Formation of chitosan-fucoidan nanoparticles and their electrostatic interactions: Quantitative analysis

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The stoichiometric distributions of both positive amino groups and negative sulfate ions loaded in chitosan-fucoidan nanoparticles (CFNs) were predicted quantitatively by correlating the separate yields of loaded chitosan and fucoidan, and a proposed relative charge density model (case 1). In addition, those distributions of both positive amino groups and negative sulfate ions loaded in CFNs were obtained by deriving the expression of their loaded concentrations directly from the experimental data (case 2). Both the model-prediction and experimental derivations were remarkably consistent with each other except at pH 2. The discrepancy between cases 1 and 2 at pH 2 was explained by an increase in the sulfate group loading because of the most intensive electrostatic (specific ion) interactions at pH 2. The ratio of the CFN-free net charge density shielded by counter-ions in the solution entrapped in CFNs to their counter-ion-crosslinking charge density was suggested to be a quantitative criterion for determining the size distribution of CFNs. The formation of CFNs ranked according to size was predicted well and explained reasonably by the suggested criterion, considering both the ionic strength of the entrapped solution in CFNs and the nonspecific binding (interaction) of the positive amino groups among the chitosan molecules. Furthermore, the fraction of nonspecifically-bound positive amino groups causing hysteresis was quantified from the positive net charged amino groups per unit-mass CFN. Thus, its magnitude was predicted to have a strong correlation with the CFN-preparation conditions, such as pH and fucoidan to chitosan mass ratio.

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[Key words: Chitosan-fucoidan nanoparticle; Electrostatic interactions; Quantitative analysis; Relative charge density model; Specific ion interaction; Nonspecific interaction; Prediction]

Polyelectrolyte complexation is used widely to produce chitosan nanoparticles. Chitosan is accompanied by only one polyanionic substance, such as tripolyphosphate (TPP) (1—9), alginate (10,11), dextran sulfate (12), cellulose-hydrolyzed carboxymethyl cellulose (13), heparin (14,15), DNA (16), glycyrrhetic acid (17), carboxymethyl konjac glucomannan and poly-gamma glutamic acid (18,19), and fucoidan (20—22). Nanoparticles have distinct advantages over microparticles, such as a relatively higher intracellular uptake. In addition to their particle size, the uptake by the intestinal epithelia appears to be affected by the nature of the nanoparticle and nanoparticle-charge properties (23). Yue et al. (24) reported that the surface charge affects the cellular uptake and intracellular trafficking of chitosan-based nanoparticles in that positively-charged nanoparticles promote internalization because the positively-charged nanoparticles prefer to interact with a negatively-charged cell surface due to electrostatic interactions. With chitosan, epithelial cells open their tight junctions to enhance the absorption of drugs through the oral routes (25). Yu et al. (21) also reported a CFN-induced tight junction opening for the transport of an anti-angiogenic sulfated polysaccharide across the Caco-2 cell monolayers. Therefore, it is essential to provide insight into the characterization of CFNs including their positively net charged properties and their size. On the other hand, few studies have correlated the experimental data on the characterization and the formation of CFNs with the extent of the ionic interactions between chitosan and fucoidan using model-approach-analysis. Under these circumstances, a previous study (26) proposed a relative charge density model of prepared CFNs to provide insight into an analysis of the ionic interactions in terms of the polyelectrolyte complexes. Using the proposed relative charge density model, the extent of the ionic interactions can be predicted in terms of the pH (pH 2—6) and the fucoidan to chitosan mass ratio (FCMR) (1:0.05 through 1:1), where positively net charged CFNs were prepared. In addition, the formation of CFNs was explained qualitatively by their size and stability. Using the results of elemental analysis and the yield, the stoichiometry of the element of CFNs was examined to determine the separate yields of chitosan (CYD) and fucoidan (FYD) loaded CFNs. In addition to chitosan-amide groups, the distribution of CYD suggested the involvement of positively charged amino groups in the electrostatic interactions among chitosan molecules (i.e., nonspecific interactions). Therefore, the authors proposed the relative charge density model for chitosan-fucoidan polyelectrolyte complexation to analyze their electrostatic interactions.

In addition to the previous study (26), the formation of CFNs and their electrostatic interactions still need to be analyzed

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quantitatively. In the present study, the stoichiometric distributions of both positive amino groups and negative sulfate ions loaded in CFNs were obtained by deriving the expression of their loaded concentrations from the experimental data. In addition, the relative charge density model suggested previously (26) was applied to predict the distribution of both positive amino groups and negative sulfate ions loaded in CFNs in terms of the pH and FCMRs. The prediction was carried out quantitatively by correlating the stoichiometry of the amino groups and sulfate groups loaded in the CFNs, and the relative charge density model. To validate the suggested relative charge density model, the predicted distribution, taking the relative charge density model into consideration, was compared with the distribution derived from the experimental data without considering the relative charge density model. In addition, some general rules were introduced to predict the distribution of the CFN-size in terms of the pH and FCMRs. The prediction of the CFN size distribution was validated by a comparison with the experimentally measured size distribution of CFNs according to the pH and FCMRs. Keen insight into their interactions, including the reversible specific ionic interactions and irreversible interactions causing hysteresis, as well as the resulting experimental characterization of CFNs prepared by polyelectrolyte complexation according to the pH-environment and FCMRs, was obtained.

MATERIALS AND METHODS

Preparation of chitosan-fucoidan complex nanoparticles   Chitosan-fucoidan complex nanoparticles were prepared using the methodology reported elsewhere (26). A 0.1 %w/v chitosan solution was prepared to a final volume of 100 ml by dissolving 100 mg of chitosan (deacetylation degree of 75–85%, viscosity (0.5% in 5% acetic acid) of 5–20 cps, case no. 0321-6250 Showa chemicals Japan) in 0.2 %w/v acetic acid (Merck, Germany). Subsequently, 5 ml of this 0.1 %w/v chitosan solution (in 0.2 %w/v acetic acid) was placed in a beaker. The pH of each 5 ml aliquot of the 0.1 %w/v chitosan solution (in 0.2 %w/v acetic acid) was adjusted to 2, 3, 5, and 6 using either 1N NaOH or HCl (DC Chemical Korea) to determine the effects of pH. 100 mg of fucoidan from *Fucus vesiculosus* (Sigma–Aldrich, case no. F5631-1G) was dissolved in deionized water to make a 0.1 %w/v fucoidan solution to a final volume of 100 ml. A dispersion of the chitosan-fucoidan complex was prepared by mixing the positively-charged chitosan and negatively-charged fucoidan using the dropping method. In this manner, 0.25 ml, 0.5 ml, 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml of a 0.1 %w/v fucoidan solution were added to the chitosan solution with continuous stirring to achieve FCMRs of 1:0.05, 1:0.1, 1:0.2, 1:0.4, 1:0.6, 1:0.8, and 1:1, respectively. After stirring for 30 min, the turbidity of the dispersion was measured using an UV-Vis spectrophotometer (UV-1601 PC Shimadzu). The mixture was then centrifuged at 13,000 g for 15 min. The pellets were re-dispersed in 3 ml of water, and transferred to empty glass tubes and freeze dried. Upon lyophilization in a freeze-dryer (Ilshin Lab Co. Ltd., FD 8512), the dried mass yield was calculated gravimetrically. The effects of the adjusted and unadjusted pH of the chitosan solutions on the formation and yield of the CFNs was analyzed at various FCMRs (0.05, 0.10, 0.20, 0.40, 0.60, 0.80, and 1.00).

Measurement of pH   The pH of the chitosan solution before and after adding the fucoidan solution was measured using a methodology reported elsewhere (26). The unadjusted pH of the 0.1 %w/v chitosan solution (in 0.2 %w/v acetic acid) and 0.1 %w/v fucoidan solution was 3.69 and 7.08, respectively. In addition, the pH of the final suspension was similar to the corresponding pH of the chitosan solution when the FCMR was 0.05. The pH, however, increased slightly (by between 0.1 and 0.2) when the FCMR was changed from 0.1 to 1.0.

Elemental analysis of nitrogen, sulfur, carbon and hydrogen   Elemental analysis was performed on nitrogen, sulfur, carbon, and hydrogen using the methodology reported elsewhere (26).

Analysis of zeta-potential and mean particle size   The zeta-potential and mean particle size were measured using the methodology reported elsewhere (26).

Prediction of relative charge density   The dissociation equilibrium models were established for both chitosan and fucoidan solutions in terms of the relative charge density according to the previous study (26). The pK$_a$ of chitosan and fucoidan are approximately 6.5 (15) and 1–2.5 (16), respectively. In the dissociation model for a chitosan solution, the protonated amino groups ($-NH_3^+$) are in equilibrium with the deacetylated amino groups ($-NH_2$). For a fucoidan solution, however, the hydrogenated sulfate groups ($-OSO_3H$) are in equilibrium with sulfate ions ($-OSO_3^-$). The errors of $[NH_4^+]$ and $[-OSO_3^-]$ due to the slight increase in pH were highest at FCMR = 1.0; the maximized errors of $[NH_4^+]$ and $[-OSO_3^-]$ were estimated to be -0.002 and $+3.5\%$ at pH 2, respectively, and -12

and $+0.004\%$ at pH 6, respectively. Under these circumstances, the pH of the final suspension was assumed to be the same as that of the chitosan solution. The relative charge density of the protonated amino groups ($\frac{[-NH_4^+]}{[TN]_o}$) and sulfated ions ($\frac{[-OSO_3^-]}{[-OSO_3H]_o}$) at a pH of 2.00, 3.00, 3.69 (unadjusted), 5.00, and 6.00 were predicted. These predictions were made in a mixed solution with a dilution effect before the occurrence of polyelectrolyte complexation. $[TN]_0$ and $[-OSO_3H]_0$ denote the sum of $[-NHCOCH_3]$ (acetylated amino groups) and $[-NH_2]_0$ (deacetylated amino groups), and the sum of $[-OSO_3^-]$ and $[-OSO_3H]$, respectively, prior to mixing. The relative charge density, ($\frac{[-OSO_3^-]}{[-OSO_3H]_o}$), of the sulfate ions was multiplied by $\frac{[-OSO_3H]_0}{[TN]_o}$ to allow a comparison with the relative charge density ($\frac{[-NH_4^+]}{[TN]_o}$) of the protonated amino groups. A degree of deacetylation of 0.8 was used to relate $[TN]_0$ with the deacetylated amino groups, ($[-NH_2]_0$). A sulfate ratio of 0.33 was applied. As a result, the applied ratio of $\frac{[-OSO_3H]_0}{[TN]_o}$ was 0.63. Each relative charge density when the chitosan solution was mixed with a fucoidan solution according to a given FCMR was then predicted at pH 2, 3, 3.69 (unadjusted), 5, and 6.

Use of the relative charge density model to predict the stoichiometry of the positive amino groups and negative sulfate groups involved in polyelectrolyte complexation (case 1)   Using the results of elemental analysis of nitrogen (%) and sulfur (%), the yield of the chitosan-fucoidan polyelectrolyte complex can be split into the yields of loaded chitosan and fucoidan, respectively. The yield of chitosan was defined as the yield of loaded chitosan mass per unit input mass (i.e., used chitosan and used fucoidan). Similarly, the yield of fucoidan was defined as the yield of loaded fucoidan mass per unit input mass. Therefore, the yield of chitosan (CYD) can be expressed as

$$CYD = [N(\%)/100](Y/100)\alpha \tag{1}$$

where $N(\%)$, Y and α denote the nitrogen (%) fraction of the CFNs, the ratio (i.e., % yield) of the formed CFNs to the sum of used chitosan mass and the used fucoidan mass, and the number (i.e., 12.07) expressing the reciprocal of the nitrogen-mass-occupied fraction from the used chitosan mass, respectively (26).

In a similar manner, the yield of fucoidan (FYD) can be expressed as

$$FYD = [S(\%)/100](Y/100)\beta \tag{2}$$

where $S(\%)$, Y and β denote the sulfur (%) fraction of CFNs, the ratio (i.e., % yield) of the formed CFNs to the sum of the used chitosan mass and used fucoidan mass, and the number (i.e., 9.11) expressing the reciprocal of a sulfur-mass-occupied fraction from the used fucoidan mass, respectively (26).

The chitosan loading efficiency was defined as the ratio of the loaded-chitosan mass in the formed CFNs to the chitosan mass used. Similarly, the fucoidan loading efficiency was defined as the ratio of the loaded-fucoidan mass in the formed CFNs to the used fucoidan mass. The chitosan- and fucoidan loading efficiencies can be expressed using the definitions of the loaded chitosan yield (CYD) and the loaded fucoidan yield (FYD), as described in Eqs. 1 and 2.

The chitosan loading efficiency (CLE) can be expressed as

$$CLE = CYD\left(1 + \frac{x}{c}\right) \tag{3}$$

where c and x denote the used chitosan mass (mg) and the used fucoidan mass (mg), respectively.

According to the experimental procedure, c is 5 mg, and $\frac{x}{c}$ is expressed as the FCMR. Therefore, CLE can be formulated as

$$CLE = CYD(1 + FCMR) \tag{4}$$

Similarly, FLE can be formulated as

$$FLE = FYD\left(1 + \frac{1}{FCMR}\right) \tag{5}$$

The ratio of the stoichiometric coefficients of the loaded nitrogen and loaded sulfur to form a CFN was reported (26) to be (i) barely dependent on the pH environment for the reaction of polyelectrolyte complexation (Fig. S1), and (ii) almost identical to that of nitrogen and sulfur used in the solution (Fig. S2). Furthermore, when the amino groups of the formed CFNs were assumed to be re-dissolved at equilibrium, the concentration of protonated-amino groups from the formed CFNs (i.e., $[NH_3^+]^*$) was calculated by multiplying $[NH_3^+]$ by CLE. Similarly, the concentration of negative sulfate ions from the formed CFNs (i.e., $[-OSO_3^-]^*$) can be calculated by multiplying $[-OSO_3^-]$ by FLE.

Therefore, $[NH_3^+]^*$ per $[TN]_O$ can be estimated as follows:

$$\frac{[NH_3^+]^*}{[TN]_O} = CLE\frac{[NH_3^+]}{[TN]_O} \tag{6}$$

Similarly, $[-OSO_3^-]^*$ per $[-OSO_3H]_0$ can be estimated as

$$\frac{[-OSO_3^-]^*}{[-OSO_3H]_O} = FLE\frac{[-OSO_3^-]}{[-OSO_3H]_O} \tag{7}$$