Analysis of Chitosan/Tripolyphosphate Micro- and Nanogel Yields is Key to Understanding Their Protein Uptake Performance

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ABSTRACT: Chitosan/tripolyphosphate (TPP) micro- and nanogels are widely explored as vehicles for protein drug and vaccine delivery. Yet, aside from the consensus that protein uptake into these particles is enhanced by stronger protein/particle binding, factors that control their uptake performance, such as differences in the chitosan, TPP and protein concentrations, remain poorly understood. Here, we show that many of the differences in the reported association efficiencies (*AE*-values) for protein uptake likely reflect the largely-ignored variability in the particle yield (X_{Agg}), which is the fraction of the added chitosan that self-assembles into particles and (like the *AE*) varies with the chitosan, TPP and protein concentrations. Factors affecting X_{Agg} are first systematically explored. The *AE* is then shown to scale almost linearly with the X_{Agg} (which increases with the TPP and protein-to-chitosan ratios) until all chitosan aggregates into particles. Remarkably, the data collected at variable TPP and protein concentrations collapses onto a single $AE \propto X_{Agg}$ curve for each protein type. Further analysis of protein/particle binding reveals this rise in *AE* with X_{Agg} to reflect: (1) an increase in binding sites within the particles; and (2) a decrease in soluble (unaggregated) chitosan molecules, which form soluble protein/chitosan complexes and compete with the chitosan/TPP particles for the unassociated protein. These findings highlight the need to carefully analyze the effects of formulation parameters on chitosan/TPP particle yields and can likely be extended to other ionically crosslinked colloidal drug carriers.

Keywords: *chitosan, tripolyphosphate, microgels, nanoparticles, yield, association efficiency (AE), drug delivery*

1. INTRODUCTION

Chitosan is a copolymer of glucosamine and N-acetylglucosamine [1, 2]. It is usually obtained through the deacetylation of chitin, a naturally-abundant polysaccharide obtained from organisms such as crustaceans, insects and fungi [1, 3, 4]. Due to its abundant feedstocks and attractive properties, such as being biocompatible [5, 6], mucoadhesive [7, 8] and enhancing molecular permeation across epithelial layers [9, 10], chitosan and its derivatives attract widespread interest in drug delivery [5, 11, 12]. Of these materials, gel-like micro- and nanoparticles prepared through ionotropic gelation of chitosan with tripolyphosphate (TPP) are among the most widely-studied [5, 13-24]. Besides the benefits of their parent chitosan molecules, these drug carriers: (1) form under very mild aqueous conditions (which helps preserve the activity of their bioactive payloads during encapsulation) [5, 25]; (2) protect proteins against denaturation during their storage and use [26]; and (3) can release drugs on demand in response to external stimuli [27, 28].

In the last two decades, many studies have been performed to elucidate factors that control chitosan/TPP micro- and nanogel size [14, 16, 18, 24, 29-31]. These revealed that the particle size distributions could be optimized by tuning the molecular weight and degree of deacetylation (DD) of the parent chitosan molecules [16, 24, 30], mixture pH, ionic strength and chitosan/TPP/drug concentration [14-16, 18, 30], and the method by which the parent chitosan and TPP solutions were mixed [32]. Yet, little attention has been devoted to understanding the effect of these parameters on the particle yield (i.e., the fraction of the chitosan chains that self-assembles into chitosan/TPP particles). For instance, though several studies have shown the particle yield to vary greatly with the TPP:glucosamine molar ratio [33-35] and insulin uptake [36], most continue to tune the TPP:glucosamine ratio to optimize the particle size while ignoring its effect on the particle yield, and often (either implicitly or explicitly) assume that

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5 virtually all of the chitosan is assembled into particles. Thus, to reliably optimize methods for
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7 chitosan/TPP micro- and nanogel preparation (by varying parameters such as TPP:glucosamine
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9 ratio, pH and ionic strength), a fuller understanding of chitosan/TPP micro- and nanogel yields
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11 should be obtained.
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14 Another challenge in optimizing chitosan/TPP particle preparation lies in maximizing the
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16 association efficiency (*AE*) of the loaded drug, which is the percentage of the added drug that is
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18 successfully loaded into the drug carrier. This *AE* varies significantly with the formulation
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20 parameters (e.g., the chitosan, TPP and drug concentrations used), but these variations remain
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22 both poorly understood and difficult to reliably predict. Though it is generally accepted that *AE*
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24 increases with the chitosan/payload binding strength, variations in *AE* with the payload, chitosan
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26 and TPP concentrations still lack a convincing explanation and, indeed, often show opposing
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28 findings. In the literature examining drug concentration effects on the *AE* of proteins, for
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30 instance, several groups reported *AE* to increase with the protein concentration used during drug
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32 uptake [15, 23, 24, 35, 37], while several others reported *AE* to decrease with the protein
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34 concentration [13, 14, 38-40].
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40 Further confusion exists regarding the effects of TPP:glucosamine molar ratio and
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42 chitosan concentration. Several groups reported the *AE* to increase with the TPP:glucosamine
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44 ratio [15, 33, 34, 37], but provide no consensus for the reasons for this effect (with one group
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46 suggesting that the enhanced *AE* at higher TPP concentrations reflects denser crosslinking [34],
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48 while another attributing it to a shift in the pH that is speculated to enhance protein/chitosan
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50 binding [15, 34]). Moreover, a report by Zhang *et al.* suggested a nonmonotonic TPP effect on
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52 *AE*, where the uptake first increases with the TPP concentration and then slightly decreases [40],
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54 which brings the generality of protein uptake increasing with the TPP:glucosamine ratio into
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5 Similarly, Gan *et al.* and Zhang *et al.* have reported a counterintuitive result where
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7 increasing the chitosan concentration during protein uptake decreased the *AE*-value [15, 40].
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9 This was regardless of whether the chitosan concentration was varied at a constant TPP
10 concentration [40] or at a constant TPP:glucosamine ratio [15], and both groups attributed the
11 reduction in uptake efficiency to increased solution viscosity. The reasons for this speculation,
12 however, are unclear and could be challenged. Indeed, a report by Yang *et al.* indicated opposing
13 findings, where the *AE* first increased drastically with the chitosan concentration, and then
14 decreased slightly with the chitosan concentration as the chitosan concentration became high
15 [41]. The variability and poor understanding of these results make it difficult to draw generalized
16 conclusions on the factors that determine protein uptake. To this end, we hypothesized that many
17 of these variations in *AE* with the protein/chitosan/TPP concentrations might reflect the
18 largely-ignored variability in the particle yield.
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33 To test this hypothesis, here we systematically explore the effects of TPP:glucosamine
34 molar ratio, pH, ionic strength and added protein on the chitosan/TPP particle yield. We then use
35 bovine serum albumin (BSA; molecular weight $\approx$ 66.5 kDa; pI $\approx$ 4.7 - 4.9 [42]) and
36 α -lactalbumin (α -LA; molecular weight $\approx$ 14.2 kDa; pI $\approx$ 4.2 - 4.5 [42]) as model proteins to
37 investigate the effect of particle yield on the protein uptake. The relationship between the *AE* and
38 the particle yield that emerges from this analysis is then used to provide essential guidelines for
39 choosing chitosan, TPP and protein concentrations for optimizing protein uptake into
40 chitosan/TPP particles. Finally, we explain the correlation between the *AE* and particle yield
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2. MATERIALS AND METHODS

2.1. Materials. Millipore Direct-Q 3 deionized water (DI water) with resistivity of 18.2 M Ω ·cm was used in all experiments. Chitosan (viscosity-average molecular weight = 154 kDa and DD = 86% as determined by capillary viscometry [17] and pH titration [43], respectively), sodium tripolyphosphate (TPP), fluorescein isothiocyanate (FITC), BSA, dimethyl sulfoxide (DMSO) and Bradford protein reagent were all purchased from Sigma-Aldrich (St. Louis, USA). α -LA was a kind gift from Davisco Foods International, Inc. (Eden Prairie, MN). Hydrochloric acid (HCl), sodium chloride (NaCl) and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Fair Lawn, NJ). All materials were used as received.

2.2. Preparation and Analysis of FITC-Labeled Chitosan. To facilitate measurement of the soluble (i.e., unaggregated) chitosan concentration before and after particle formation, the chitosan was labeled with FITC. Specifically, 5.7 mL of FITC solution in DMSO (1 mg/mL) were added to 150-mL batches of 0.2 wt% chitosan solution (adjusted to pH 4.0 using HCl) and allowed to react for 3 h in the dark at room temperature. The resulting FITC-labeled chitosan (FITC-chitosan) was then dialyzed for 24 h against DI water three times. By quantifying the unreacted FITC concentration in the dialysate, it was determined that roughly 0.3% of the chitosan amine groups were labeled with FITC. Once dialyzed, the purified FITC-chitosan solution was freeze dried and stored until use at -18 °C.

Because FITC absorbs UV and visible light, dissolved FITC-chitosan can be quantified by UV-Vis spectrometry. Here, since the UV-Vis spectra of FITC-chitosan solutions was pH-dependent, an isosbestic wavelength of $\lambda = 463$ nm was chosen to quantify FITC-chitosan concentrations at pH ≥ 4.0 and 0 mM NaCl (where $\epsilon = 0.944$ mL mg⁻¹ cm⁻¹ remained constant with the pH). When parent solution pH was reduced to 3.0, however, ϵ at $\lambda = 463$ nm became

pH-dependent and the FITC-chitosan concentration was quantified at $\lambda = 440$ nm ($\varepsilon = 0.791$ mL mg^{-1} cm^{-1}).

The UV-Vis spectra of FITC-chitosan solutions also varied with the ionic strength. Thus, at pH > 4.7 and NaCl concentrations of 150 and 1000 mM, the dissolved FITC-chitosan was quantified at $\lambda = 463$ nm (where $\varepsilon = 1.002$ mL mg^{-1} cm^{-1} and 1.324 mL mg^{-1} cm^{-1} , respectively). Conversely, when the parent solution pH was 4.0, the FITC-chitosan in 150 and 1000 mM NaCl solutions was quantified at $\lambda = 440$ nm (where $\varepsilon = 0.776$ mL mg^{-1} cm^{-1} in 150 mM NaCl and 1.025 mL mg^{-1} cm^{-1} in 1000 mM NaCl).

2.3. pH, Ionic Strength and TPP:Glucosamine Ratio Effects on Micro- and Nanogel Yield. To

study the pH and TPP:glucosamine ratio effects on the fraction of FITC-chitosan that assembled into particles, the pH of parent FITC-chitosan solutions was adjusted to either 4.0 or 5.5 using HCl and NaOH. This enabled the pH to be tuned with little (no more than a few mM) effect on the ionic strength. To prepare FITC-chitosan/TPP micro- and nanogels at variable TPP:glucosamine ratios, 1 mL of variably concentrated TPP solutions, 0 - 0.30 wt% (0 - 8.2 mM) at pH 4.0 and 0 - 0.15 wt% (0 - 4.1 mM) at pH 5.5, were added dropwise into 5 mL of pH-matched 0.1 wt% FITC-chitosan solution (which was 5.2 mM in its glucosamine monomer units). The resulting chitosan/TPP particle dispersions were then equilibrated for 30 min. To make the final chitosan and TPP concentrations here the same as those used in the protein uptake experiments (vide infra), an additional 2 mL of protein-free water (which was the volume of protein solution added during protein uptake) with corresponding pH-values were then added dropwise to the FITC-chitosan/TPP mixtures. Moreover, to ensure proper mixing during each addition step, the chitosan/TPP mixture was stirred with a 12 mm $\times$ 4 mm magnetic stir bar at 800 rpm throughout this entire procedure.

After stirring for an additional 30 min, the dispersions (which were composed of polydisperse 40 - 1000 nm particles; Supplementary Data, Fig. S1) were centrifuged in a Beckman Coulter ultracentrifuge (Ann Arbor, MI; Motor model: SW 55Ti) at 50,000 rpm (303,000 g) and 20 °C for 1.5 h. This centrifugation force and time was sufficient to separate almost all of the particles from solution while keeping molecular chitosan and unencapsulated BSA dissolved in the supernatant. The FITC-chitosan that remained dissolved in the supernatant (i.e., the FITC-chitosan that was not aggregated into particles) was then quantified by UV-Vis spectrometry at the wavelengths described above. Based on these measurements, the particle yield, defined here as the fraction of chitosan formed into chitosan/TPP particles (X_{Agg}), was determined as:

$$X_{Agg} = \frac{C_{CS}^i - C_{CS}^f}{C_{CS}^i} \quad (1)$$

where C_{CS}^i was the initial chitosan concentration and C_{CS}^f was the free chitosan concentration remaining in the supernatant after particle formation.

To explore the ionic strength effect on X_{Agg} , chitosan/TPP particles were prepared by titrating TPP solutions into FITC-chitosan solutions (and then adding 2 mL of pH-matched water) as described in the preceding paragraphs. The ionic strength, however, was now varied by adding either 0, 150 or 1000 mM NaCl to all parent FITC-chitosan, TPP and pH-matched water solutions (so that the particles were now formed in the presence of either 0, 150 or 1000 mM of added NaCl). To confirm that the salt effects were qualitatively similar regardless of the preparation of pH, two different pH-values (4.0 and 5.5) were used in this experiment. Moreover, to improve reproducibility, all the X_{Agg} measurements were repeated three times.

2.4. *Protein Uptake Effects on Micro- and Nanogel Yield.* To probe the effect of protein addition on X_{Agg} , 0.2 - 0.6 wt% BSA solutions at pH-values of either 4.0 or 5.5, and 0.12 - 0.36 wt% α -LA solutions at pH 6.0 were prepared (where a higher α -LA solution pH was used due to the insufficient α -LA solubility at lower pH-levels). Besides the protein concentration effect, the effect of the uptake method used (i.e., incubation versus incorporation [15]) was investigated. In the incubation method, 1 mL of variably concentrated TPP solutions (0 - 0.30 wt% at pH 4.0, 0 - 0.15 wt% at pH 5.5 and 0 - 0.12 wt% at pH 6.0) were added dropwise into 5 mL of pH-matched 0.1 wt% FITC-chitosan solution, to produce particles with variable TPP:glucosamine ratios. After stirring for 30 min, 2 mL of pH-matched protein (either BSA or α -LA) solution were added dropwise, whereupon the dispersions were stirred for another 30 min (to allow uptake to occur) before being centrifuged. Thus, protein was taken up into preformed particles. Conversely, in the incorporation method, 2 mL of protein solution was added to the 5 mL of FITC-chitosan solution before the TPP addition. After stirring the chitosan/protein mixtures for 30 min, 1 mL of variably concentrated TPP solution (at the same concentration and pH-values used in the incubation method) was added dropwise into the mixtures, and reacted for 30 min (i.e., causing the protein uptake and particle formation to occur simultaneously). Once protein uptake was completed, the X_{Agg} -values were determined from the dissolved FITC-labeled chitosan concentration remaining after centrifugation as described above. Each condition was tested in triplicate.

2.5. *Formulation and Uptake Method Effects on AE.* Protein-loaded chitosan/TPP particles were prepared as described in the preceding section, except without FITC-labeling the chitosan. The protein concentration remaining in solution after uptake was then determined using the Bradford assay (the accuracy of which was insensitive to the presence of chitosan; Supplementary Data, Fig. S2) after centrifuging out the particles. The protein *AE* was then calculated as:

$$AE = \frac{C_p^i - C_p^f}{C_p^i} \times 100\% \quad (2)$$

where C_p^i is the initial (or overall) protein concentration achieved after mixing the parent protein, chitosan and TPP solution together, and C_p^f is the free protein concentration remaining in the supernatant after uptake and centrifugation. All AE measurements were repeated three times.

2.6. DLS and TEM Analysis of Protein/Molecular Chitosan Binding. To improve understanding of protein uptake into chitosan/TPP particles, protein complexation with molecular (TPP-free) chitosan was also probed by dynamic light scattering (DLS), using a Zetasizer Nano ZS (Malvern, UK) dynamic and electrophoretic light scattering instrument. Two mL of either 0.60 wt% BSA or 0.36 wt% α -LA solution were mixed with 5 mL 0.1 wt% chitosan solution at pH 6.0 for 30 min, whereupon (instead of the TPP solution) 1 mL of pH-matched water was added to the chitosan/protein mixture. After 30 min of stirring, the chitosan/protein mixtures were ultracentrifuged via the procedure used for the protein uptake experiments, and the apparent volume-weighted size distributions were then obtained by DLS (for both chitosan/protein mixtures, and uncomplexed chitosan and protein controls) using a non-negative least squares (NNLS) algorithm.

Further, the DLS data on protein complexation with molecular (TPP-free) chitosan was confirmed by transmission electron microscopy (TEM), using a Hitachi HD-2300 scanning transmission electron microscope. Here, chitosan/BSA mixtures (and chitosan and BSA solution controls) were prepared and centrifuged as described above. The resulting solutions were then deposited onto carbon grids (carbon type A; Ted Pella, Redding, CA), dried for 10 min and imaged using a 200 kV acceleration voltage. To prevent dust contamination, all parent protein

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5 and chitosan solutions used for the DLS and TEM analyses were passed through 0.8- μ m
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7 (Sartorius Minisart NML) cellulose acetate syringe filters before being mixed to form
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9 protein/chitosan complexes; and, as with other experiments, at least three replicate samples were
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11 used at each condition.
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14 15 16 17 **3. RESULTS AND DISCUSSION**

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19 *3.1. TPP:Glucosamine Ratio and pH Effects on the Micro- and Nanogel Yield.* Chitosan/TPP
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21 micro- and nanogel yield depended strongly on the TPP:glucosamine molar ratio. The X_{Agg}
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23 increased sigmoidally with the TPP:glucosamine molar ratio (see Fig. 1). This increase was
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25 qualitatively consistent with previous reports, where the particle yield also increased with the
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27 TPP:chitosan ratio [33-35]. When X_{Agg} reached 1.0, however (at the 0.32:1 TPP:glucosamine
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29 molar ratio at pH 3.0; black squares in Fig. 1), further TPP addition led to rapid particle
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31 aggregation and precipitation. This precipitation likely reflected the presence of free TPP ions
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33 which, once all the chitosan molecules were aggregated into particles, became available to bridge
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35 existing micro- and nanogels [18, 30].
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Fig. 1. Comparison of X_{Agg} versus TPP:glucosamine molar ratio curves obtained by titrating TPP solutions at (■) pH 3.0, (●) pH 4.0 and (▲) pH 5.5 into 0.1 wt% chitosan solutions with matching pH-levels, and (▼) TPP solutions at their natural pH (pH 9.6) into 0.1 wt% chitosan solutions at pH 4.0. The error bars are standard deviations and the lines are guides to the eye.

The rate at which X_{Agg} increased with the TPP:glucosamine ratio depended on the solution pH. As the pH of the parent chitosan and TPP solutions was increased from 3.0 to 5.5, the TPP:glucosamine molar ratio where the particles became fully formed decreased from 0.32:1 to 0.16:1 (Fig. 1). This reflected changes in the chitosan and TPP protonation. The protonation of chitosan (whose effective pK_a is roughly 6.0 - 6.5 [44]) decreased as the pH was raised and diminished the cationic chitosan charge. Conversely, TPP is a polyprotic acid with multiple pK_a 's ($pK_{a,3} = 2.8$, $pK_{a,4} = 6.5$ and $pK_{a,5} = 9.2$ [45]), and became more-negatively charged at higher pH-levels. This simultaneous increase in TPP negative charge and reduction in chitosan positive charge enhanced the TPP:chitosan charge stoichiometry and decreased the TPP requirement for achieving high chitosan/TPP micro- and nanogel yields.

The pH effect was most significant as the pH was varied between 4.0 to 5.5 and was weak when the pH was varied between 3.0 and 4.0 (Fig. 1). Additionally, because the pH of parent TPP solutions is not adjusted by many groups, we also probed the evolution in X_{Agg} upon the

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5 addition of TPP (its sodium salt) at its natural/unadjusted pH (of roughly 9.6) to a pH 4.0
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7 chitosan solution. Not surprisingly, this yielded X_{Agg} -values that were intermediate to those
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9 obtained when both chitosan and TPP solutions were at pH 4.0 and 5.5 (Fig. 1). This was
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11 because the high pH of the natural TPP solution elevated the acidic chitosan solution to higher
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13 pH-values (which, when the initial chitosan solution pH was 4.0, rose to pH 7.1 at the highest
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15 TPP:glucosamine molar ratio).
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19 Furthermore, even when pH-matched TPP solution was added to the chitosan solution, a pH
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21 drift occurred (Supplementary Data, Fig. S3). This pH drift reflected changes in the acid-base
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23 equilibria of the chitosan and TPP upon their association, which have also previously been
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25 reported in poly(allylamine)/TPP mixtures [46]. When the chitosan was not fully protonated (e.g.,
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27 at pH 5.5), the complexation of its ionized amine groups with TPP favored its further protonation
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29 (as predicted by Le Chatelier's principle). This led to a depletion of free protons in solution and
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31 an increase in pH. Conversely, when TPP was not fully deprotonated (e.g., at pH 4.0 most TPP
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33 ions bore two protons), its binding to chitosan favored its further deprotonation (i.e., its
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35 neutralization by chitosan shifted its acid-base equilibria towards more ionized/deprotonated
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37 forms). Thus, upon binding to chitosan, TPP released protons and lowered the pH.
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43 The extents to which these competing effects altered the mixture pH depended on the initial
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45 protonation states of the chitosan and TPP. At pH 5.5, where the chitosan was not fully
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47 protonated, the proton uptake by chitosan was more dominant than their release by TPP. The pH
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49 therefore rose from 5.5 to 6.8 as the TPP:glucosamine ratio was increased from 0 to 0.16:1
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51 (Supplementary Data, Fig. S3). When particles were prepared from solutions at pH 4.0, on the
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53 other hand, the chitosan was more-fully protonated and the pH dropped by 0.1 - 0.2 units (data
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55 not shown), because proton release from TPP ions outweighed their uptake by the chitosan.
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58 These pH drifts (especially the larger one that occurred when the parent solutions were at pH 5.5)
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might have further affected X_{Agg} -values – e.g., they might have promoted further particle formation when the mixture pH drifted upwards.

3.2. Ionic Strength Effect on the Micro- and Nanogel Yield. Several studies have demonstrated that ionic strength (i.e., monovalent salt concentration) affects chitosan/TPP particle formation and stability [17-22]. Due to the competition between monovalent and multivalent ions, a high monovalent salt concentration inhibits chitosan/TPP binding [18]. Nonetheless, adding moderate (150 mM) NaCl concentrations to the parent chitosan and TPP solutions appeared to have no impact on the TPP:glucosamine ratio where X_{Agg} first reached unity (see black squares and red circles in Fig. 2). Moreover, at lower TPP:glucosamine ratios, X_{Agg} was actually higher in 150 mM NaCl solutions than in solutions without added NaCl (regardless of whether the parent solution pH was 4.0 or 5.5). This surprising increase in particle yield might reflect a more-uniform TPP distribution between the chitosan chains. In the absence of added NaCl, the ionic crosslinking is very rapid (and occurs within milliseconds) [18, 19]. Thus, the TPP might be consumed before being thoroughly mixed, which would limit the ionic crosslinking to only a fraction of the chitosan chains. Conversely, in the presence of NaCl the ionic crosslinking is much slower [18, 19]. This might enable more uniform mixing of TPP with the receiving chitosan solution prior to its binding and, consequently, may lead to a higher fraction of the chitosan chains being ionically crosslinked.

Fig. 2. Comparison of X_{Agg} versus TPP:glucosamine molar ratio curves obtained from (a) pH 4.0 and (b) pH 5.5 chitosan and TPP solutions containing (■) 0 mM, (●) 150 mM and (▲) 1000 mM NaCl. The error bars are standard deviations and the lines are guides to the eye.

Another notable NaCl effect was that, unlike the particles formed in the absence of added salt, no precipitation occurred when the TPP:glucosamine ratio exceeded the point where X_{Agg} first reached unity. This was consistent with previous findings that NaCl stabilizes chitosan/TPP dispersions in the presence of excess TPP [18] and indicated that NaCl addition might lower the risk of aggregating chitosan/TPP particles when operating at TPP:glucosamine molar ratios that optimize the particle yield. When the monovalent salt concentration was increased to very high levels, however (e.g., 1000 mM NaCl), the chitosan/TPP binding was inhibited and, consistent with light scattering observations reported earlier [18], very little chitosan aggregation occurred (see blue triangles in Fig. 2). This suggests that X_{Agg} -values are optimized at intermediate ionic strengths.

3.3. Protein Uptake Effects on the Chitosan/TPP Micro- and Nanogel Yield. To study the effect of protein uptake on particles yield, BSA and α -LA were used as model protein drugs. At

pH-values above their isoelectric points, these proteins were negatively charged and were able to electrostatically bind to the cationic chitosan/TPP particles. This binding has been reported to yield insoluble chitosan/protein particle formation even without TPP [47, 48] and increased X_{Agg} at every TPP:glucosamine molar ratio (see Figs. 3a and b). Consequently, as protein was added, the TPP:glucosamine molar ratios where X_{Agg} reached unity and the particles started to aggregate and precipitate diminished – i.e., the onset of precipitation shifted from 0.16 to 0.13:1 TPP:glucosamine molar ratio when BSA was added (where the parent solution pH was 5.5) and from 0.11 to 0.08:1 when α -LA was added (where the parent solution pH was 6.0).

Fig. 3. Comparison of X_{Agg} versus TPP:glucosamine molar ratio curves obtained: (a) at pH 5.5 in the presence of (■) 0, (●) 0.05, (▲) 0.10 and (▼) 0.15 wt% BSA loaded using the incubation method; and (b) at pH 6.0 in the presence of (■) 0, (●) 0.03, (▲) 0.06 wt% and (▼) 0.09 wt% α -LA loaded using the incubation method; (c) comparison of X_{Agg} -values obtained in the presence of BSA using the two uptake methods (incubation versus incorporation); and (d) comparison of X_{Agg} versus TPP:glucosamine molar ratio curves obtained in the presence of (■) 0, (●) 0.05 and (▲) 0.15 wt% BSA at pH 4.0 and (▼) 0, (◆) 0.05 and (★) 0.15 wt% BSA at pH 5.5. The error bars are standard deviations and the lines are guides to the eye.

Proteins are large molecules and their diffusion in and out of chitosan/TPP particles could be inhibited. Due to this possibility, effects of two uptake methods were compared: (1) the incubation method, where the protein was loaded into preformed particles; and (2) the

incorporation method, where the particles were formed in the presence of protein and the protein was loaded during their formation (such that potential problems with protein diffusion into the particles were minimized) [15]. Surprisingly, the uptake method used had virtually no impact on the X_{Agg} -values (see Fig. 3c and Supplementary Data, Fig. S4).

Furthermore, to probe the pH effect on particle yield in the presence of protein drugs, X_{Agg} versus TPP:glucosamine molar ratio plots were obtained at various BSA concentrations at pH 4.0 and 5.5 (Fig. 3d). At pH 4.0, the X_{Agg} -values were independent of the BSA concentration and indicated that BSA had little impact on particle formation when the pH was low. This insensitivity may have reflected the positive net charge of BSA ($pI \approx 4.8$ [42]) at pH 4.0, which largely eliminated its electrostatic binding to the chitosan (vide infra). Conversely, at pH 5.5 (i.e., above the pI of BSA) the negatively charged protein bound to the chitosan and, consequently, increased the X_{Agg} (Fig. 3d). This increase in chitosan/TPP particle yield with the chitosan-binding protein uptake (shown here for both BSA and α -LA) has also been reported to occur upon the encapsulation of insulin [36], which suggests this increase in X_{Agg} to be a general effect.

3.4. Protein Concentration, Uptake Method and pH Effects on AE. Protein uptake into chitosan/TPP particles was investigated using parent chitosan, TPP and protein solutions at pH 5.5 for BSA and 6.0 for α -LA (with the pH of α -LA measurements being slightly higher due to its limited solubility at lower pH-levels). Despite there being fewer unoccupied cationic amine groups on the chitosan chains at higher TPP:glucosamine ratios, the *AE* of BSA within chitosan/TPP particles rose from 10 - 20% to roughly 80 - 90% as the TPP:glucosamine molar ratio was increased from 0.025 to 0.13:1 (i.e., to the point where X_{Agg} reached near-unity and further TPP addition led to precipitation; Fig. 4a). As the TPP:glucosamine ratio was increased

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5 beyond this point, however, the *AE* dropped sharply and was again below 20% when the
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7 TPP:glucoamine ratio exceeded 0.2:1. These BSA uptake trends were qualitatively similar to
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9 those obtained for α -LA (cf. Figs. 4a and b). α -LA uptake, however, was less efficient with its
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11 peak *AE* below 60% and (just like the points where X_{Agg} in these samples first reached near-unity;
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13 see Figs. 3a and 3b) this peak α -LA *AE* occurred at a slightly lower TPP:glucosamine ratio than
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15 that for BSA. Furthermore, up to the TPP:glucosamine ratios where the *AEs* reached their
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17 maximum values, the *AE*-values were slightly higher when higher protein concentrations were
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19 used (Figs. 4a and b).
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Fig. 4. AE dependence on the TPP:glucosamine molar ratio for dispersions at: (a) pH 5.5 loaded with (■) 0.05, (●) 0.10 and (▲) 0.15 wt% BSA by the incubation method; and (b) pH 6.0 loaded with (■) 0.03, (●) 0.06, (▲) 0.09 wt% α -LA by the incubation method; (c) comparison of AE-values for BSA obtained using the two uptake methods (incubation and incorporation); and (d) BSA AE dependence on the TPP:glucosamine molar ratio at (■) pH 4.0, (●) pH 5.5. The error bars are standard deviations and the lines are guides to the eye.

The initial increase in AE with the TPP:glucosamine ratio was consistent with previous reports by Gan *et al.* [15] and Hu *et al.* [30]. These authors speculated that the enhanced protein uptake likely reflected an increase in the negative BSA charge, which strengthened the

electrostatic chitosan/TPP binding and resulted from the pH increase that occurred when alkaline sodium TPP was added to the chitosan. This pH effect on protein/particle association was also confirmed by Mattu *et al.*, who showed that the *AE* of BSA increased with the dispersion pH [49]. Similarly, as discussed previously, the pH-value increased with the TPP addition even when the parent chitosan, TPP and protein solutions were at the same pH (Supplementary Data, Fig. S3). Yet, though the pH/increasing binding strength effect proposed in the previous studies has served as a plausible explanation for the increasing *AE*, data in Figs. 1 and 3 suggests that it might not be the only possible interpretation. This is because X_{Agg} , which increases the mass of chitosan/TPP particles available for protein uptake, also increases with both the TPP:glucosamine ratio and pH. Thus, the increase in *AE* might also reflect an increase in X_{Agg} . This alternative interpretation may also explain the increase in *AE* with the protein content (since X_{Agg} also increases with added protein; see Figs. 3a and b) and will be discussed in more detail in the following section.

Once the chitosan was fully aggregated (i.e., X_{Agg} reached near-unity) and the particles started precipitating, the *AE* of both BSA and α -LA decreased with the TPP:glucoamine ratio. This was despite the continued increase in pH (cf. Figs. 4a and b and Fig. S3) and likely reflected the strong competitive binding of TPP ions to the chitosan which, instead of forming new particles (as they did at lower TPP:glucoamine ratios), started displacing the bound proteins from the chitosan/TPP matrix. Furthermore, consistent with observation on the protein effects on X_{Agg} (see Fig. 3c), the *AE*-values obtained at each BSA concentration and TPP:glucosamine ratio were independent of the protein loading method used (i.e., incubation versus incorporation; see Figs. 4c and S3). This insensitivity suggested that protein uptake was governed by the equilibrium binding of proteins to chitosan/TPP particles and, interestingly, opposed the data of Calvo *et al.*, which showed the *AE* of BSA to be much higher when loaded via incorporation [13].

To further probe the role of binding on protein uptake, *AE*-values obtained by loading BSA (via incubation) at pH 4.0 and 5.5 were compared (see Fig. 4d). At pH 5.5, the BSA had a net negative charge and, not surprisingly, was able to bind to chitosan efficiently. At pH 4.0, however, BSA had a net positive charge. This largely eliminated its electrostatic attraction for the chitosan and its *AE* remained below 10% for every TPP:glucosamine value examined (despite the apparent chitosan/TPP particle ζ -potential at pH 4.0 being higher than that at pH 5.5; Supplementary Data, Table S1). This very low protein uptake was consistent with the pH effect on X_{Agg} (Fig. 3d), where X_{Agg} increased with BSA addition at pH 5.5 but was insensitive to BSA at pH 4.0. It also confirmed that protein/particle affinity (which can be tuned by varying the pH) is essential to obtaining a high *AE*.

3.5. Relationship between AE and X_{Agg} . In addition to affecting the protein/particle affinity, we hypothesized that the effects of the various formulation parameters on *AE* were strongly linked to variations in X_{Agg} . To investigate this relationship, the *AE*-values obtained at TPP:glucosamine molar ratios up to the precipitation point were plotted versus X_{Agg} . Regardless of whether the BSA was loaded through incubation or incorporation (cf. Figs. 5a and b), the *AE* versus X_{Agg} data obtained at each protein concentration collapsed onto a single line. A linear regression on this collapsed data yielded the correlation $AE = 80.2X_{Agg}$ ($R^2 = 0.965$) for the incubation method and $AE = 83.9X_{Agg}$ ($R^2 = 0.968$) for the incorporation method, confirming that the uptake method had almost no impact on *AE*. To test whether this nearly linear scaling applies to other chitosan-binding proteins, the same correlation was plotted for α -LA loaded using the incubation method. (Fig. 5c). This again yielded a linear function, where $AE = 60.0X_{Agg}$ ($R^2 = 0.955$). The linear relationships obtained in each of these experiments show that, for affinity-driven protein uptake, X_{Agg} is a key determinant of *AE*, irrespective of the protein concentration or loading procedure.

Fig. 5. The scaling of AE with X_{Agg} for particles loaded with (■) 0.05, (●) 0.10 and (▲) 0.15 wt% BSA via the (a) incubation and (b) incorporation method; and (c) for particles loaded with (■) 0.03, (●) 0.06 and (▲) 0.09 wt% α -LA via the incubation method. The error bars are standard deviations and the lines are linear regression fits (to data at all three protein concentrations) and the error bars are standard deviations.

Moreover, to test whether the increase in AE with the TPP:glucosamine ratio (and the X_{Agg}) might also be affected by the upward pH drift (Supplementary Data, Fig. S3), AE and X_{Agg} data obtained as described above was compared with that measured after lowering the dispersion pH back to the initial values of the parent chitosan, protein and TPP solutions (pH 5.5 for BSA uptake and 6.0 for α -LA uptake). This pH reduction had little impact the AE and X_{Agg} , and the points obtained at both pH levels fell on the same AE versus X_{Agg} lines (Supplementary Data, Fig. S5). Given that protein uptake appears to be governed by equilibrium partitioning (i.e., its insensitivity to the uptake method used), this invariance of the AE versus X_{Agg} lines with the dispersion pH (at least when the pH exceeds the protein pI) strongly suggests that the increase in AE with the TPP:glucosamine ratio primarily reflects the increase in X_{Agg} and not the upward drift in the pH.

3.6. Model Analysis of Protein Uptake into Chitosan/TPP Particles. The trends in Fig. 5, where (for a given X_{Agg} -value) the fraction of protein that partitions into the particles is essentially independent of the total protein content, suggest that the protein uptake data can be described by the linear binding isotherm:

$$q = K_{eff} C_P \quad (3)$$

where q is the extent of protein binding (mg of bound protein per mg of particulate chitosan), K_{eff} is the effective binding constant and C_P is the supernatant protein concentration. To confirm this, q was plotted versus C_P by converting the AE -values into q -values as:

$$q = \frac{C_P^{Tot} AE}{C_{CS}^{Tot} X_{Agg}} \quad (4)$$

where C_p^{Tot} is the total protein concentration and C_{CS}^{Tot} is the total chitosan concentration in each chitosan/TPP/protein mixture. Repeating this analysis at each TPP:glucosamine ratio and protein concentration (and neglecting the small increases in X_{Agg} with the protein content) revealed binding isotherms for both BSA and α -LA which, at least to a first-order approximation, could indeed be modeled as linear (Figs. 6a and b).

Fig. 6. Binding isotherm data showing: (a) BSA uptake into particles with TPP:glucosamine molar ratios of (■) 0.026, (●) 0.053, (▲) 0.079, (▼) 0.106 and (◆) 0.132:1; (b) α -LA uptake into particles with TPP:glucosamine molar ratios of (■) 0.021, (●) 0.042, (▲) 0.063 and (▼) 0.084:1; and K_{eff} versus $(1 - X_{Agg})$ plots for (c) BSA and (d) α -LA. In (a, b) the dashed lines are linear fits of each data set to Eqn. 3, while the solid lines are the theoretical predictions of all data sets based on Eqns. 3 and 6 (and fitted parameters K and $\tilde{K}$). The lines in (c, d) are linear fits experimental data to Eqn. 6. All error bars are standard deviations.

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5 Interestingly, the slopes of these isotherms increased with the TPP content. In other words,
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7 even when corrected for differences in particulate chitosan mass at variable TPP:glucosamine
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9 ratios (and, accordingly, variable X_{Agg} -values), protein partitioning into the particles was more
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11 favorable at higher X_{Agg} -values. Thus, higher AE -values at higher TPP:glucosamine ratios (and
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13 higher X_{Agg} -values) could not be explained by an increase in protein binding sites on the particles
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15 alone. This observation suggested that the presence of unassociated chitosan might interfere with
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17 protein uptake into the particles. Consequently, we hypothesized that the dissolved
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19 (uncrosslinked) chitosan molecules might compete for the protein molecules with the
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21 TPP-crosslinked chitosan chains within the particles.
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26 This view is consistent with previous work on protein/polyelectrolyte interactions, which
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28 showed that soluble protein/polyelectrolyte complexes (including those based on chitosan [50,
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32 presence of the soluble protein/chitosan complexes was confirmed through the DLS analysis of
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34 TPP-free chitosan/BSA and chitosan/ α -LA mixtures at pH 6.0. Without chitosan, the
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36 volume-weighted size distribution showed a single peak at 4 - 5 nm for BSA and 3 nm for α -LA
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38 (see Figs. 7a and b), which were on the same order as the literature hydrodynamic diameters of
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40 those proteins (which were roughly 7 nm and 3 nm, respectively [55]). Similarly, the apparent
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42 volume-weighted size distribution obtained for protein-free chitosan solution at pH 6.0 produced
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Fig. 7. Representative volume-weighted size distributions obtained by DLS for (a) 0.15 wt% BSA and (b) 0.09 wt% α -LA: (■) protein, (●) 0.063 wt% chitosan and (▲) mixtures of protein with 0.063 wt% chitosan after ultracentrifugation.

When the chitosan and proteins were mixed, however, and ultracentrifuged to remove any insoluble chitosan/protein complexes, the apparent volume-weighted size distributions obtained by DLS differed from those of the dissociated chitosan and protein molecules (green triangles in Figs. 7a and b). The apparent size distributions in these mixtures were typically bimodal and composed of: (1) a large peak at near 20 nm; and (2) a small peak in the roughly 100 - 1000 nm range (whose coexistence was also confirmed by TEM; see Supplementary Data, Section G). Though the origin of the latter, smaller peak remains uncertain, the presence of these peaks (at apparent diameters larger than those of chitosan and protein alone) strongly suggests the formation of “soluble” protein/chitosan aggregates that are not removed from the solvent through ultracentrifugation. Likewise, the dominant peak at $d_H \approx 20 - 30$ nm in the DLS data agrees well with the hydrodynamic diameters of soluble protein/polyelectrolyte complexes reported in the previous works [54, 56]. The presence of these small aggregates confirms that, when X_{Agg} is smaller than 1.0, competitive protein binding to uncrosslinked chitosan chains likely limits protein uptake.

The inference of this competitive protein binding enables further modeling of the protein uptake data, which might explain the near-linear scaling of AE with X_{Agg} . First, it indicates that the supernatant protein concentration measured by UV-Vis spectroscopy reflects the combined concentrations of: (1) free unbound protein; and (2) protein bound to soluble chitosan chains (i.e., protein bound to chitosan chains that are not removed through ultracentrifugation). Accordingly, the linear partitioning model can be modified to account for the competitive binding effect by expressing the total supernatant protein concentration as:

$$C_P = \tilde{C}_P + \tilde{K} \tilde{C}_P C_{CS}^{Tot} (1 - X_{Agg}) \quad (5)$$

where $\tilde{C}_P$ is the free (unbound) protein concentration, $\tilde{K}$ is the equilibrium constant for the protein/molecular chitosan binding, and the product $\tilde{K} \tilde{C}_P C_{CS}^{Tot} (1 - X_{Agg})$ quantifies the concentration of protein bound to the soluble chitosan. This analysis again assumes that the mass of protein bound per mass of soluble chitosan, $\tilde{q}$, can be approximated by the partitioning expression $\tilde{q} = \tilde{K} \tilde{C}_P$, while the mass of protein bound per mass of particulate chitosan can be approximated as $q = K \tilde{C}_P$, where K is the equilibrium constant for the protein binding to the particulate chitosan. Combining the latter q definition with Eqns. 3 and 5 yields an expression for K_{eff} :

$$K_{eff} = \frac{K}{1 + \tilde{K} C_{CS}^{Tot} (1 - X_{Agg})} \quad (6)$$

These K_{eff} -values can be experimentally obtained by fitting the binding data obtained at each TPP:glucosamine ratio (dashed lines in Figs. 6a and b) to Eqn. 3. Furthermore, once the K_{eff} variation with the X_{Agg} (which depends on the TPP:glucosamine ratio) is known, the magnitudes of K and $\tilde{K}$ can be obtained.

This was easily achieved by plotting K_{eff} versus $(1 - X_{Agg})$ as shown in Figs. 6c and d, and fitting this data to Eqn. 6 (see Table 1). These fits revealed the magnitudes of the K - and $\tilde{K}$ - values to be very close to one another (i.e., within 30% of one another for both protein species) and indicated that, while X_{Agg} remained below 1.0, TPP did not hinder protein binding to the chitosan. Furthermore, the fitted values of K and $\tilde{K}$ for both proteins suggest that the linear partitioning model given by Eqns. 3 and 6 can (at least at typical protein loading conditions) describe the experimental binding isotherms quite well – i.e., protein uptake data at every TPP:glucosamine ratio can be fitted using K and $\tilde{K}$ as the only adjustable parameters (see solid lines in Figs. 6a and b).

Table 1. Fitted equilibrium constants for BSA and α -LA binding to chitosan/TPP particles and soluble chitosan.

Protein	K (mL/mg)	$\tilde{K}$ (mL/mg)	$K/\tilde{K}$	R^2
BSA	13.6	18.0	0.76	1.000
α -LA	2.72	2.72	1.00	0.987

Based on the above analysis, it is tempting to derive a binding isotherm-based equation that predicts the linear AE versus X_{Agg} correlations in Fig. 5. Since AE is defined as the protein fraction that becomes loaded into the particles, it can be rewritten as:

$$AE = \frac{K_{eff} C_{CS}^{Tot} X_{Agg}}{1 + K_{eff} C_{CS}^{Tot} X_{Agg}} \quad (7)$$

which is obtained by expressing the encapsulated protein concentration as $K_{eff} C_{CS}^{Tot} X_{Agg} C_P$ (or $q C_{CS}^{Tot} X_{Agg}$) and the unencapsulated protein concentration as C_P . Since K_{eff} is a function of X_{Agg} , the linearity of this expression can be checked by substituting Eqn. 6 for K_{eff} , which yields:

$$AE = \frac{KC_{CS}^{Tot} X_{Agg}}{1 + C_{CS}^{Tot} [\tilde{K} + (K - \tilde{K}) X_{Agg}]} \quad (8)$$

Though this expression is not strictly linear, it becomes so when K and $\tilde{K}$ are roughly equal like they are in Table 1, and (for $0.6 \leq K/\tilde{K} \leq 1.5$; see Supplementary Data, Section H) simplifies Eqn. 8 to the linear expression:

$$AE = \frac{KC_{CS}^{Tot}}{1 + K' C_{CS}^{Tot}} X_{Agg} \quad (9)$$

where K' is the weighted average binding constant (defined as $K' = 0.75K + 0.25 \tilde{K}$) and is used in the denominator instead of K to minimize errors caused by small differences between K and $\tilde{K}$ (see Supplementary Data, Section I for the derivation). This simplified linear expression is consistent with the shapes of the AE versus X_{Agg} correlations in Fig. 5, where the slope obtained for the stronger-binding BSA was higher than for the weaker-binding α -LA. Moreover, the slope of the AE versus X_{Agg} line defined by this equation increases with the protein/chitosan binding strength and the total chitosan concentration, and (for systems where $K = \tilde{K}$) approaches the value of one (or 100%) when $K' C_{CS}^{Tot} \gg 1$ (Supplementary Data, Fig. S7).

Notably, the sensitivity of this slope to the total chitosan concentration (which is varied in many studies) depends on the protein/chitosan binding strength. When $K' C_{CS}^{Tot} \gg 1$, this slope is predicted to be practically independent of C_{CS}^{Tot} . Conversely, at lower $K' C_{CS}^{Tot}$ -values, the slope is predicted to become more sensitive to C_{CS}^{Tot} . These model predictions were confirmed experimentally by varying the parent chitosan solution concentration between 0.05 and 0.15 wt%

(such that, upon the addition of TPP and protein solution C_{CS}^{Tot} ranged between 0.31 and 0.94 mg/mL). The AE versus X_{Agg} curves obtained for the stronger-binding BSA (for which $K'C_{CS}^{Tot}$ ranged from 4.59 to 13.8) showed almost no variation, just as predicted by Eqn. 9 (see Fig. 8a). Those for the weaker-binding α -LA, however (for which $K'C_{CS}^{Tot}$ ranged from 0.85 to 2.55), increased noticeably with the overall chitosan concentration (Fig. 8b).

Fig. 8. The scaling AE with X_{Agg} for particles prepared using (■) 0.31, (●) 0.63 and (▲) 0.94 mg/mL overall chitosan concentrations and loaded via the incubation method with (a) 0.05 wt% BSA and (b) 0.03 wt% α -LA. The error bars are standard deviations and the lines are the model predictions based on Eqn. 9 for mixtures containing (—) 0.31, (---) 0.63 and (····) 0.94 mg/mL chitosan, respectively.

In addition to relating AE to X_{Agg} , the above analysis allows q (i.e., the amount of protein taken up per mg of particulate chitosan, or the particle “loading capacity”) to be predicted as a function of C_P^{Tot} and C_{CS}^{Tot} . This can be achieved by combining Eqn. 4 with either Eqn. 8 or 9.

For instance, if K and $\tilde{K}$ have similar values, Eqn. 9 can be used to yield:

$$q = \frac{KC_P^{Tot}}{1 + K'C_{CS}^{Tot}} \quad (10)$$

which predicts the concentration of protein within the particles to increase with the total protein concentration used in the encapsulation process and the protein/chitosan binding strength, and to decrease with the total chitosan concentration used. Notably, because for equal K and $\tilde{K}$ the protein distributes itself evenly between dissolved and particulate chitosan (and q is already normalized by the chitosan mass within the particles), q is predicted to be nearly independent of X_{Agg} when plotted as a function of C_P^{Tot} . This linear dependence of q on C_P^{Tot} is confirmed in Figs. 9a and b, where the data for each protein from Fig. 6 (BSA in Fig. 9a and α -LA in Fig. 9b) collapses onto the lines predicted by Eqn. 10.

Fig. 9. Plots of protein uptake as function of total protein used during the encapsulation procedure for: (a) BSA loaded at (■) 0.026, (●) 0.053, (▲) 0.079, (◆) 0.106 and (▼) 0.132:1 TPP:glucosamine molar ratios; and (b) α -LA loaded at (■) 0.021, (●) 0.042, (▲) 0.063 and (◆) 0.084:1 TPP:glucosamine molar ratios. Both plots are obtained based on data in Fig. 6. The lines are model predictions obtained using Eqn. 10 and the error bars are standard deviations.

3.7. Further Discussion. The sensitivity of X_{Agg} to the TPP:glucosamine ratio, pH, ionic strength and protein concentration (Figs. 1 - 3) clearly shows chitosan/TPP micro- and nanogel yield depends strongly on the compositions used during their preparation. This indicates that, when these mixture compositions are adjusted (e.g., to optimize particle size distribution or stability), their effect on particle yield must be taken into account. Further, consistent with previous studies

on how particle yield varies with the TPP:glucosamine molar ratio [33-35], it confirms that the assumption that nearly all of the chitosan self-assembles into particles (which not infrequently appears in the literature) is often incorrect and should be used with caution.

More importantly, this study clearly shows that protein *AE* depends strongly on the chitosan/TPP micro- and nanogel yields, and that X_{Agg} should be optimized to ensure high *AE*-values. It also provides possible explanations for many of the trends that have been reported previously. Contrary to the literature explanations discussed in the Introduction [15, 34], the primary reason for the increase in *AE* with the TPP:glucosamine ratio [15, 33, 34, 37] appears to be the increase in X_{Agg} . Similarly, the reduction in *AE* with the chitosan concentration [15, 40] likely reflects a reduction in X_{Agg} , which occurs regardless of whether the chitosan concentration is increased at a constant TPP concentration [40] or a constant TPP:glucosamine ratio [15]. When the TPP concentration is kept constant, X_{Agg} decreases because the TPP:glucosamine ratio decreases. Likewise, when the chitosan is increased at a constant TPP:glucosamine ratio, X_{Agg} likely decreases due to a decrease in the BSA:chitosan ratio (i.e., as the number of BSA molecules binding to each chitosan chain decreases the protein-mediated increase in X_{Agg} becomes less pronounced).

The observations made in this study also explain the opposing findings reported by Yang *et al.*, where the *AE* first sharply increased with the chitosan concentration, and then decreased with the chitosan concentration when the chitosan concentration became high [41]. In Yang's work, the BSA:chitosan ratio was kept constant and the BSA and chitosan concentrations both increased while the TPP concentration was fixed [41]. Thus, the rise in chitosan and BSA concentrations corresponded to a decrease in the TPP:glucosamine molar ratio. At low chitosan/BSA concentrations, the TPP:glucosamine molar ratio was high and the chitosan was fully assembled into particles (i.e., X_{Agg} was 1.0). Under these conditions, *AE* decreases with the

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5 TPP:glucosamine molar ratios (cf. Figs. 3a and 4a). Hence, not surprisingly, the AE increased as
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7 the TPP:glucosamine ratio decreased with the chitosan/BSA concentrations. At high
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9 chitosan/BSA concentrations, however, TPP:glucosamine ratios became much lower, such that
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11 X_{Agg} was likely lower than 1.0. Under these conditions, both X_{Agg} and AE increase with the
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13 TPP:glucosamine ratio (Figs. 3a and 4a). Thus, as TPP:glucosamine ratio decreased with the
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15 chitosan/BSA concentrations at high chitosan/BSA concentrations, AE also decreased.
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19 The effect of added protein on X_{Agg} (see Fig. 3) also explains the increase in AE that has been
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21 reported to occur at higher protein concentrations [15, 23, 24, 35, 37]. Yet, there are also several
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23 reports that indicate an opposite trend [13, 14, 38-40]. The easiest of these opposing results to
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25 explain is that by Jarudilokkul *et al.* [38], who used a TPP:glucosamine molar ratio well above
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27 0.2:1. Under this condition, X_{Agg} had already reached unity and TPP ions were displacing protein
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29 from the particles (see Figs. 4a and b). Thus, at this high TPP:glucosamine ratio, further protein
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31 addition did not enhance the particle yield and AE decreased with the protein concentration due
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33 to a saturation of available chitosan binding sites.
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37 In contrast, the other four studies that showed AE to decrease with the protein concentration
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39 used at TPP:glucosamine ratios of around 0.1:1, where Fig. 3 shows protein addition might still
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41 increase X_{Agg} -values. Interpretation of these opposing literature results, however, is unfortunately
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43 complicated by the fact that none of these studies provide complete information on the pH-values
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45 of their parent chitosan and TPP solutions, and final chitosan/TPP particle dispersions. Since pH
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47 is a critical determinant of X_{Agg} (see Fig. 1), this makes it difficult to postulate on how X_{Agg}
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49 changed with the protein concentrations and to explain the trends reported in these works. We
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51 therefore emphasize that detailed pH information is essential to include in reports on
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53 chitosan/TPP particles. Another cautionary note on performing X_{Agg} and AE analyses is on
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55 selecting proper centrifugations procedure for removing the particles from the supernatant.
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Though many groups report centrifuging the dispersions for 30 min at 20,000 to 40,000 g, X_{Agg} measurements at variable centrifugation speeds and times (data not shown) revealed that nearly-quantitative particle removal requires much more extensive centrifugation (such as described in Section 2.3).

More broadly, the findings reported herein highlight the need to carefully analyze the effects of formulation parameters on chitosan/TPP micro- and nanogel yield and show (both experimentally and through model analysis) that this particle yield is a key determinant of AE . The relationship between AE and X_{Agg} revealed in this work provides essential guidelines for optimizing protein uptake in these colloidal drug carriers, explains hitherto poorly-understood literature results, and can likely be extended to protein encapsulation within other ionically crosslinked colloidal drug carriers (e.g., those based on alginate [57]).

4. CONCLUSIONS

This study has systematically examined the relationship between chitosan/TPP micro- and nanogel yields and their protein uptake properties. Spectroscopic analysis of chitosan aggregation confirmed chitosan/TPP particle yields to be acutely sensitive to the formulation parameters used in their preparation; where, in addition to increasing with the TPP:glucosamine ratio [33-35] and added protein [36] as reported previously, particle yields (which were defined here as the fractions of aggregated chitosan, or X_{Agg} -values) unambiguously increased with the pH, and varied (nonmonotonically) with the ionic strength. This variability indicated that, when formulation parameters are optimized (e.g., to tune the particle size or stability), their effects on the particle yield must be carefully considered.

Besides affecting the overall particle preparation efficiency, chitosan/TPP particle yields were discovered to have a critical impact on their protein uptake performance. For

chitosan-binding proteins, the *AE* increased almost linearly with the particle yield (up until the point where all chitosan aggregated into particles), indicating that, to maximize protein uptake, the particle yield must also be maximized. Such favorable particle yields and *AE*-values were easily achieved by raising the TPP:glucosamine molar ratio. When the TPP:glucosamine ratio exceeded that needed to aggregate all the chitosan into particles, however (such that $X_{Agg} = 1.0$), the excess TPP displaced the loaded protein from the particles and (due to the strong, competitive chitosan/TPP binding) caused the *AE* to decrease with further TPP addition. This revealed that there is an optimal TPP:glucosamine molar ratio for protein uptake which, for the chitosan used for this work (i.e., 86% DD chitosan with a viscosity-average molecular weight of 155 kDa), is near the 0.13:1 TPP:glucosamine molar ratio when the particles are formed from parent chitosan, TPP and protein solutions at pH 5.5, and near the 0.08 - 0.09:1 TPP:glucosamine molar ratio when the particles are formed from solutions at pH 6.0.

The nearly linear relationship between the particle yields and *AE*-values (which occurred when $X_{Agg} < 1.0$), on the other hand, apparently reflected two effects: (1) that higher particle yields provided more particulate binding sites for the protein uptake; and (2) that the dissolved chitosan (which was not crosslinked into particles) competed with the chitosan/TPP particles for the protein. Remarkably, the *AE*-values obtained at variable TPP:glucosamine ratios and protein concentrations collapsed onto a single *AE* versus X_{Agg} curve, whose insensitivity to the protein loading procedure suggests that protein uptake is governed by simple equilibrium partitioning. As a first order approximation, these near-linear *AE* versus X_{Agg} relationships can be modeled based on linear binding isotherms, which successfully predict variations in both *AE* and particle protein content. Overall, these findings show that knowledge of the chitosan/TPP micro- and nanogel yield is essential to understanding and optimizing their protein uptake performance,

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5 explain many of the trends in the literature *AE* data, and can likely be extended to other ionically
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7 crosslinked colloids that are currently being explored.
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12 **Acknowledgment.** We are grateful to the National Science Foundation (CBET-1133795) for
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14 supporting this work.
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Graphical Abstract