herein. Of course, the informational material can also be provided in any combination of formats.

In addition to the nanoparticles, the kit can include other ingredients, such as a solvent or buffer, a stabilizer, or a preservative. The kit can also include other agents, e.g., a second or third agent, e.g., other therapeutic agents. The components can be provided in any form, e.g., liquid, dried or lyophilized form. The components can be substantially pure (although they can be combined together or delivered separate from one another) and/or sterile. When the components are provided in a liquid solution, the liquid solution can be an aqueous solution, such as a sterile aqueous solution. When the components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. The solvent, e.g., sterile water or buffer, can optionally be provided in the kit.

The kit can include one or more containers for the nanoparticles or other agents. In some cases, the kit contains separate containers, dividers or compartments for the nanoparticles and informational material. For example, the nanoparticles can be contained in a bottle, vial, or syringe, and the informational material can be contained in a

plastic sleeve or packet. In other situations, the separate elements of the kit are contained within a single, undivided container. For example, the nanoparticles can be contained in a bottle, vial or syringe that has attached thereto the informational material in the form of a label. In some cases, the kit can include a plurality (e.g., a pack) of individual containers, each containing one or more unit dosage forms (e.g., a dosage form described herein) of the nanoparticles. The containers can include a unit dosage, e.g., a unit that includes the nanoparticles. For example, the kit can include a plurality of syringes, ampules, foil packets, blister packs, or medical devices, e.g., each containing a unit dose. The containers of the kits can be air tight, waterproof (e.g., impermeable to changes in moisture or evaporation), and/or light-tight.

The kit can optionally include a device suitable for administration of the nanoparticles, e.g., a syringe or other suitable delivery device. The device can be provided pre-loaded with nanoparticles, e.g., in a unit dose, or can be empty, but suitable for loading.

The invention is further illustrated by the following examples. The examples are provided for illustrative purposes only. They are not to be construed as limiting the scope or content of the invention in any way.

Examples

Example 1: Preparation of Stable
Nanocolloids of Poorly Soluble
Drugs

Stable colloids of poorly soluble drugs were prepared in order to increase their solubilization and bioavailability. To do this, high power sonication of poorly soluble drug aqueous dispersions is used with simultaneous LbL-nanocoating. Such coating reverses and enhances a particle surface charge which prevents re-aggregation of the drug and allows for getting smaller and smaller drug colloids (proportionally to the sonication time).

A simultaneous application of powerful sonication and adsorption of opposite charged polyelectrolytes caused a systematic decrease of insoluble drug particle size to nano-scale in the following process (depicted schematically in Figure 6.1). Sonication energy initially cleaves and cracks the bulk drug, and polyelectrolytes immediately fix this sub-dividing, preventing re-aggregation of the pieces. Longer sonication times allowed for smaller and smaller particles (to about 100 nm diameter) which are stable in water due to an adsorbed monolayer of polyelectrolytes. Further build-up of an organized multilayer shell through LbL architecture (alternate adsorption of polycations and polyanions) caused the formation of thicker shells of about 5 nm to about 30 nm, which controlled the drug release rate.

Methods

Materials and Instruments

The poorly soluble and potent anti-cancer drugs tamoxifen (TMF) and paclitaxel (PCT) were used in these experiments (solubility below 1 µg/mL). All polyelectrolytes

used for the LbL assembly were used at a concentration of 2 mg/mL. Poly (allylamine hydrochloride) (PAH), FITC-labeled PAH, and poly (dimethyldiallylamide ammonium chloride) (PDDA) were used as positively charged polyelectrolytes. Sodium poly (styrene sulphonate) (PSS) was used as a negatively charged polyelectrolyte. Deionized water and PBS at pH 7.4 were used as solvents. Drug crystal disintegrations were performed using an Ultra Sonicator 3000 (Misonix Inc, Farmingdale, NY) at 3-18 W for 10-30 minute. To prevent sample overheating during the sonication and to keep the temperature in the range of 20-30°C, liquid nitrogen was used to cool the sample tubes. The thickness of the polyelectrolyte multilayer was measured using a Quartz Crystal Microbalance (9 MHz QCM, USI-System, Japan). Surface potential (zeta potential) and particle size measurements were performed using ZetaPlus Microelectrophoresis (Brookhaven Instruments). A Field Emission Scanning Electron Microscope (Hitachi) was used for particle imaging. A Laser Scanning Confocal Microscope (Leica TCS SP2 from Leica Microsystems Inc.) was also used to control shell formation and to follow colloid stability.

LbL Assembly and Properties of Nanoparticles

Initially, all drug samples were disintegrated using ultrasonication with cooling at 18 W for up to 30 minutes in 1 mL volume before any polyelectrolyte was added. The size of the drug particles formed was periodically measured. Prior to the addition of the first layer of polyelectrolyte, the zeta potential reading was also taken. Polycations were used to form the first surface layer, since drug nanoparticles of both drugs were found to bear an intrinsic negative charge. Drug samples were then centrifuged at 14,000 rpm for 7 minute, washed, and re-suspended in either water or PBS to remove excess

polyelectrolyte. Zeta potential readings were then taken. The coating process was repeated using the polyanion polymer but without ultrasonication. Zeta potential measurements were taken after each layer was added.

Images of colloidal particles formed were taken both immediately and at 48 hrs following LbL assembly to analyze the stability of the colloids formed. Dry samples were prepared for SEM imaging using 5 μL -10 μL of the colloidal suspension obtained. Sample droplets on bare silicon wafers were dried by heating them at 50°C for 1 hr or by storing them overnight at RT. Drug colloids were kept in a low volume of saturated solution to prevent drug release.

Drug Release from Colloidal Particles at Sink Conditions

To determine the release rate of different drugs from the colloidal particles prepared using LbL assembly, samples prepared using differing numbers of coating cycles were placed in 1 mL horizontal diffusion chambers made of cellulose acetate membrane. The samples were then stirred in a large volume of PBS, pH 7.2, to mimic the sink conditions expected in vivo. The concentrations of the released drugs were measured by HPLC.

Attachment of Ligand Moieties to the LbL Nanocolloids of Poorly Soluble Drugs

To prepare nanocolloids with a "reactive" surface suitable for covalent attachment of various ligands, PAH containing free amino groups was used to form the outer layer on the drug particles. Paclitaxel was used as the drug in this series of experiments. The monoclonal nucleosome-specific 2C5 antibody (mAb 2C5) was conjugated to LbL paclitaxel nanoparticles. This antibody recognizes a broad variety of cancer cells via

cancer cell surface-bound nucleosomes [41, 42]. The antibody was conjugated in two steps (Figure 6.2). In the first step, the carboxylate groups on mAb 2C5 were activated using 1-ethyl-3-carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS), rendering the antibody amine-reactive. In the second step, the activated antibody was added to LbL paclitaxel nanoparticles coated with polyamino-containing PAH polymer. All reactions were carried out in HBS, pH 7.4, at 4°C with continuous stirring in the presence of argon gas. The modified particles were centrifuged at 12 rpm for 10 minute and re-suspended twice using PBS to remove unconjugated antibody.

The amount of paclitaxel in the nanoparticle preparations was measured by reversed phase HPLC. A D-7000 HPLC system equipped with a diode array (Hitachi, Japan) and Spherisorb ODS2 column, 4.6 mm × 250 mm (Waters, Milford, MA, USA) was used. The particles were dissolved with the mobile phase prior to loading into the HPLC column. The column was eluted with acetonitrile/water (65:35%, v/v) at 1.0 mL/min. A Paclitaxel peak was detected at 227 nm. Injection volume was 50 μL. All samples were analyzed in triplicate.

Antibody Activity Preservation on the Surface of LbL Drug Nanoparticles

To verify the preservation of mAb 2C5 specific activity after the conjugation with LbL-paclitaxel nanoparticles, a standard ELISA was performed. Briefly, ELISA plates pretreated with 40 μg/ml polylysine solution in TBS, pH 7.4, were coated with 50 μL of 40 μg/mL nucleosomes (the water-soluble fraction of calf thymus nucleohistone, Worthington Biochemical, Lakewood, NJ) and incubated for 1 hr at RT. The plates were then rinsed with 0.2% casein, 0.05% Tween 20 in TBS (casein/TBS), pH 7.4. To these plates, serial dilutions of mAb 2C5-containing samples were added and incubated for 1 hr

at RT. The plates were extensively washed with casein/TBS and coated with horseradish peroxidase goat anti-mouse IgG conjugate (ICN Biomedical, Aurora, OH), diluted according to the manufacturer's recommendation. After 1 hr incubation at RT, the plates were washed with casein/TBS. Bound peroxidase was quantified by the degradation of its substrate, diaminobenzidine, supplied as a ready-for-use solution, Enhanced K-Blue TMB substrate (Neogen, Lexington, KY). The intensity of the color developed was analyzed using a Labsystems Multiscan MCC/340 ELISA reader at 492 nm (Labsystems and Life Sciences International, UK).

Cytotoxicity of Targeted Paclitaxel LbL Nanoparticles

The cytotoxicity of various concentrations of LbL-paclitaxel nanoparticles against MCF-7 and BT-20 cells was studied using an MTT test. A ready-for-use CellTiter 96® Aqueous One solution of MTT (Promega, Madison, WI) was used according to the manufacturer's protocol. Formulations with paclitaxel concentration of up to 200 ng/mL dispersed in Hank's buffer were added to cells grown in 96-well plates to about 40% confluence. After 48 hr or 72 hr of incubation at 37°C, 5% CO_2, plates were washed three times with Hank's buffer. Next, 100 µl of media and 20 µl of CellTiter 96® Aqueous One solution were added to the plates, and the plates were incubated for 1 hr at 37°C, 5% CO_2. The cell survival rate was then estimated by measuring the color intensity of the MTT degradation product at 492 nm using an ELISA plate reader. Untreated cells were considered as 100% growth.

Results

LbL-Stabilized Drug
Nanoparticles and Surface Zeta
Potential

To find optimal sonication conditions, initial experiments were performed with tamoxifen crystals at a concentration of 2 mg/ml in the suspension. As shown in Figure 6.3, particle size could be controlled by the duration of sonication, and decreased with increased sonication time. After 30 minutes of sonication at 18 W, particles of about 100 nm were obtained (polycationic PDDA was added prior to the size measurement to prevent particle re-aggregation). When similar sonication conditions were applied to paclitaxel crystals, particle sizes of about 100 nm were also obtained. Increasing the sonication time further did not result in a significant decrease in drug particle size.

As depicted in Figure 6.4, no surface charge for tamoxifen was observed after normal bath sonication, but a strong negative charge was obtained just after 2.5 sec pulse power sonication. Sequential addition of layers of PAH and PSS resulted in nanoparticles having positive and negative charges, respectively.

Zeta potential was measured during the process of sequential PDDA/PSS adsorption onto tamoxifen cores. After the addition of PDDA, the initially negatively charged nanoparticles were recharged to a positive potential of about +45 mV. The addition of PDDA formed a stable colloidal solution when sonication was terminated. The polyanion PSS was then added to the PDDA-coated tamoxifen nanoparticles, in the presence of sonication, to perform LbL assembly. PSS polyanion adsorption added one more monolayer to the shell, and again reversed the surface potential to a negative value (-17 mV). Next, the PDDA polycation was added again, which resulted in tamoxifen

particles that were positively charged (around +80 mV). Addition of a fourth polymer layer of the polyanion PSS resulted in tamoxifen particles that were again negative. Alternating layers of PDDA and PSS were added to the tamoxifen particles until tamoxifen nanoparticles were formed that were coated with an organized multilayer shell with the composition $(PDDA/PSS)_3$.

Sonicated paclitaxel particles were also initially negatively charged (Figure 6.5). When paclitaxel was coated with either PAH or PDDA, the surface charge was reversed after sonication (Figure 6.5). When the polyanion PSS was subsequently added to paclitaxel/PAH nanoparticles, the resulting nanoparticles had a negative zeta potential. Further assembly using alternating additions of PAH and PSS under sonication resulted in nanoparticles having corresponding changes in zeta potential values, until paclitaxel nanoparticles were formed having a composition of $(PAH/PSS)_2$.

In separate experiments using quartz Crystal Microbalance (QCM) monitoring of the PDDA/PSS or PAH/PSS assembly on quartz resonator, a single polycation/polyanion bilayer was determined to have a thickness of 1.5 nm in the dry state. As polyelectrolyte multilayer thickness doubles in water [22, 28], the thickness of the $(PDDA/PSS)_3$ shells was estimated to be around 4.5 nm in the dry state and around 9 nm in aqueous solution. $(PAH/PSS)_2$ shell thickness was estimated to be around 3 nm in the dry state and 6 nm in aqueous solution.

Nanoparticle Imaging and Some Properties

Scanning electron microscopy (SEM) and confocal fluorescence microscopy were used to confirm the sizes of the nanoparticles formed by the LbL technology described herein. After tamoxifen was sonicated for 20 minutes in the presence of 2 mg/ml PAH,

nanoparticles were obtained that were mainly spherical in shape and had a diameter of 120 ± 30 nm (Figures 6.6 and 6.7). The nanocolloids were stable in water, since SEM images taken after 48 hrs still showed individual non-agglomerated nanosized particles. Adding a first layer of polyanion PSS did not result in tamoxifen size decrease even after 20 minute of sonication.

For paclitaxel, nanoparticles having a (PAH-PSS)$_2$ shell composition were produced having particle sizes of about 91 nm and about 300 nm (Figures 6.9C and 6.10). However, aggregation of some paclitaxel nanoparticles to about 1.5 μm diameter particles was observed. Reducing the initial paclitaxel concentration to 1 mg/ml resulted in nanoparticles having an elongated rod-like shape with dimensions of about 50 nm x about 120 nm, which did not aggregate.

The SEM images were obtained after drying the samples, and during the drying process the nanoparticles become partially aggregated. To demonstrate that this aggregation was the result of SEM sample preparation and that the nanoparticles did not aggregate in aqueous suspension, images of the samples were obtained using confocal fluorescence microscopy.

Tamoxifen nanoparticles were prepared by coating tamoxifen with a layer of FITC-labeled PAH. Fluorescence imaging of these LbL-coated tamoxifen particles in suspension did not reveal any aggregation (Figure 6.11). Paclitaxel nanoparticles coated with FITC-labeled PAH also did not aggregate. Further assembly of PAH-coated tamoxifen nanoparticles, through alternate sequential adsorption of PSS and PAH to build a multilayer was performed, in which the last PAH layer was labeled with FITC.

Figure 6.11 depicts a confocal image of a tamoxifen nanoparticle demonstrating effective LbL encapsulation within a three layer shell.

In other experiments, SEM and confocal images were obtained 2-7 days after sample formation, demonstrating the stability of aqueous drug nanocolloids.

Given that the thickness of a single polymeric layer was about 1.5 nm in dry state, the amount of drug in the stable nanocolloidal particles was calculated to be from about 85% by weight (for tamoxifen particles with the triple PDDA/PSS bilayer coating) to about 90% by weight (for paclitaxel particles with the double PAH/PSS layer coating). Further, colloidal suspensions of both drugs were completely stable during the two weeks of observation.

Drug Release From LbL Nanoparticles

LbL technology can be used to control the drug release rate from polymer-stabilized colloidal nanoparticles by changing the thickness or composition of the nanoparticles. Accordingly, the release of tamoxifen from LbL nanocolloidal particles containing 2 mg/mL tamoxifen and having a single PDDA coating or a coating composition of (PDDA/PSS)$_3$ was measured in standard sink conditions (PBS buffer at pH 7.4). Curves were produced from the experimental data using Peppas' model of exponential approximation [43]. As depicted in Figures 3.9 and 4.4, slower release rates were observed as the number of polyelectrolyte layers in the shell increased. At sink conditions (PBS buffer at pH 7.4), non-coated tamoxifen crystals (both without and with sonication) were solubilized within about 2 hrs. PDDA- and (PDDA/PSS)$_3$-coated nanoparticles were estimated to solubilize at around 10 hrs. Similar results were obtained for paclitaxel. Slower release rates were obtained using LbL coatings containing

different polycations and polyanions and varying the number of shells. Similar results were seen for paclitaxel nanoparticles.

Surface Modification of LbL-coated Drug Nanoparticles and Cytotoxicity Analysis

To demonstrate the ability to derivatize the LbL-coated drug nanoparticles, paclitaxel-containing nanoparticles were produced having one layer of PAH, as described above. The tumor-specific mAb 2C5 was then attached to the PAH-coated paclitaxel nanoparticles via free amino groups on the surface layer of PAH. It was depicted that 2C5-modified LbL-coated paclitaxel nanoparticles specifically recognized the target antigen (i.e., nucleosomes).

The cytotoxicity of the mAb 2C5-modified paclitaxel-containing nanoparticles was determined using MCF-7 cells and BT-20 cells, as described above. Paclitaxel nanoparticles having a single layer of PAH, but without the 2C5 modification, were used as control. After incubating MCF-7 cells for 48 hrs or 72 hrs in the presence of 100 ng/mL unmodified paclitaxel nanoparticles, about 95% of the cells were alive. However, when MCF-7 cells were incubated in the presence of 100 ng/ml 2C5-modified paclitaxel-containing nanoparticles, around 30% of the cells were killed. Similar results were seen when BT-20 cells were incubated in the presence of 30 ng/ml of paclitaxel nanoparticles.

Example 2: Preparation of Stable Nanoparticles of *meso*-Tetraphenylporphyrin and Camptothecin

LbL nanoparticles of meso-tetraphenylporphyrin and camptothecin were prepared as described in Example 1. As depicted in Figure 6.12, *meso*-tetraphenylporphyrin nanoparticles were produced using a coating of FITC-labeled PAH, which reversed the

surface charge from negative to positive. SEM analysis demonstrated particle sizes ranging from about 83 nm to about 194 nm (Figure 6.12).

LbL nanoparticles of camptothecin were also prepared. Optimization of the first polycation coating was performed. Three polycations (PAH, PEI and PDDA) and one polyanion (PSS) were used. In the presence of PSS, which has the same charge as the drug core, no particle size decrease was observed (Figure 6.13). All the polycations were able to reduce the particle size, and the smallest particles were obtained with polylysine treatment. SEM images of camptothecin after 30 minutes of sonication with cationic poly L-lysine detected particles of about 390 nm, whereas sonication with PSS resulted in larger particles.

Some representative results are summarized below in Table 1. The release time for tamoxifen was about 6 hrs.

Table 1. Physical characteristics and dimensions of drug nanoparticles.

Drugs	Particle Size	Coating Thickness	Colloidal Stability
Tamoxifen	125 ± 30 nm	5 nm	<= 7 Days
Paclitaxel	110 ± 30 nm	5 nm	<= 7 Days
meso-Tetraphenylporphine	140 ± 50 nm	5 nm	<= 7 Days
Camptothecin	390 ± 50 nm	5 nm	<= 7 Days

Example 3: Preparation of Stable Nanoparticles of Paclitaxel Using Biocompatible Coatings

LbL drug nanoparticles of paclitaxel were prepared as described in Example 1, but biocompatible materials were used in the coatings. Paclitaxel-containing nanoparticles were prepared with a first layer of protamine sulfate (PS) followed by subsequent

coatings of human serum albumin (HSA). Smaller nanoparticles were obtained with 30 minute sonication + LbL coating with protamine sulfate.

Figure 4.3 depicts zeta potential readings of paclitaxel LbL by biocompatible PS and BSA. As demonstrated, the charge alternates between positive and negative values with each subsequent addition of PS and HSA, respectively.

To determine the release of paclitaxel from these nanoparticles, the release rates through 200 nm membranes over 2 hrs were measured, as described in Example 1. As shown in Figure 4.4, at 2 hrs, 12.06 % paclitaxel was released from naked paclitaxel with sonication, 9.7% of paclitaxel was released from particles with 1 layer of PDDA, 7.41% paclitaxel was released from particles having two (PS-HSA) bilayers, and 3.44% paclitaxel was released from particles having three (PDDA-PSS) bilayers.

Figure 6.14 depicts the sustained release curve for paclitaxel coated with 3 bilayers of biocompatible PS and HSA for 8 hrs at sink conditions at pH 7.3. As demonstrated, these nanoparticles have sustained release for over 500 minutes.

Brief Description of the Drawings

Figures 6.1 through 6.14 are presented for the purpose of illustration only, and are not intended to be limiting.

I. Formation of colloidal nanoparticles

Insoluble drug
crystals

Ultra sonication of the
drug in polyelectrolyte
solution

II. Alternate coating of nanoparticles with polymeric layers

Subsequent drug release
from the final LbL
nanoparticle
(at sink conditions)

Formation of
multibilayered
nannoshell

Formation of the second
layer by the oppositely
charge of polyelectrolyte
(assembly of the first layer)

Drug nanoparticle
stabilized with the first
layer of polyelectrolyte
after the removal of
ultrasound

Figure 6.1 A diagrammatic representation of a method for making a nanoparticle of the invention.

Antibody containing
Carboxylate groups

Sulpho-NHS

EDC, HBS pH 7.4

Carboxyl-activated antibody

Drug nanoparticle with
outer amino layer

Antibody conjugated to drug nanoparticles
through amide bond formation
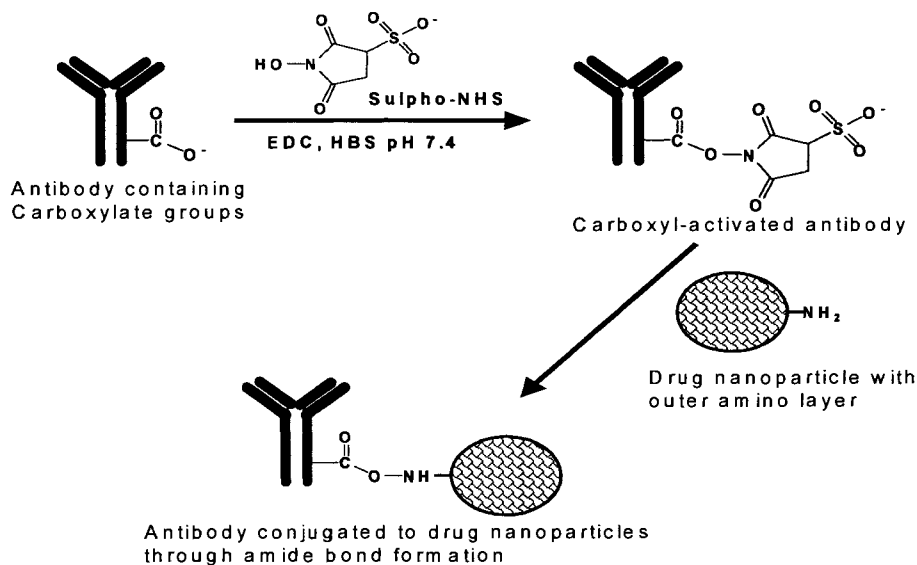
Figure 6.2 A diagrammatic representation of a method of conjugation of an antibody to a nanoparticle of the invention. (Adapted from the book Bioconjugate Techniques by Greg Hermanson)
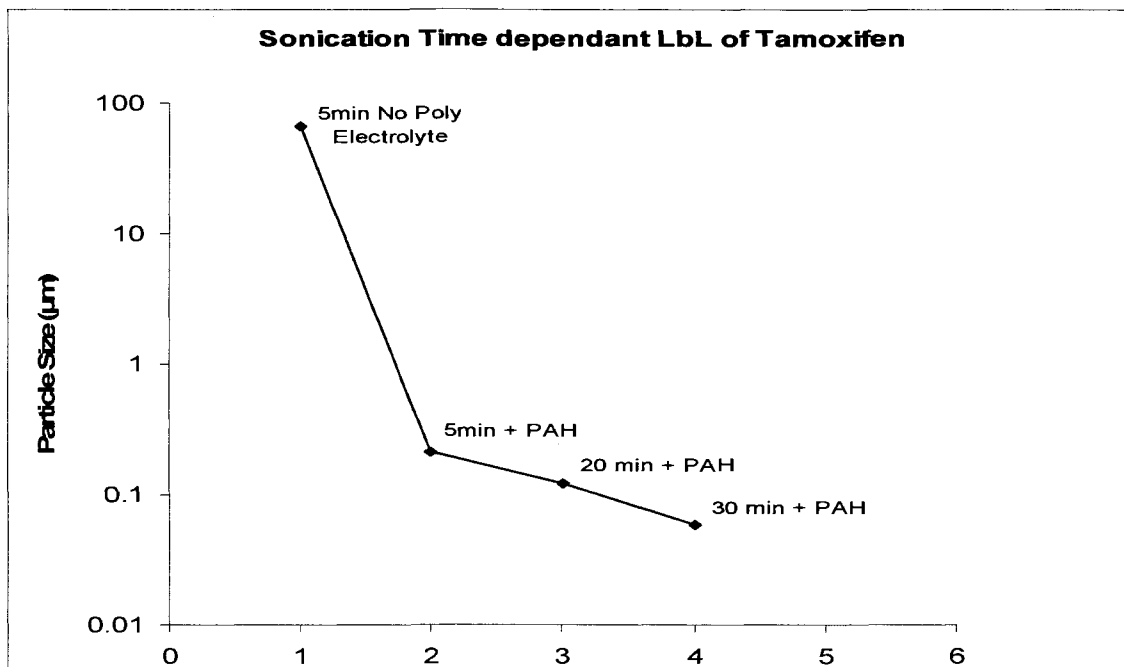
Figure 6.3 A graphic representation of the particle size of nanoparticles containing tamoxifen or paclitaxel particle size following various durations of sonication.
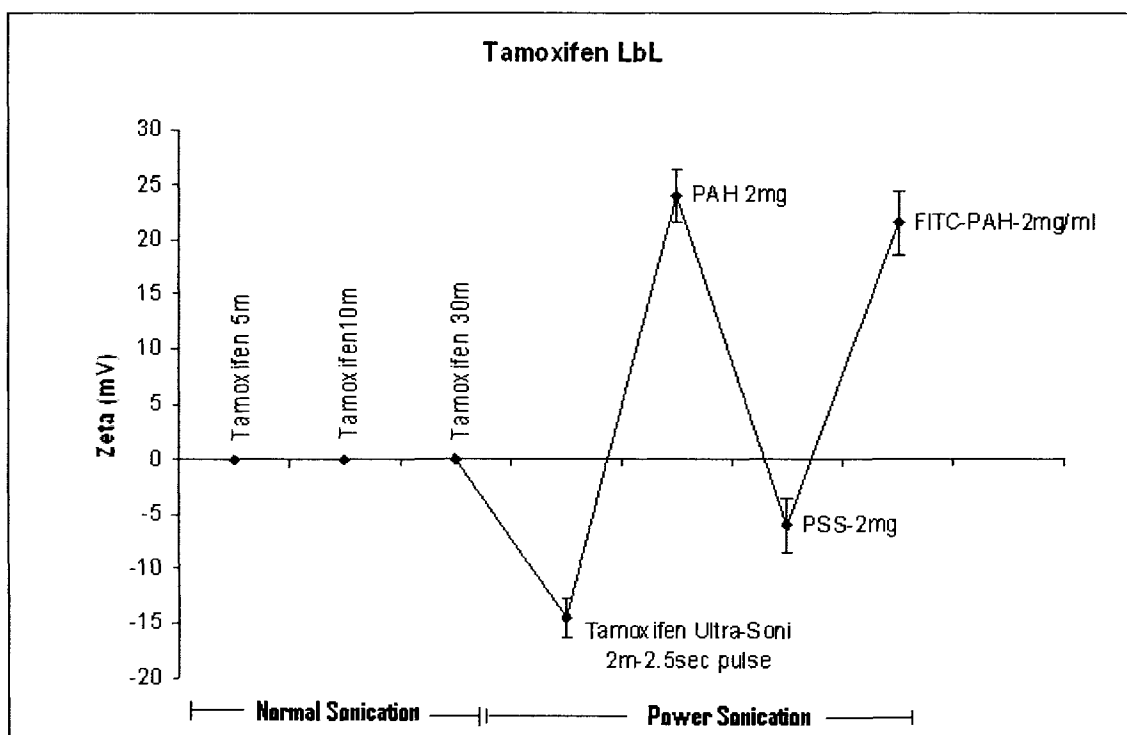
Figure 6.4 A graphic representation of the zeta potential obtained from tamoxifen particles (5 mg/ml) after normal water bath sonication or pulse power sonication.
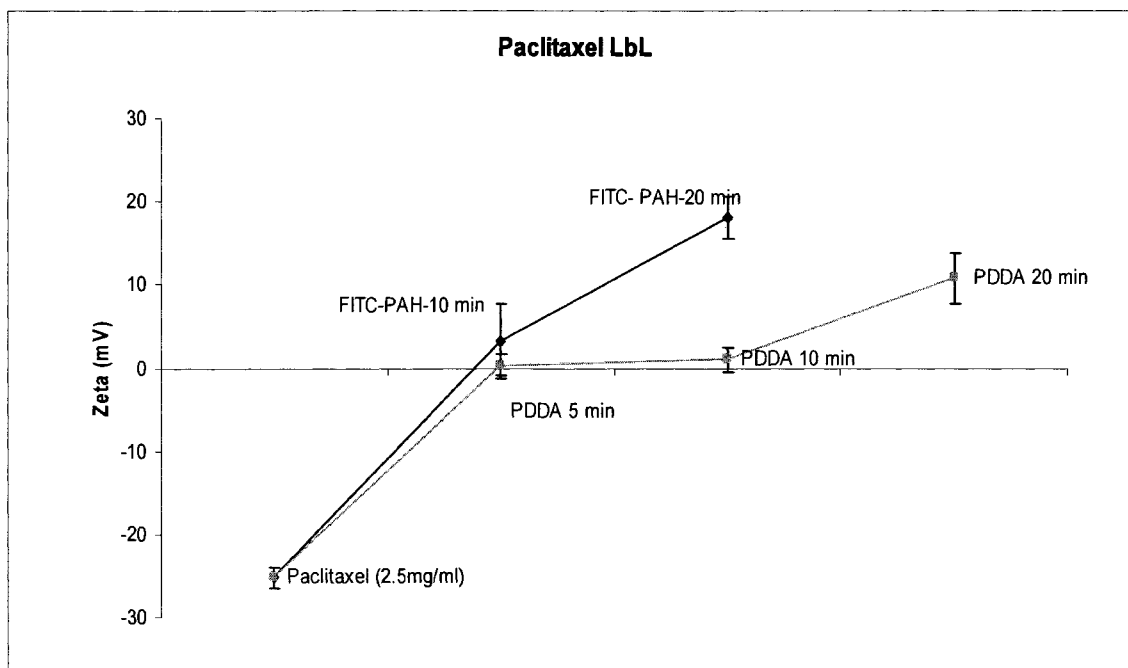
Figure 6.5 A graphic representation of zeta potentials obtained from the addition of PAH and PDDA onto paclitaxel (2.5 mg/ml) containing nanoparticles.
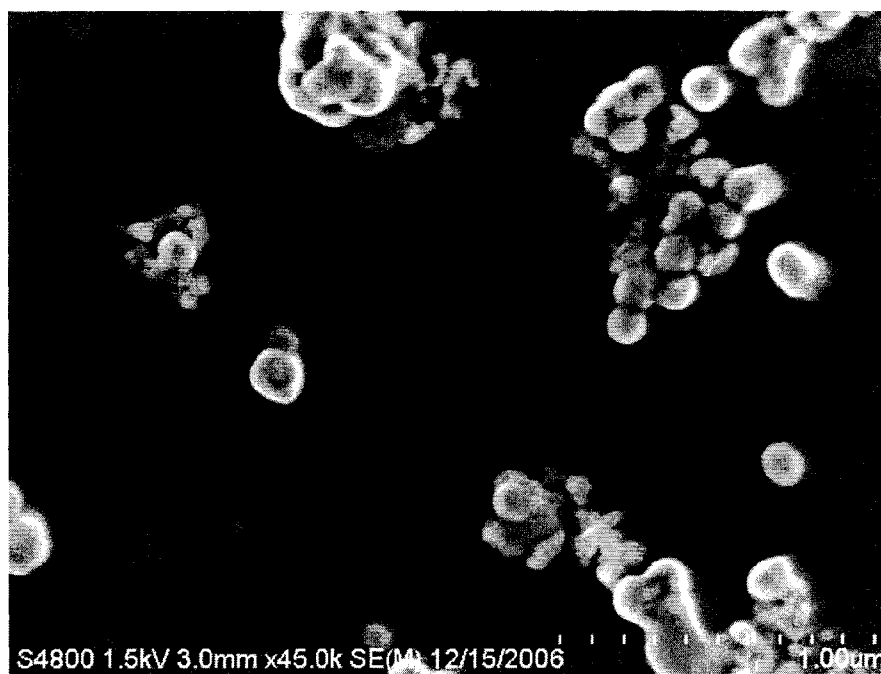
Figure 6.6 A representation of a scanning electron microscopy (SEM) image of tamoxifen-containing nanoparticles with 2 mg/ml PAH at low magnification.
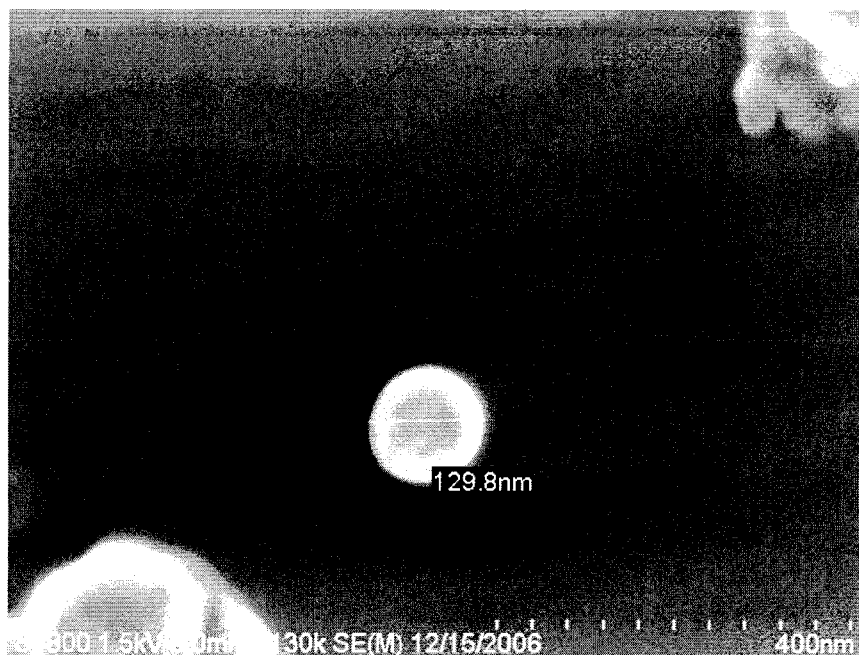
Figure 6.7 Representations of SEM images of tamoxifen-containing nanoparticles at higher magnification.

a)   b)
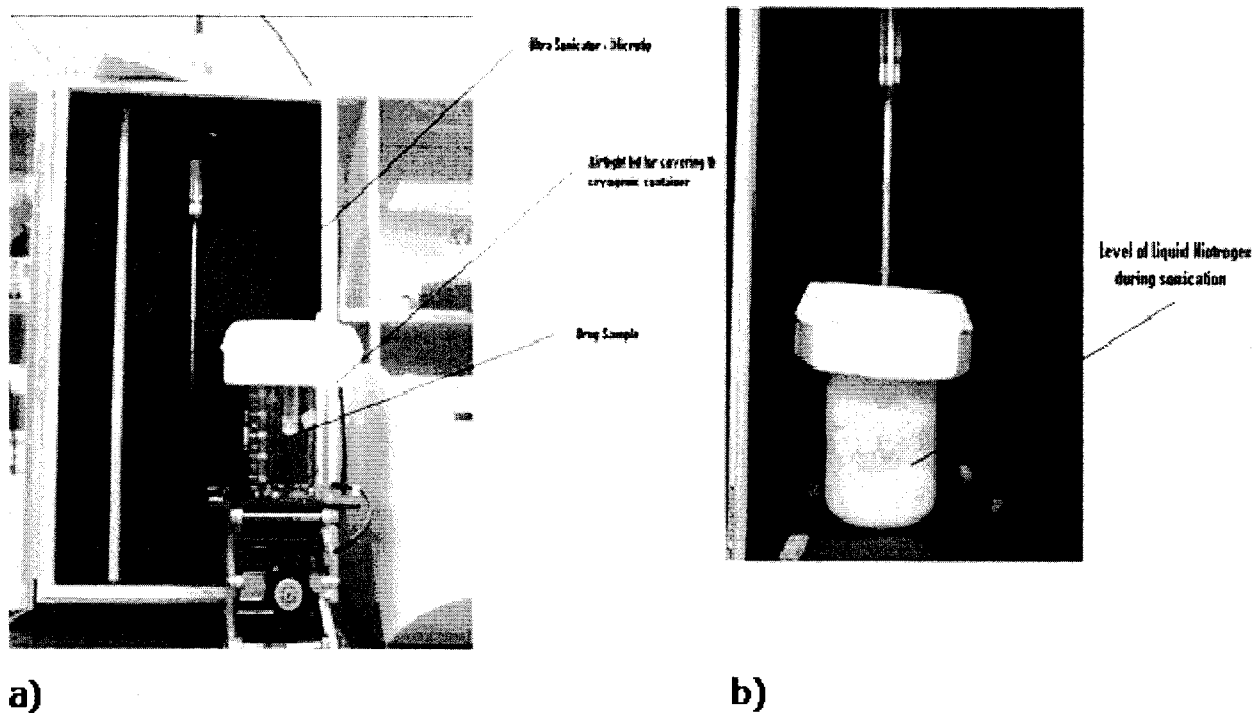
Figure 6.8 (a) Apparatus for crypgenic sonication and (b) picture taken during cryogenic sonication with Liquid Nitrogen surrounding the drug sample. The sample temperatures after sonication were measured with an Infra-Red Thermometer Gun.

a)

b)
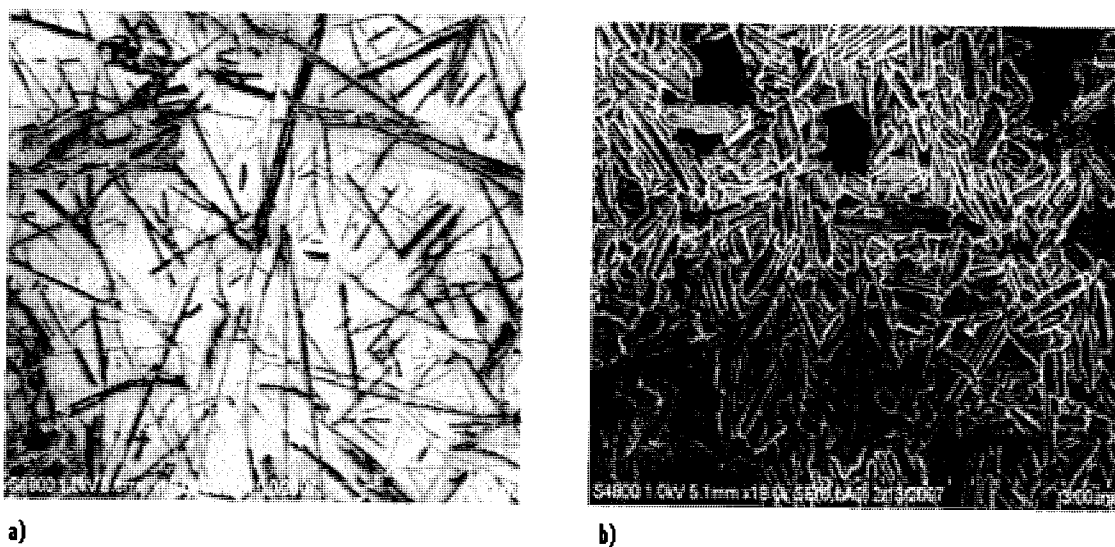
Figure 6.9 (a) Representation of an SEM image of paclitaxel (2 mg/ml) sonicated for 10 minute at 18 W on ice without any polyelectrolyte. (b) Representation of an SEM image of paclitaxel (2 mg/ml) sonicated for 10 minute at 18 W surrounded by liquid nitrogen without any polyelectrolyte.

a)

b)
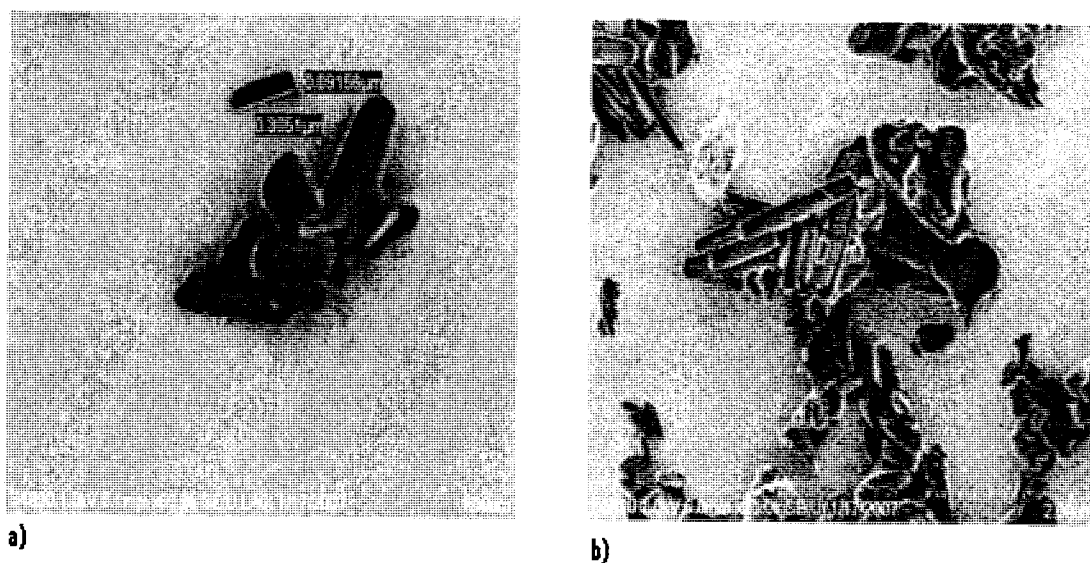
Figure 6.10 (a)-(b) Representation of an SEM image of paclitaxel (2 mg/ml) particles obtained after two bilayer deposition $(PAH-PSS)_2$.

a)   b)
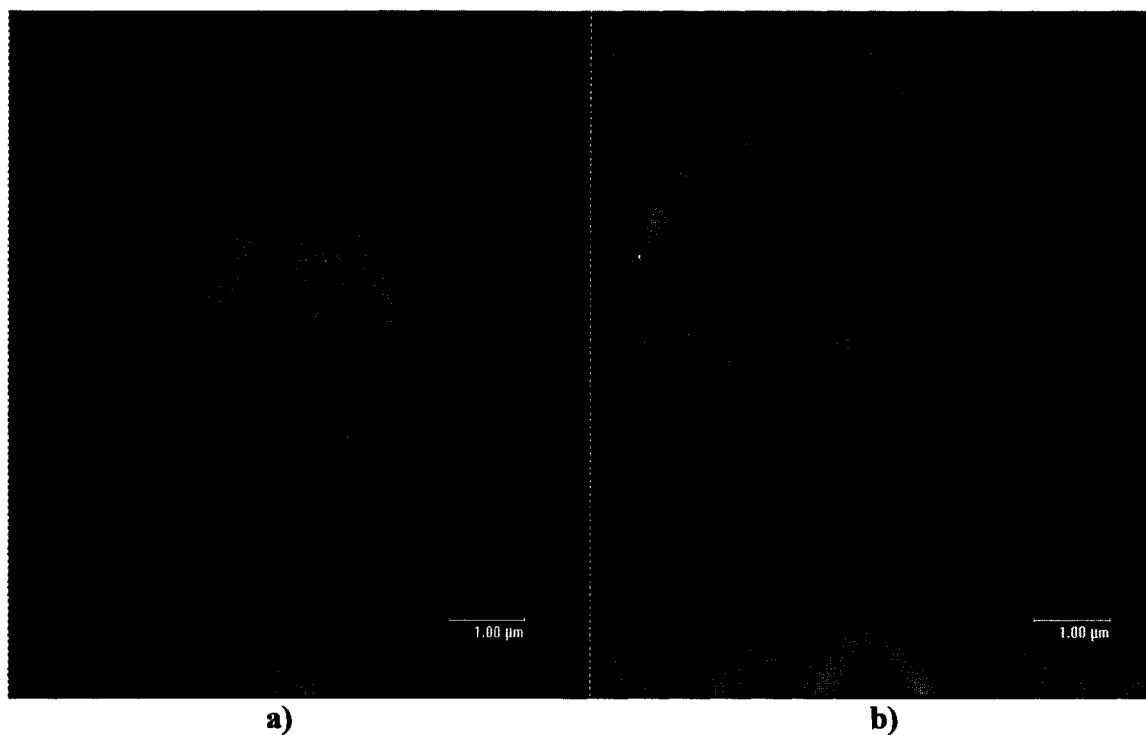
Figure 6.11(a) Representation of a confocal fluorescent image of an aqueous dispersion of tamoxifen-containing nanoparticles coated with FITC-labeled PAH along with (b) transmission image.

a)   b)
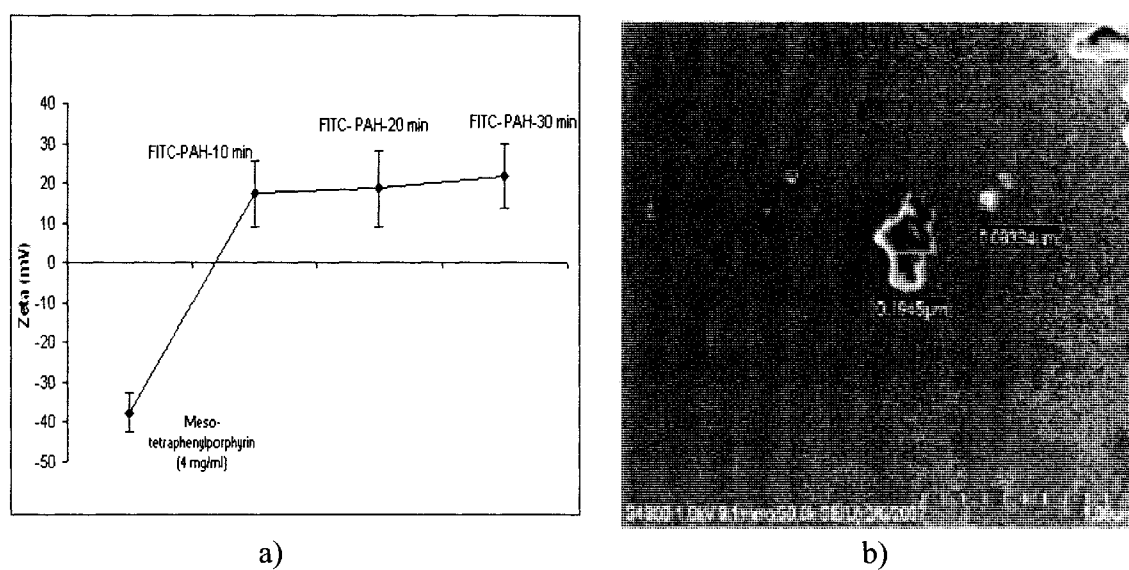
Figure 6.12 A graphic representation of (a) zeta potentials of *meso*-tetraphenylporphyrin containing nanoparticles coated with FITC-PAH (b) showing SEM image of the drug with particle size from 83 to 194 nanometers.
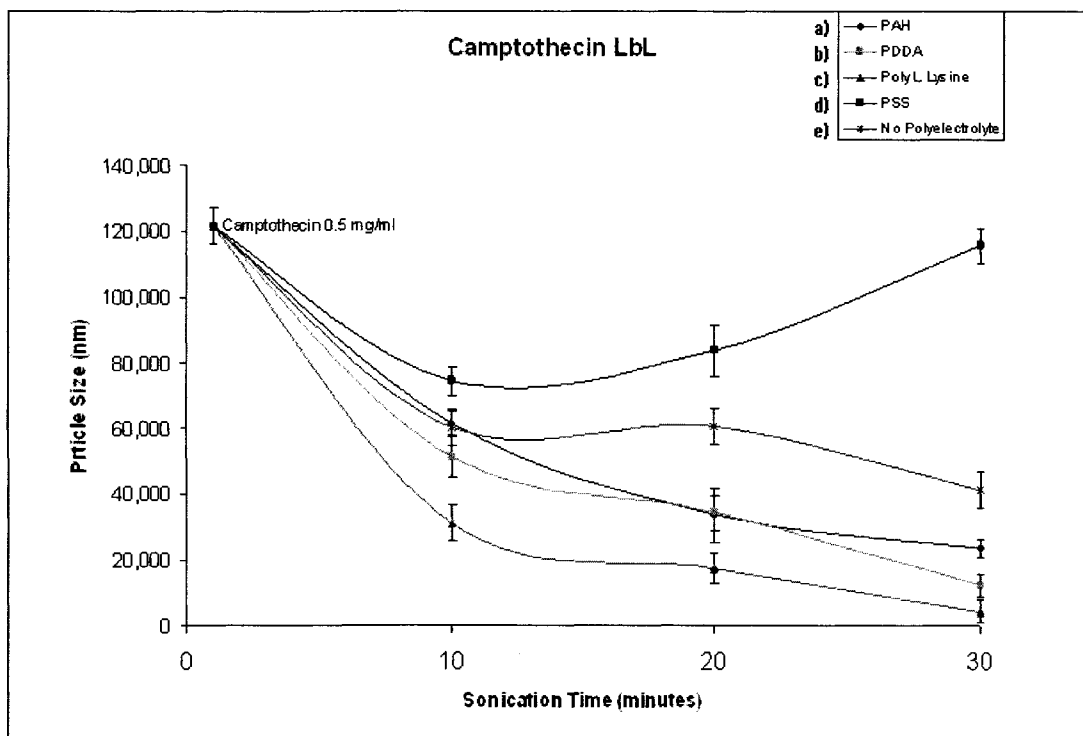
Figure 6.13 A graphic representation of particle size of camptothecin-containing nanoparticles coated with PAH, PDDA, poly L-lysine, PSS, or uncoated.
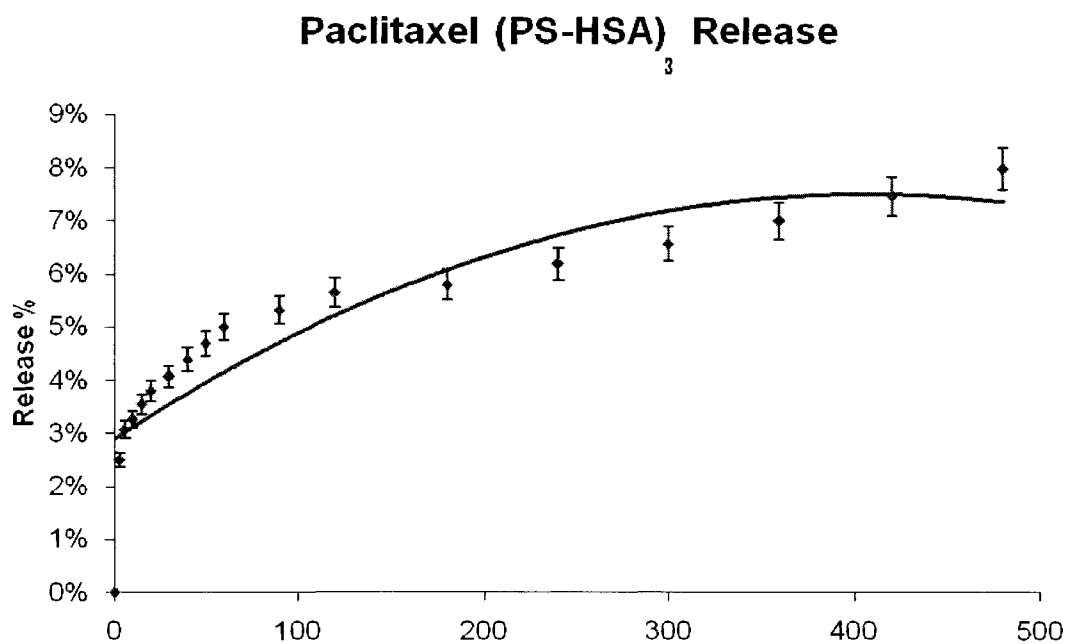
Figure 6.14 A graphic representation of paclitaxel release over time in hrs from paclitaxel-containing nanoparticles coated with (PS-HSA)₃ layers.

Equivalents

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

Claims

1. A stable nanoparticle comprising:

    (a) a compound;

    (b) a first defined solid polymeric layer comprising a first polymer, the first layer surrounding the compound; and

    (c) a second defined solid polymeric layer comprising a second polymer, the second layer surrounding the first layer,

    the first polymer and the second polymer having opposite charges, and the nanoparticle having a diameter of about 100 nm to about 500 nm.

2. The nanoparticle of claim 1, wherein the compound is present at about 5% by weight to about 95% by weight.

3. The nanoparticle of claim 1, wherein the first polymeric layer and the second polymeric layer have a combined thickness of about 5 nm to about 30 nm.

4. The nanoparticle of claim 1, wherein the first polymer is positively charged and the second polymer is negatively charged.

5. The nanoparticle of claim 1, further comprising a third polymeric layer surrounding the second polymeric layer, the third polymeric layer comprising a third polymer having an opposite charge from the second polymer.

6. The nanoparticle of claim 5, wherein the first polymer and the third polymer are the same.

7. The nanoparticle of claim 1, wherein the solubility of the compound is very low.

8. The nanoparticle of claim 1, wherein the second polymeric layer is modified with a targeting agent.

9. The nanoparticle of claim 5, wherein the third polymeric layer is modified with a targeting agent.

10. The nanoparticle of claim 8, wherein the targeting agent is an antibody.

11. The nanoparticle of claim 9, wherein the targeting agent is an antibody.

12. The nanoparticle of claim 1, wherein the nanoparticle does not contain a detergent or a surfactant.

13. The nanoparticle of claim 1, wherein the compound is released from the nanoparticle at a rate dependent on the number of LbL coatings

14. A nanoparticle comprising:

(a) a compound; and

(b) a polymeric coating comprised of alternating polymeric layers of oppositely charged polymers; the nanoparticle having a diameter of about 100 nm to about 500 nm.

15. The nanoparticle of claim 14, wherein the nanoparticle comprises two, three, four, five, or six polymeric layers of oppositely charged polymers.

16. The nanoparticle of claim 14, wherein the compound is present at about 5% by weight to about 95% by weight.

17. The nanoparticle of claim 14, wherein the polymeric layers have a combined thickness of about 5 nm to about 30 nm.

18. A method of making a stable nanoparticle, the method comprising:

subjecting a water-insoluble compound to ultrasonication; and adding a first polymer to the compound in the presence of ultrasonication, the polymer being added at a concentration sufficient to form a stable first polymeric layer around the compound.

19. The method of claim 18, wherein after ultrasonication, the water-insoluble compound has a negative charge in the absence of the polymer.

20. The method of claim 18, wherein the polymer added to the compound has a positive charge.

21. The method of claim 18, wherein the ultrasonication is performed at about 20 °C to about 30 °C.

22. A method of treating a subject having a tumor, the method comprising administering to the subject a nanoparticle in an amount sufficient to reduce tumor size or number of tumor cells, wherein the nanoparticle comprises:

(a) a compound;

(b) a first defined solid polymeric layer comprising a first polymer, the first layer surrounding the compound; and

(c) a second defined solid polymeric layer comprising a second polymer, the

second layer surrounding the first layer, the first polymer and the second polymer having

opposite charges, and the nanoparticle having a diameter of about 100 nm to about 500

nm.

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VITA

Dr. Anshul Agarwal has been associated with Louisiana Tech University for more than 7 years. After completeing his medical school in 2002 from University of Mumbai, India he joined Louisiana Tech University and in 2004 he got a MS degree in biomedical engineering for his work on recombinant hemoglobin for artificial blood substitutes. Then he worked on cancer research as a clinical research associate at Louisiana State University Health Science Center and Fiest Weiller Cancer Center in Shreveport. In 2006, he joined Louisiana Tech Biomedical Engineering PhD program and began working on cancer drug nanocapsules in Dr. Yuri Lvov's group. He developed new technology for 100-nm diameter polyelectrolyte capsules for paclitaxel and tamoxifen targeted delivery. These achievements were recognized by two publications in prestigious international journals and in 1998 applied for US patent together with Louisiana Tech and Northeastern University, Pharmacy Department where some pre-clinical testing of these new drug formulations were performed. During studying at Louisiana Tech University, he got approval of his Medical Doctor degree obtained earlier in India (by US Education Commission for Foreign Medical Graduates) and became a practicing physician. Over the last two years, Dr. Anshul Agarwal has been working as a medical house officer at the Department of Family Medicine at clinics and in-patient ward service, at Louisiana State University Health Science Center, Shreveport. With his

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research work performed in the last three years in parallel at Louisiana Tech and at Louisiana State University Health Science Center, Dr. Agarwal lived his dream of an ideal Biomedical Engineer, a person who is a professional both in medicine and engineering.

Intellectual Property

Licensing: Through Louisiana Tech Research Foundation to Nemucore Medical Innovations Inc, Wellesley, MA, "Therapeutic Stable Nanoparticles," October 18, 2008.

Patenting: Y. Lvov, **A. Agarwal**, V. Torchilin, "Stable Nano-Colloids of Poorly Water Soluble Drugs," US patent application # 60/959,728 (with Northeastern University, Boston, MA), July 16, 2008.

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1. **A. Agarwal**, Y. Lvov, R. Sawant, V. Torchilin, "Stable Nanocolloids of Poorly Soluble Drugs with High Drug Content Prepared Using Sonicated Layer-by-Layer Technology." *J. Controlled Release,* v.128, 255-260, 2008.

2. Y. Lvov, **A. Agarwal**, R. Sawant, V. Torchilin, "Sonication Assisted Polyelectrolyte Nanoencapsulation of Paclitaxel and Tamoxifen." *Pharma Focus Asia,* v.7, 36-40, 2008.

Papers Presented at International Conferences

1. Y. Lvov, **A. Agarwal**, V. Torchilin, "Stable Colloids of Poorly Soluble Drugs Using Layer-by-Layer Nanocoating of Drug Nanoparticles." 34th Controlled Release Society Meeting, Long Beach, CA, July 7-11, 2007.

2. Y. Lvov, **A. Agarwal**, "Layer-by-Layer Nanocoating for Pharmaceuticals: from Microcapsules to Nanoshells." 5th International Nanomedicine and Drug Delivery Symposium, Boston, November 2-3, 2007.

3. **A. Agarwal**, E. Toledo, Y. Lvov, "Nanoengineered Gadolinium- an Approach Towards Developing a Novel Contrast Agent for High Precision MRI." 25th Annual Houston Conference on Biomedical Engineering Research, February 7, 2008.

4. **A. Agarwal**, Y. Lvov, T. Levchenko, V. Torchilin. "Stable Nanocolloids of Paclitaxel through Sonication Assisted Nanoassembly," 83rd ACS Colloid & Surface Science Symposium and 13th Intern Conference on Surface Science, New York, June 14-19, 2009.