

CALCIUM PHOSPHATE COATED LIPOSOMES: A NOVEL APPROACH FOR DRUG TARGETTING



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andhi college of Pharmaceutical sciences, Jaip Abstract

Cancer is a major public health problem in the world. Cancer continues to be a major life-threatening disease. The most limiting factors in treatment of cancer are mutation and drug resistance in the cancer cells, poor selectivity and immunosuppressive action, side effect of the anticancer drugs, and low penetration in solid tumor. Liposomes have raised considerable interest as drug carriers in cancer chemotherapy because of their ability to alter the biodistribution by selectively targeting the drug to the tumor and control the release rate of encapsulated drugs. Such formulation and drug targeting strategies enhance the effectiveness of anticancer chemotherapy and reduce at the same time the risk of toxic side-effects. In spite of desirable feature of liposome as carrier, they suffer from disadvantages such as their rapid clearance from systemic circulation leading to short duration of action, less stability and drug leakage resulting into loss of drug before it reaches the target site. Such draw backs can be easily overcome by coating the liposome with materials such as silica and calcium phosphate. Calcium phosphate coated liposome has excellent biocompatibility, absorbability and high or binding affinity for variety of molecules. Dissolution Profile of calcium phosphate play an important role in drug release. Calcium phosphate has low solubility at pH 7.4 and thus it is expected that there would be negligible dissolution and hence the drug release from the liposomes while in systemic circulation. Calcium phosphate dissolves to an appreciable extent at acidic pH and thus is expected to dissolve and release the drug at the pH of the tumor interstitium (4.5 to 5.5).

INTRODUCTION

In the field of targeted drug delivery has grown rapidly in the last three decades. A target-oriented drug delivery system may best supply drug selectively to its site(s) of the action(s) in a manner that provides maximum therapeutic activity (through controlled and predetermined drug release kinetics), preventing degradation or inactivation during transit to the target sites, and protecting the body from adverse reactions because of inappropriate disposition. Most of the anticancer drugs have a low therapeutic index (ratio of toxic dose to therapeutic dose), target drug delivery may provide an effective treatment at a relatively low drug concentration.

Basically three strategies have been used to achieve drug targeting. These include-

- Magic bullet approach use of site specific pharmacologically active molecules.
- Pro drug approach preparation of pharmacologically inert agents that are activated only at the active site.

Magic gun/missile (drug-carrier) approach - use of biologically inert carrier system that selectively direct the drug to a specific site in to the body.

CALCIUM PHOSPHATE NANOSHELL AS DELIVERY SYSTEM

Inorganic particles are an emerging area in biomolecules entrapping and has potential application in Material science (Avni et.al,1994) as their advantage include no microbial attack, excellent storage stability and in expensive. These inorganic compounds have various biomedical application in Vaccination process, Carrier for protein, Drug delivery and Gene therapy vector (Luo and Saltrmann, 2000; Cui and Mumper, 2003).

Among various inorganic particles, calcium phosphate salt is extensively developed and studied as delivery system. Calcium phosphate is considered as the model compound for inorganic constituent of the bone and teeth. Blood serum is considered as aqueous solution, supersaturated with respect to a calcium phosphate due to presence of protein and enzyme in biological fluid extensive complexation of free calcium takes place, thus reducing the actual super saturation (Dalas et. al, 1991).

Calcium plays an important role in endocytosis which is the major route for cellular internalization of gene delivery vectors. Calcium phosphate has been used for manufacturing various forms of implants due their to excellent biocompatibility (Zhang and Gonsalves, 1997). lt has an advantage of absorbability and high binding affinity for variety of molecules (Yu et. al., 1992) and has been used as delivery vehicle for various proteins such as insulin delivery (Morcol et al., 2004), sustain release of growth factors (Matsumoto et al.,2004) and carrier material for antibiotic. macromolecule and contraceptives(Joosten et al., 2004; Paul and Sharma, 1999).

Liquid-filled liposomes stabilized bv calcium phosphate are promising materials. The reinforcing coating temporarily protects the inherently fragile liposome and its contents while in the bloodstream, and provides a platform on which to construct a surface capable of chemical recognition and the ability to elude the reticuloendothelial system. Calcium phosphate coated liposomes may have a unique usefulness for the delivery

of bone, dental and anti-neoplastic therapeutics.

Liposome- coated calcium phosphate may be suited for delivery of hydrophilic drug substances. The liposome-templated design can be more flexible than drugcoated powders, or randomly coprecipitated materials because their load capacity can be manipulated by the solubility of the encapsulant and the liposome size, without affecting the selfassembly of the organic coating. Variation of coating thickness and stoichiometry may be one means of manipulating the dissolution kinetics of the inorganic coating to control the release of materials. Dissolution encapsulated kinetics of these particles is likely to be determined by the coating thickness, stoichiometry of the mineral phase, pH, and the activity of phosphatases.

Technical Challenges in Calcium Phosphate Nanoshell Development

The development process of a novel calcium phosphate nanoshell that can be used as either a biosensor, engineered contrast agent, targeted drug delivery vehicle, and as an artificial oxygen carrier. Development of these nanoshell requires

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addressing the following synthesis, functionalization, and analysis challenges.

Synthesis: A novel synthesis must be developed, which involves reproducible preparation of the nanoshell liquid core templates (liposome), reliable coating of the template with calcium phosphate, control over the size and thickness of shells, salt and protein aggregation, and concentrating the shells.

Functionalization: A protocol for the encapsulation of functional molecules like fluorescent dyes or therapeutic agents, and the attachment of enzymes and antibodies to the shell surface must be established.

Analysis: Quantitative and well understood analysis methods sensitive to the relatively low concentration of shell suspensions (~1 nM), sub micron size, and heterogeneity of the particles must be validated.

Synthesis of the nanoshells is the most important issue to address in this study since it is controls the properties of the final material. Functionalization of the shells is critical in determining the feasibility of using this newly developed material in the biomedical applications. Studying the physical and chemical characteristics of the shells is complicated by both the diminutive size of the nanoshells which affects the type of techniques that can be used for detection, the heterogeneous nature of the suspension complicates analysis of individual shells by bulk techniques and the low concentrations of particles which affects absorbance, fluorescence, chemical, and crystallographic analysis.

ISSUES IN SYNTHESIS OF THE NANOSHELLS

Preparation of Templates (Liposomes)

Convenient template for the formation of calcium phosphate nanoshells is liposomes. The goal for template preparation is to obtain a defect free, monodisperse spherical template suspension with narrow size distribution that is stable to changes in ionic strength when the inorganic salts are added to the reactant mixture. Liposomal templates normally begin as lyophilized powder that is hydrated to swell the lipid bilayer and eventual budding to form a spherical liquid crystalline lipid bilayer enclosing an aqueous pocket.

Size Control of Templates (Sizing)

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If prepared by simple mixing, liposomes have an irreproducible and wide size distribution. In order to make the suspension more monodisperse size reduction and homogenization methods are used. The size of the templates is important in particle transport, loading capacity, and colloidal stability and is influenced of by the type homogenization. Stirring, sonicating, and extrusion are accepted methods for manipulating the size distribution of liposomes.

Sonication effectiveness depends on the position of the sample in the sonicating bath and the level of water because this affects the intensity of the pressure waves and sonication geometry. The time of sonication is also important.

Extrusion through a porous filter produces particles sizes that depend critically on the extrusion filter pore diameter, the number of extrusion passes, the extrusion pressure and temperature.

Maintaining Template Structural Stability During Synthesis

There are two principle mechanisms bywhichliposomesreorganize:

coalescence/aggregation and bursting from osmotic pressure gradients. Newly formed liposomes with phosphatidic acid headgroups resist coalescence sterically through the hydration layer present on their exterior in aqueous systems and electrostatically by their pH dependent surface charge resulting from the ionizable protons from the phosphate headgroups. Coalescence can be negatively impacted by an increased salt concentration or a decrease in pH, resulting in a decreased net electrostatic charge on the liposome surface allowing the liposomes to approach close enough to each other to coalesce and reform.

Reorganization of the template prepared in distilled water can occur upon introduction to a reaction solution which includes calcium salts, phosphoric acid, sodium hydroxide and other salts at a different tonicity than the stock. This phenomenon can be understood by considering the internal energy and chemical potential of the system. While precipitation of calcium and phosphate ions onto existing template seeds is thermodynamically favoured over homogeneous crystallization, collisions between particles and the speed of the

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precipitation reaction can lead to irreversible particle aggregation during the reaction. This effect increases as the collision Frequency increases. The two parameters, supersaturation and collision frequency, to consider when trying reducing nanoshell aggregation.

Issues in Functionalizing the Nanoshells:

Functionalization of a particle can be achieved by encapsulating a substance within the shell or attaching a molecule on the outside of the particle. Issues regarding these two methods are described in greater detail below.

Loading Calcium Phosphate Nanoshell Templates with Functional Molecules

Nanoshells alone have limited biomedical usefulness, and they need to be enhanced by a functional molecule like a fluorescent dye for tracking, a reactive dye for monitoring, a contrast agent for imaging, an oxygen carrier, a protein or enzyme etc. Maximal loading of a suspension of shells with a functional molecule is limited by the

Concentration of particles in the suspension, the solubility of the solute in the particle phase(s), the size of the

shells, and the ability of the particle to retain the solute. There are various methods used to encapsulate solutes (drugs, fluorophores, biomolecules, etc) into liposomes including freeze thaw, lipid film hydration, cross flow injection method, dehydration rehydration. Of these, lipid film hydration is the most direct involving only hydrating a freeze dried lipid film with a solution of the solute to be encapsulated.

Determination of the Concentration and Condition of Encapsulated Solutes via Spectroscopy

The absorbance and fluorescence spectrophotometric techniques providean accessible and effective way to determine the concentration of species in solutions. The analysis of encapsulated solutes is complicated by the interactions of the analytical technique with the shell itself. This problem is mainly in the form of scattering of the incident or fluorescent light Absorbance of light in the UV-visible region occurs from the excitation of outer electrons from a ground to excited state.

SYNTHESIS

Three types of synthesis were investigated and are presented in the order they were developed

 the dropwise synthesis relied on manual titrations of calcium and phosphate salts

2) the one step supersaturation synthesiswas performed by simultaneous additionof all ingredients to the reaction

3) the stepwise supersaturation synthesis is a hybrid of the two methods and utilizes

Dropwise Synthesis

The reaction flask was filled with appropriate DI water and sonicated liposome solution. Strong ammonia was added under gentle stirring to the solution dropwise until the pH was 11.5. Once the pH becomes constant, calcium solution was added and precursor allowed to stir for 10 minutes followed by the first phosphate precursor solution, which stirred for 10 additional minutes. Alternating additions continued in this manner for two more additions then the stirring was increased to improve agitation and the alternating additions were spaced from 10 minutes to 2 minutes. Shell suspensions were rested

for 1 day to reach equilibrium before any characterization was performed.



Figure : Schematic diagram of Drop-wise synthesis method.

Dropwise synthesis required inconvenient reaction and preparation conditions that led to difficulty in obtaining reproducible results. This method also had potential complication in future applications and scale-up:

- The pH of 11 required to get the calcium phosphate to condense could denature labile biomolecules intended for future surface attachment or encapsulation thus a more neutral pH would be desired.
- The high pH required the reaction to be nitrogen purged to avoid carbonate formation from the solubilization of atmospheric CO₂

- The manual burette titration apparatus did not give accurate control over titration volume and timing, resulting in a product that was difficult to duplicate.
- The liposomes used as nanoshell templates were prepared via sonication which is a convenient method of making unilamellar liposomes but the resulting suspension size was difficult to reproduce, and sonication could also lead to lipid oxidation and degradation of sensitive molecules.
- Ammonia was used for pH control and as the counter ion for the phosphate species and would need to be removed before introduction of the shells in vivo.
- In addition, the assumption was made that the liposomes would behave as rigid seed crystals that would act as a nucleation site for calcium phosphate growth when in fact they are liquid crystals and are sensitive to the ionic strength gradients present during introduction to the hypertonic inorganic reaction solution. This ionic shock caused the liposomes to

deform, shrink, and pinch off daughter liposomes and other random lipid structures that could act as templates which adversely affected the quality of the nanoshell product.

One Step Supersaturation Synthesis

The dropwise synthesis results were difficult to reproduce and an alternative method relying on an improved understanding of solution supersaturation was developed.

To coat the liposomes with calcium phosphate using this method, DI water were mixed with phosphate precursor and NaOH (pH ~10). Calcium precursor and prepared liposomes were added to this solution within few seconds of each other using volumetric pipette while the suspension was stirred magnetically at room temperature, typically 25° C. To generate a set of samples with different thicknesses, CEPA solution was pipetted into stirring solution at specific time interval after reaction began to halt stabilize reaction and the shell suspension. Following CEPA addition, the suspension was allowed to stir for at least 1 day before any post of processing.



Figure : Schematic diagram of one step supersaturation synthesis.

The one step super saturation method which offers several advantages over the drop wise synthesis-

- All the reactants were added at once eliminating the need for titrations and automation of the synthesis by relying on self assembly.
- Extrusion could be used to prepare the liposomes and improved the reproducibility of liposome templates over sonication. Extrusion allowed highly monodisperse liposome suspensions by selection of the proper polycarbonate membrane.
- The counter ions of calcium, base, and phosphate used (Ca2+ and Na+) are more biofriendly than ammonium and nitrate used previously.
- The reaction was performed at lower pH (8 to 10) than the stepwise addition method.

- Nitrogen purge was unnecessary for this synthesis as it was carried out at lower pH where calcium carbonate formation was less of a problem.
- The thickness of the shell could be changed by stopping the calcium phosphate reaction on the shell surface by adding CEPA at specific times during the reaction, as before.

The improvements listed above made the one step supersaturation synthesis superior to the dropwise synthetic method for liposome templated calcium phosphate nanoshell preparation. In this method, the process for crystallizing calcium phosphate on the liposome began with the development of a supersaturated calcium phosphate solution. When the liposomes were added, the growth began instantly and continued until the saturated amount of calcium and phosphate had precipitated and reached an equilibrium conondition. The growth process could still be interrupted with the addition of CEPA to obtain nanoshells with varying thickness.

Multistep Supersaturation Synthesis

One-step synthesis provide shell thickness control but was cumbersome

because the addition of a surface stabilizer (CEPA) had to be timed. To allow for simplified thickness control, an improved method was developed to more accurately control the supersaturation combining by the stepwise and one step supersaturation synthesis methods. Liposomes were added to a Pyrex beaker with DI water and stirred magnetically at room temperature. Calcium and phosphate precursor adjusted to pH 7.0 with NaOH were added via a volumetric pipette to the reaction and stirred.

After 10 hours, when the solution calcium concentration stabilized , the average particulate size distribution was measure using dynamic light scattering size and TEM grid, after which another Calcium and phosphate precursor adjusted to pH 7 with NaOH were added to the reaction vessel and stirred for another 10 hours. This process was repeated until the desired shell size was obtained. After the final addition, CEPA was added to the reaction to coat and stabilize the shells.



Figure : Schematic Diagram of the multistep supersaturation synthesis

There were two principle drawbacks to the one step supersaturation method:

- Size control was less accurate even when using CEPA to stop nanoshell growth.
- Linearly increasing concentrations of anionic lipid, calcium, phosphate, and NaOH to achieve a higher concentration of shells/ml was not successful (no shells, high flocks, high crystals seen via TEM). Scale up of nanoshell concentration to a higher shells/ml would require concentrating a number of reactions and is not desirable.

These shortcomings prompted the development of a further improved synthetic method that expanded the concept of supersaturation control of the reaction. The size control and scalability

issues of the one step supersaturation synthesis are addressed by hybridizing it with the dropwise synthesis. This allows a measured of more control supersaturation by adding small amounts of calcium and phosphate, allowing them to grow, then adding another small amount of calcium and phosphate. Due to consumption of calcium phosphate during the interim period between additions, the saturation at the final addition was lower than what it would be if all three additions were added simultaneously. This prevented homogeneous nucleation from outpacing shell growth as it would if the total amount of calcium and phosphate was added simultaneously. Using this stepwise saturation approach, calcium phosphate could be added to vary the final thickness of liposomes and still obtain a more concentrated shell suspension without the need for CEPA as a capping agent. This synthetic method is promising for coating liposomes with calcium phosphate.

CHARACTERIZATION

Particle Sizing and Zeta Potential :

Dynamic light scattering (DLS) is used as a bulk sizing method to complement

the direct observations from microscopy. To measure the particle size distribution and zeta potential of the nanoshell suspensions, filter through a 0.45 μ m polycabonate filter to remove dust. Then Zeta Particle Sizer with a He-Ne laser and a detector angle of 90 degrees is used for particle size measurements.

TEM Microscopy:

Carbon coated 300-mesh copper grids with a Formvar support (Ted Pella) is used for particle visualization using a transmission electron microscope. After the reaction has been completed a 1-2 µl drop of shaken solution is placed in the center of the grid and allowed to air dry.

AFM Microscopy:

Atomic force microscopy is used to confirm the rigid spherical morphology of

the particles, as well as to confirm the particle sizes against those obtained from DLS.

Conductivity, Calcium, and pH monitoring

Three methods were used to estimate the total reaction time necessary for all the

Calcium and Phosphate to react:

1) The concentration of free calcium as a function of time

The pH measurement using pH electrode calibrated using a three point calibration

(pH 4, 7, 10). Both calcium concentration and pH were recorded with conductivity meter.

 Conductivity measurement using conductivity meter

Measurement of Solution Stability

For the shells to be used for practical applications in life sciences they need to resist aggregation while in physiological salt (up to 140 mM) and in the presence of Proteins. When 1 ml of a dialyzed nanoshell suspension using any of the three methods and coated with CEPA containing approximately 1 nM shells was introduced to a 0.2 M NaCl solution, the mean size of the nanoshell suspension did not vary by more than 7 % of the initial value of 124 nm. When added to a 2.5 g/dL, (half of the physiological level) of albumin, the mean distribution size of

the suspension decreased due to scattering contributions of the small protein molecules to the unimodal analysis which shifted the mean to a lower value.

CONCLUSION:

Encapsulation of Antineoplastic agent into physiologically biocompatible and biodegradable calcium phosphate coated liposome is expected to:

- Decrease the distribution of drug to the tissues other than the tumor because of prevention of leakage from the liposomes.
- Preferentially deliver drug to tumor region because of pH dependent solubility of calcium phosphate and also due to EPR effect.
- Improve pharmacokinetic and pharmacodynamic fate of а compound by enhancing its uptake via clathrin coated pits mediated endocytosis. (Stephanie M. Schmidt et al., 2007) and facilitate endosomal release, and making the drug available in cytoplasm.

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