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Controlling chitosan-based encapsulation for protein and vaccine delivery

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ABSTRACT

Chitosan-based nano/microencapsulation is under increasing investigation for the delivery of drugs, biologics and vaccines. Despite widespread interest, the literature lacks a defined methodology to control chitosan particle size and drug/protein release kinetics. In this study, the effects of precipitation–coacervation formulation parameters on chitosan particle size, protein encapsulation efficiency and protein release were investigated. Chitosan particle sizes, which ranged from 300 nm to 3 μm, were influenced by chitosan concentration, chitosan molecular weight and addition rate of precipitant salt. The composition of precipitant salt played a significant role in particle formation with upper Hofmeister series salts containing strongly hydrated anions yielding particles with a low polydispersity index (PDI) while weaker anions resulted in aggregated particles with high PDIs. Sonication power had minimal effect on mean particle size, however, it significantly reduced polydispersity. Protein loading efficiencies in chitosan nano/microparticles, which ranged from 14.3% to 99.2%, were inversely related to the hydration strength of precipitant salts, protein molecular weight and directly related to the concentration and molecular weight of chitosan. Protein release rates increased with particle size and were generally inversely related to protein molecular weight. This study demonstrates that chitosan nano/microparticles with high protein loading efficiencies can be engineered with well-defined sizes and controllable release kinetics through manipulation of specific formulation parameters.

1. Introduction

Chitosan is under investigation for a wide variety of biomedical applications including drug delivery, gene delivery, wound healing, antimicrobial applications, tissue engineering and vaccine delivery [1–6]. The use of chitosan in these diverse applications is supported by its exceptional versatility. Chitosan can be used in solutions, hydrogels and/or nano/microparticles, while an endless array of chitosan derivatives with customized biochemical properties can be prepared through facile conjugation of side chain moieties to solvent-accessible amine and hydroxyl groups. As a result, chitosan is currently among the most well-studied biomaterials; nearly 2000 publications in the biomedical literature used chitosan as a keyword in 2013. For reference, other ubiquitous biomaterials, polylactic-co-glycolic acid (PLGA) and polycaprolactone (PCL) were keywords in less than 800 and 400 publications, respectively, last year.

Chitosan nano/microparticulate systems, in particular, are under development for facilitating the delivery of genes, protein biologics and antigens [7–12]. When developing nano/microparticulate systems for drug/protein delivery applications it is important to be able to control characteristics, such as particle size, size distribution, and surface charge, as these can significantly influence release kinetics. For example, increasing particle size has been shown to increase release rates of encapsulated drugs/proteins [13,14]. In addition, particle size has been shown to influence uptake by immune cells in vaccine applications. Particles with sizes similar to pathogens, such as viruses (5–300 nm) and bacteria (1–5 μm), are readily taken up and processed by antigen presenting cells (APCs) which leads to enhanced vaccine responses. Our recent studies demonstrated that 1 μm chitosan particles were optimal for uptake by both dendritic cells and macrophages, however, APC activation was highest with 300 nm chitosan particles [12].

It is clear that different biomedical applications will require unique chitosan particles with well-defined dimensions and release

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Table 1
Effect of formulation factors on particle size, size distribution and protein loading efficiency.

Parameter	Level	Mean size (nm)	PDI	Protein loading efficiency (%)
Precipitant salt	$Na_3C_6H_5O_7$	490 ± 18.3	0.154	83.7 ± 3.5
	Na_2SO_4	427 ± 7.6	0.113	54 ± 2.6
	Na_3PO_4	1621 ± 188.0	0.667	14.6 ± 1.7
	NaCl	–[b]	–	–
	KCl	–[b]	–	–
	$MgSO_4$	445 ± 7.7	0.124	47.5 ± 2.0
	$(NH_4)_2SO_4$	551.9 ± 3.5	0.135	62.5 ± 6.1
Chitosan viscosity[a] (molecular weight)	<20 cPs	418 ± 16.2	0.104	50.3 ± 2.3
	20–200 cPs	424 ± 7.7	0.160	56.5 ± 3.4
	200–600 cPs	605 ± 13.3	0.190	56.5 ± 2.6
	600–1200 cPs	728 ± 15.0	0.212	53.5 ± 4.3
	1200–2000 cPs	854 ± 26.2	0.385	58.5 ± 4.2
Chitosan concentration	0.5 mg/ml	334 ± 27.5	0.093	58.5 ± 3.4
	1 mg/ml	418 ± 3.5	0.113	57.3 ± 2.5
	2 mg/ml	521 ± 2.8	0.259	59.9 ± 6.8
	3 mg/ml	587 ± 10.6	0.182	73.6 ± 2.2
	5 mg/ml	656 ± 13.6	0.277	92 ± 0.8
Sonication power	0 W	922 ± 79.2	0.657	92.3 ± 1.8
	10 W	438 ± 3.9	0.182	88.3 ± 5.3
	20 W	418 ± 9.6	0.156	74.2 ± 6.3
	40 W	421 ± 4.1	0.098	56.5 ± 2.6
Precipitant salt addition rate	0.2 ml/min	774 ± 33.2	0.580	71.9 ± 7.0
	1 ml/min	574 ± 9.1	0.350	67.3 ± 3.2
	3 ml/min	495 ± 3.6	0.250	65.3 ± 3.6
	5 ml/min	444 ± 5.6	0.200	62 ± 2.8
	8 ml/min	424 ± 2.1	0.121	58.1 ± 2.2
Protein	Insulin	411 ± 19.3	0.195	98.3 ± 0.5
	Ova	423 ± 3.6	0.178	96.5 ± 1.1
	BSA	424 ± 7.7	0.160	58.1 ± 2.5
	Con A	410 ± 5.5	0.113	53.5 ± 3.6

[a] Viscosity range of chitosan was indicated by the manufacturer as obtained at a concentration of 1 mg/ml (w/v) in 1% acetic acid.
[b] No particles formed.

characteristics. Thus, there exists a need to develop a methodology for effective control of chitosan particle size, protein loading efficiency and protein release. Chitosan particles have been manufactured via a variety of chemistries including crosslinking, ionotropic gelation and precipitation–coacervation [15–18]. These last two methods are particularly attractive for the delivery of labile polypeptides as encapsulation is accomplished under mild aqueous conditions.

In this study, we utilized the precipitation–coacervation technique to comprehensively characterize the effects of various formulation factors, such as chitosan concentration, precipitant salt concentration, chitosan molecular weight, rate of precipitant addition, protein size and sonication power on chitosan particle size, polydispersity, and protein loading efficiency. A range of precipitant salts with varying strengths of hydration in the Hofmeister series were explored. In vitro release studies were performed to determine the effect of particle size, precipitant salt, and encapsulated protein on protein release from chitosan nano/microparticles. An analysis of the binding between proteins and chitosan explains the impact of protein–chitosan interactions during encapsulation and release. The results obtained in this study will be useful in the standardization of protocols for the preparation of chitosan-encapsulated proteins.

2. Materials and methods

2.1. Reagents

Chitosans with viscosities of 20, 20–200, 200–600, 600–1200, and 1200–2000 cPs, were purchased from Primex (Siglufjordur, Iceland) and purified via filtration in hydrochloric acid and precipitation in sodium hydroxide. Pluronic F-68, acetic acid, sodium chloride (NaCl), potassium chloride (KCl), sodium sulfate (Na_2SO_4), ammonium sulfate ($(NH_4)_2SO_4$), magnesium sulfate ($MgSO_4$), potassium sulfate (K_2SO_4), sodium citrate ($Na_3C_6H_5O_7$), trisodium phosphate (Na_3PO_4), fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA), unlabeled BSA and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO). FITC-OVA, FITC-insulin and FITC-concanavalin A (FITC-ConA) were purchased from Life Technologies (Green Island, NY).

2.2. Preparation and characterization of chitosan particles

Chitosan particles were prepared using the precipitation–coacervation method developed by Berthold et al. with slight modifications [19,20]. In brief, chitosan was dissolved in a 2% (v/v) acetic acid solution. Chitosan particles were formed by adding 50 mM precipitant salt solution drop wise to the chitosan solution using an infusion pump (Pump 11 Elite, Harvard Apparatus, Holliston, MA). Nonionic stabilizer Pluronic F-68 was added and particles were allowed to stabilize for 2 h under constant stirring and intermittent sonication using a sonicator (S4000; Misonix Inc., Farmingdale, New York). Chitosan particles were collected after centrifugation at 25,000 g for 10 min at 4 °C and freeze dried (FreeZone, Labconco, Kansas City, MO) before further use. Chitosan particles loaded with various fluorescence-labeled proteins including FITC-BSA, FITC-Insulin, FITC-OVA, and FITC-ConA, were prepared in a similar manner except that proteins were dissolved in chitosan solution before adding the precipitant salt solution. Size and polydispersity index (PDI) of the resultant particles were determined by dynamic light scattering (DLS) (Zetasizer, NanoZS90, Malvern).

2.3. Effect of formulation factors on particle properties

The influence of formulation factors, including chitosan concentration, molecular weight, rate of precipitant addition, protein size, and sonication power, on chitosan particle size and size distribution was determined. Preliminary experiments indicated that precipitant concentration, stabilizer concentration, pH, and chitosan:protein ratio had negligible effect on chitosan particle size (data not shown). Therefore, an experimental design using 6 factors including chitosan concentration (0.5–5 mg/ml), chitosan viscosity (<20 to 1200 cPs), precipitant salt composition, infusion rate of precipitant (0.2–8 ml/min), sonication power (0–40 W), and proteins of different sizes (insulin – (6 kDa), ovalbumin – (45 kDa), BSA – (66.5 kDa), and ConA – (105 kDa)) was utilized. A constant chitosan-to-protein mass ratio of 10:1 was maintained for all experiments. The experimental design is shown in Table 1.

2.4. Protein loading and release studies

Protein loading efficiency was calculated indirectly. Briefly, the amount of FITC-conjugated protein remaining in the supernatant after removing the protein-loaded chitosan particles was determined through spectrofluorimetry. The difference between the initial and the unencapsulated protein concentrations was divided by the initial concentration (equation (1)).

$$\text{Percentage loading efficiency} = \frac{(\text{Initial conc.} - \text{Unencapsulated conc.})}{\text{Initial conc.}} \times 100 \quad (1)$$

For protein release studies, FITC-BSA loaded chitosan particles with discrete sizes (300 nm, 1 μm, and 3 μm) (see Table 2) and salt groups (Na_2SO_4, $(NH_4)_2SO_4$, $MgSO_4$, K_2SO_4, and $Na_3C_6H_5O_7$) (see Table 3) were prepared using data gathered from previous experiments. FITC-BSA loaded chitosan particles were suspended in deionized water or phosphate buffered saline (PBS) and incubated in total darkness at 37 °C. To determine the effect of protein size on release profile, chitosan particles loaded with different proteins, i.e. FITC-labeled insulin, OVA, BSA, and ConA (see Table 4), were suspended in PBS and incubated at 37 °C. Supernatants were collected and replaced by fresh deionized water or PBS at regular intervals for 2 weeks. Samples were stored in the dark at −20 °C until batch analysis on a spectrofluorometer (excitation wavelength: 490 nm, emission wavelength: 540 nm). Percent protein released was determined by dividing the amount of released protein by total encapsulated protein. For all release studies, the starting amount of encapsulated protein was fixed at 100 μg for all groups.

Table 2
Experimental design for protein release studies: effect of particle size.

Nominal size	Chitosan conc.	Precipitant salt	Protein	Salt addition rate	Actual mean size (nm)	Protein loading efficiency (%)
300 nm	0.5 mg/ml	Na_2SO_4	FITC-BSA	8 ml/min	318.2 ± 11	58.1 ± 3.7
1 μm	3 mg/ml	Na_2SO_4	FITC-BSA	1 ml/min	1133 ± 54.1	83.5 ± 8.3
3 μm	5 mg/ml	Na_2SO_4	FITC-BSA	0.2 ml/min	2871 ± 216	96.6 ± 0.6