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Fabrication of Lipid-Coated Chitosan Nanoparticles

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Authors' contributions

This work was carried out in collaboration between all authors. Author SFG carried out fabrication and physicochemical assessment of lipid-coated chitosan nanocomposites. Authors GNF and SST contributed to the design of this study. Author GMP conceived the study, coordinated experimental designs and assisted in the preparation of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Conventional chitosan nanoparticles (CSNPs) exhibit high encapsulation efficiency for hydrophilic drugs but lack substantial payload capacity for lipophilic drugs. This study explores fabrication of a novel lipid/chitosan nanocomposite suitable for combination therapy using hydrophilic and lipophilic drugs.
Methodology: Lipid coating of prefabricated CSNPs that were prepared by ionotropic gelation with tripolyphosphate (TPP) was accomplished in 0.1 M acetate buffer, pH 5.3, using an equimolar mixture of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and L-α-dipalmitoylphosphatidyl glycerol (DPPG) or DPPC only. Dynamic laser light scattering (DLS) was used to monitor particle size distribution and zeta potential.
Results: Rapid addition of TPP to chitosan (CS) solution prepared in acetate buffer at a final TPP/CS = 0.3:1 (g/g) reproducibly resulted in CSNPs with a mean hydrodynamic diameter of 82.8±1.7 nm and a zeta potential of +20.5±1.2 mV. Hydration of dried phospholipid films using this CSNP suspension progressively increased mean particle size of colloids up to 613.5±13 nm depending on lipid composition and lipid concentration applied. Zeta potential of DPPC/CS nanocomposites was significantly reduced to +8.7±0.1 mV, whereas surface charge of

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(DPPC/DPPG, 50:50)/CS nanocomposites remained unchanged between +18.8 and +21.6 mV, respectively.

Conclusion: Physicochemical assessment of lipid/CS nanocomposites prepared by thin film hydration suggests successful surface immobilization of zwitterionic DPPC on prefabricated CSNPs. The presence of this additional lipid layer surrounding the hydrophilic CS core is predicted to facilitate effective encapsulation of lipophilic drugs enabling combination therapy with hydrophilic and hydrophobic payloads using a single nanodelivery system.

Keywords: Ionotropic gelation; thin film hydration; phospholipids; nanocomposites.

ABBREVIATIONS

CS = Chitosan; CSNPs = Chitosan Nanoparticles; DPPC = 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPG = L-α-dipalmitoylphosphatidyl Glycerol; DLS = Dynamic Laser Light Scattering; TPP = Tripolyphosphate.

1. INTRODUCTION

Novel nanotechnology applications in biomedical research are expected to improve patient outcomes due to more effective targeting of desired cell types, which can be achieved by selectively tailoring surface properties of drug-containing nanocolloids [1]. For complex diseases, including cancer, simultaneous administration of two or more medications is required in order to successfully interfere with molecular pathways underlying the pathogenesis of the disease and/or limit development of drug resistance [2]. With the advent of biologics as potent, but inherently unstable, therapeutic agents in the treatment of various diseases, the focus of biomedical nanotechnology has expanded to include protection of encapsulated payload from premature degradation in addition to highly selective targeted drug delivery approaches with the objective to limit undesired side effects [3].

The availability of hydrophilic and hydrophobic drugs for the management of the same disease state (e.g., hydrophilic antibodies and lipophilic chemotherapeutic agents in cancer treatment) has increased the demand for drug delivery systems suitable to accommodate therapeutic agents exhibiting different physicochemical properties. To date, liposomes still represent the most promising drug delivery system for simultaneous administration of hydrophilic and hydrophobic drugs despite their limited physical and chemical stability [4]. In comparison, polymeric nanoparticles are generally more stable but have not been extensively explored for combination therapy with hydrophilic and lipophilic drugs due to vastly different encapsulation efficiencies [5]. Outside the biomedical field, polymeric nanoparticles are also highly desirable due to their large intrinsic surface area. Examples of such applications include catalysis where chitosan acts as a biosupport for catalytic surface reactions [6,7], dental support [8], and tissue engineering [9].

To enable clinical utilization of polymeric nanoparticles as carriers for both hydrophilic and hydrophobic drugs, various research groups have investigated a specialized core/shell architecture using biocompatible phospholipids [10-13]. The dual-component structure of these systems facilitates combination drug delivery where hydrophilic drugs are encapsulated in the polymeric core and lipophilic drugs are contained within the surface-immobilized lipid shell. Lipid-coated nanoparticles were fabricated using various inorganic materials, including silica [14,15] and iron oxide [16]. More recently, organic materials such as polystyrene were explored [17].

The focus of this research is to evaluate the feasibility of fabricating lipid-coated chitosan nanoparticles (CSNPs). The natural polymer chitosan (CS) has been extensively used in pharmaceutical technology to effectively encapsulate hydrophilic payload without the use of organic solvents and high shear forces [18]. Due to the facile fabrication technology using ionotropic gelation, CSNPs have been explored for encapsulation of therapeutic proteins. Subsequent coating of CSNPs with phospholipids was demonstrated to improve stability of encapsulated payload after gastrointestinal and pulmonary administration [19-22]. The aim of the present study was to investigate the impact of phospholipid composition and overall lipid concentration on

physicochemical properties of this novel CS/lipid nanocomposite. To assess the consequences of the lipid net charge on particle size and surface charge of lipid-coated nanoparticles, the zwitterionic1,2-dipalmitoyl-*sn*-glycero-3 phosphocholine (DPPC) and the negatively charged equimolar mixture of DPPC and *L*-α-dipalmitoylphosphatidyl glycerol (DPPG) were used during the fabrication process. The results from this research are anticipated to enable clinical development of CS/lipid nanocomposites as future drug delivery systems for combination cancer therapy.

2. MATERIALS AND METHODS

2.1 Materials

Low-molecular weight chitosan (CS, 50-190 kDa, deacetylation degree 93.1%), glacial acetic acid (99.98%), and sodium acetate trihydrate were obtained from Sigma-Aldrich (St. Louis, MO). Sodium tripolyphosphate (TPP) was purchased from Thermo-Fischer Scientific (Pittsburgh,PA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and *L*-α-dipalmitoylphosphatidyl glycerol (DPPG) were obtained from NOF America (White Plains, NY). All other chemicals were of analytical grade and used as received.

2.2 Methods

2.2.1 Chitosan nanoparticle preparation

CSNPs were prepared using the ionotropic gelation method as described in detail by Calvo and coworkers [20]. Briefly, CS and TPP solutions (0.75 mg/ml) were prepared in 0.1 M or 0.01 M acetate buffer, pH 5.3. Various TPP volume fractions ranging from 0.1 to 5 ml were rapidly added under magnetic stirring (500 rpm) to 10 ml of the CS solution. To explore TPP/CS mass ratio > 0.5, a 1.5 mg/ml TPP solution was used. Samples were stirred for 15 min at room temperature before physicochemical parameters of resulting colloids were assessed. Experimental variables explored during this CSNP fabrication process include TPP addition rate, TPP/CS mass ratio, and ionic strength of the acetate buffer.

2.2.2 Lipid-coated chitosan nanoparticle fabrication

Lipid-coated nanoparticles were prepared using the conventional thin film hydration method [21]. Briefly, DPPC or DPPC/DPPG (1:1, mol/mol) were dissolved in chloroform. 100 µl of this lipid solution was transferred into a 14x120 mm glass tube and evaporated to dryness using a CentriVap benchtop vacuum concentrator (Labconco, Kansas City, MO). Dried lipid film was hydrated with 1ml of prefabricated CSNP suspension that was preheated to 55℃ in a water bath. To facilitate immobilization of a phospholipid layer onto the surface of CSNPs, the suspension was exposed for 20 min to ultrasonic waves at 80 Hz, followed by a 30 min stabilizing period at 4℃.

2.2.3 Physicochemical properties of chitosan nanoparticles

Particle size distribution, polydispersity index (PDI), and zeta potential of uncoated and lipid-coated CSNPs were determined by dynamic laser light scattering (DLS) using the Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) as described previously by this laboratory [16]. Particle size values reported in this manuscript correspond to hydrodynamic diameters.

2.4 Statistical Analysis

All experiments were performed at least in triplicate. A statistical difference between experimental groups was assessed by one-way analysis of variance (ANOVA) or two-sided Student´s *t*-test for pairwise comparison. A probability value of $P < .05$ was considered statistically significant (Prism 6.0, GraphPad, San Diego, CA).

3. RESULTS

3.1 Optimization of Chitosan Nanoparticles Fabrication

To limit variability in physicochemical properties of desired CS/lipid nanocomposites, it was imperative to identify critical fabrication variables that facilitated reproducible preparation of small CSNPs of unimodal size distribution prior to the lipid coating step. Conventional dropwise addition of TPP to a CS solution resulted in a homogenous colloidal dispersion with a mean particle size of 136.3±12.5 nm. Interestingly, rapid combination of the two solutions at the same TPP/CS mass ratio (TPP/CS = 0.3) produced significantly smaller particles (82.8±1.7 nm, $P = .04$) while maintaining unimodal distribution (PDI <0.15). Consequently, fast addition of TPP to the CS solution was adopted as standard fabrication procedure.

The effect of ionic strength on fabrication of CSNPs was measured. It was predicted that the presence of ionic components in the fabrication vehicle may affect the ionotropic gelation efficiency. The data included in Table 1 compare physicochemical properties of CSNPs fabricated at TPP/CS = 0.3 using 0.1 M or 0.01 M acetate buffer, pH 5.3. These data underline a significant effect of ionic strength on the interaction between the positively charged CS and the negatively charged TPP. Interestingly, the mean particle size was smaller in the presence of greater salt concentration suggesting possible interference of water molecules in the ionic stabilization of TPP/CS association complexes.

The effect of different TPP/CS mass ratios on physicochemical properties of CSNPs is summarized in Fig. 1A. Initially, increasing amounts of TPP appear to induce more effective ionotropic gelation, thereby consistently reducing mean particle size of colloids to 80-90 nm at TPP/CS = 0.3-0.5. The PDI of these preparations ranged from 0.13-0.18 suggesting monodispersed systems. Further addition of TPP to a same CS amount, however, resulted in larger particles with a PDI > 0.5. DLS size distribution analysis of these preparations revealed the presence of several particle populations (i.e., multimodal distribution) that also included large aggregates >1 µm (data not shown).

The effect of changing TPP/CS mass ratios was less pronounced on the zeta potential. As demonstrated in Fig. 1B, the surface charge of CSNPs remained around +20 mV when using TPP/CS mass ratios ≤0.3. Particles prepared in the presence of additional TPP exhibited a significantly reduced zeta potential indicating more effective neutralization of the positive charge density of CS.

Table 1. Effect of ionic strength on physicochemical properties of chitosan nanoparticles fabricated at TPP/CS = 0.3 in acetate buffer

Ionic strength	0.1 M	0.01 M
Particle size [nm]	82.8 ± 4.6*	104.3± 0.1
Zeta potential [mV]	18.6 ± 1.2*	32.5 ± 1.1

**Significantly different from nanoparticles prepared in 0.01 M acetate buffer, pH 5.3 (P < .05)*

3.2 Lipid Coating of Prefabricated Chitosan Nanoparticles

Deposition of a lipid layer on the surface of prefabricated CSNPs is hypothesized to enable increased encapsulation efficiency for a hydrophobic drug, thus, generating a novel nanocomposite suitable for synchronous combination therapy with hydrophilic and hydrophobic drugs. Using the lipid film hydration method, CSNPs were combined with increasing amounts of an equimolar DPPC/DPPG mixture that carries an overall negative net charge. Particle size and zeta potential data are summarized in Table 2. In the presence of 0.01 mM total lipid, the mean particle sizes significantly increased to 260.4 ± 67.3 nm suggesting successful immobilization of

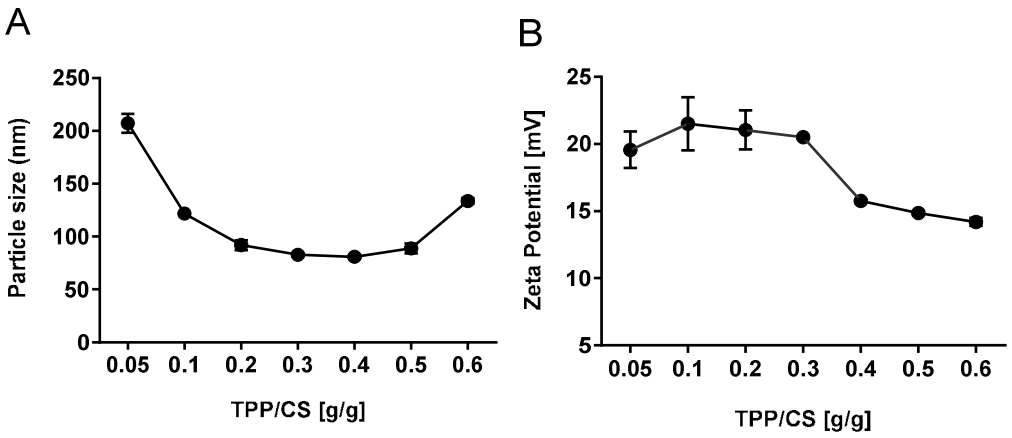

Fig. 1. Effect of TPP/CS mass ratio on physicochemical properties of CSNPs. Nanoparticles were prepared by ionotropic gelation as outlined in Materials and Methods. Panel A shows the mean particles size of fabricated CSNPs using various TPP/CS mass ratios. Panel B summarizes the corresponding zeta potential values of fabricated particle populations

Results are shown as mean ± SD (n=3). TPP: Tripolyphosphate, CS: Chitosan, CNPS= Chitosan nanoparticles

Table 2. Physicochemical properties of lipid-coated chitosan nanoparticles

Lipid composition	Final lipid concentration [mM]	Mean particle size [nm]	Zeta potential [mV]
CSNPs	0	82.8 ± 1.7	+20.5 ± 1.2
(DPPC/DPPG, 1:1)/CS	0.01	260.4 ± 67.3	+21.6 ± 2.8
(DPPC/DPPG, 1:1)/CS	0.1	214.6 ± 68.9	+21.8 ± 2.4
(DPPC/DPPG, 1:1)/CS	0.25	370.9 ± 66.9	+18.8 ± 0.3
(DPPC/DPPG, 1:1)/CS	0.5	436.1 ± 40.7	+21.2 ± 2.3
DPPC/CS	0.25	613.5 ± 13.1	+8.69 ± 0.1

CSNPs= chitosan nanoparticles, DPPC=1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DPPG=L-α-dipalmitoyl-phosphatidyl glycerol. All formulation were prepared at TPP/CS = 0.3 mass ratio. Data are shown as mean ±SD (n=3)

the lipid mixture onto the surface of CSNPs. However, the zeta potential of particle population was not significantly different from uncoated control CSNPs (+20.5 ± 1.2 mV vs. +21.6 ± 2.8 mV) although control vesicles prepared from the DPPC/DPPG (1:1, mol/mol) film in the absence of CSNPs clearly exhibited strong negative surface charge of -45.2±0.9 mV. Further increase in total lipid progressively resulted in larger colloids with diameters up to 436.1±40.7 nm (0.5 mM total lipid). In parallel, PDI increased to 0.4 implying less effective interaction of the lipid mixture with prefabricated CSNPs.

To assess whether lipid net charge influences coating efficiency of CSNPs, lipid films where prepared with the zwitterionic DPPC. After hydration with CSNP suspension, DLS revealed the presence of a single particle population with a mean diameter of 613.5±13.0 nm. Similar to the results with equimolar DPPC/DPPG, the dramatically increased particle size suggested deposition of a lipid layer onto the CSNP surface. In this preparation, however, electrophoretic mobility data indicated a significant reduction in zeta potential to +8.7±0.1 mV, which was comparable to the surface charge of DPPC control vesicles prepared in the absence of CSNPs.

4. DISSCUSION

The experimental conditions established for fabrication of CSNPs using ionotropic gelation with TPP are predicted to facilitate effective electrostatic interactions between protonated amine groups within CS and anionic phosphate groups present in TPP at pH 5.3. The resulting positive surface charge of the nanocomplexes underlines incomplete charge neutralization at the TPP/CS mass ratios studied [22]. In contrast to previous reports [23-25], our experiments demonstrated that rapid combination of the TPP solution with dissolved CS results in smaller particle size than conventional dropwise TPP addition. It is conceivable that a slower increase in TPP concentration thermodynamically facilitates extended condensation of positively charged CS polymer strands, thus, leading to larger association complexes stabilized by ionic interactions [26]. Future studies using isothermal titration calorimetry may assist in delineating the underlying thermodynamic aspects of ionotropic TPP/CS gelation.

When the TPP/CS mass ratio was < 0.2, larger particles were observed. The high positive charge density associated with dissolved CS may have resulted in increased electrostatic repulsion between polymer strands leading to multimolecular assemblies that scatter the laser light similar to large colloids [24]. As more TPP was added, electrostatic neutralization of CS facilitated compact condensation of extended polymer strands due to ionic interactions. The constant sizes ranges observed between TPP/CS ratios = 0.2-0.5 suggest the presence of a stable charge equilibrium between repulsive forces of CS amino groups and attractive ionic forces experienced by TPP molecules. Further addition of TPP removes CS strands through formation of electrostatically stabilized association complexes and, thus, leads to an imbalance of the fragile charge equilibrium [24].

Consistent with an earlier report, the zeta potential decreases as TPP/CS mass ratio increases, which may be the consequence of an increased number of ionic interactions with CS amino groups [22]. However, the findings that particle size decreases if ionic strength increased also suggests that ionic interactions between CS and TPP can be hindered by the presence of an extensive hydration shell surrounding ionized functional groups [26].

Immobilization of a lipid layer on the surface of prefabricated nanoparticles using lipid film hydration has been successfully accomplished for different inorganic and organic material [12-16]. According to various studies completed to date, an exterior lipid layer not only facilitates incorporation of hydrophobic drug moieties for combination therapy, it also increases biocompatibility of the nanocomplex with cell membranes and allows simple surface modification (e.g., PEGylation or attachment of cell-specific targeting ligands) to control pharmacokinetic and pharmacodynamics behavior [27-29]. Previously, Grenha and coworkers reported successful lipid coating of CSNPs using the negatively charged DPPC/DMPG lipid mixture [21]. The authors hypothesized that the negative charge associated with the lipid mixture facilitated effective surface coating of prefabricated CSNPs due to stabilizing electrostatic interactions with the positively charged amino groups. The negative zeta potential associated with DPPC/DPPG vesicles mixture that were prepared in 0.1 mM acetate buffer, pH 5.3, experimentally confirmed the presence of a negative charge of the lipid mixture selected for this study. Surprisingly, coating of prefabricated CSNPs with equimolar DPPC/DPPG was not associated with significant changes in zeta potential. It is conceivable that the increased particle size measured after hydration of the DPPC/DPPG lipid film with CSNP suspension corresponds to formation of larger CSNP aggregates connected via a negatively charged lipid “bridge” as proposed by Huang and coworkers [26].

The results summarized in Table 2 also demonstrate that inclusion of DPPG effectively reduces the mean particle size even if total lipid concentration was kept constant. Based on previous studies performed in our laboratory with lipid-coated iron oxide nanoparticles [16], we hypothesize that buffer components used during surface immobilization of lipid layer on solid nanoparticles may substantially contribute to the formation of the above-mentioned negatively charged lipid “bridge”. The positive surface of CSNPs is predicted to attract negatively charged buffer ions that shield cationic centers from interacting with the negatively charged lipids. The ammonium groups present in the zwitterionic DPPC may divert negatively charged acetate ions from the buffer system, thus, enabling more frequent interactions between the negatively charged phosphate groups of DPPC with the positively charged CS surface. This theory could explain why hydration of a DPPC film with a CSNP suspension increased mean particle size of fabricated nanoassemblies with a simultaneous decrease in zeta potential. Further experimental exploration of the ultrastructure of fabricated lipid/CS nanocomplexes using sensitive surface analysis techniques such as high-resolution transmission electron microscopy, X-ray photoelectron spectroscopy, and static time-of-flight secondary ion mass spectrometry will be required to delineate the supramolecular structure of these CS nanocomposites prepared by hydration of dried lipid films.

5. CONCLUSION

Physicochemical assessment of lipid/CS nanocomposites prepared by thin film hydration suggests successful surface immobilization of zwitterionic DPPC on prefabricated CSNPs. The presence of this additional lipid layer surrounding the hydrophilic CS core is predicted to facilitate effective encapsulation of lipophilic drugs enabling combination therapy with hydrophilic and hydrophobic payloads using a single nanodelivery system.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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