



Relative charge density model on chitosan–fucoidan electrostatic interaction: Qualitative approach with element analysis

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This paper proposes a relative charge density model of prepared chitosan-fucoidan nanoparticles (CFNs) to provide insight into an analysis of the ionic interactions in terms of polyelectrolyte complexes. Using the relative charge density model, the extent of the ionic interactions is predicted in terms of the pH (2 through 6) and used fucoidan to chitosan mass ratio (FCMR) (1:0.05 through 1:1), through which the formation of CFNs can be controlled to be ranked qualitatively according to size and stability. It was confirmed by the measurements of their zeta potentials and sizes and by the analysis of their decay with time. Moreover, the relative charge density model was validated to predict the isoelectric condition of a polyelectrolyte complexed suspension of CFNs. Elemental analysis with a proper mass-conversion showed that the ratio of the stoichiometric coefficients of sulfate groups to amino groups in CFNs formed were almost consistent to that of the sulfate groups to amino groups in a chitosan solution mixed with a fucoidan solution prior to the occurrence of polyelectrolyte complexation. In a pH 2-environment, there were locally intensive electrostatic interactions with a low yield to form sulfate group-rich CFNs. In contrast, in a pH 6-environment, extensive electrostatic interactions occurred to form sulfate group-poor CFNs with a high yield. In addition to the chitosan-amide groups, the separate yield-distribution of loaded chitosan indicated the possible involvement of positively charged amino groups in the electrostatic interactions among chitosan molecules.

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Chitosan derived from the deacetylation of chitin is a linear polysaccharide composed of randomly distributed β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine with a high ratio of glucosamine to acetyl-glucosamine units (Fig. 1A). Aqueous acids often dissolve chitosan because its solubility depends on the protonation of amino groups in chitosan molecules (1). As a result of protonation, chitosan carries a positive charge to interact with negatively-charged polymers and macromolecules. Because of this property, the polyelectrolyte complexation of chitosan with a polyanionic polymer has been studied widely in drug delivery and biomedical fields, such as hydro-gels (2,3), films (4), beads (5), microparticles (6), and nanoparticles (7–10). Fucoidan is a negatively charged polysaccharide occurring naturally in many species of brown algae and many marine invertebrates (Fig. 1B). Nevertheless, the only commercially available form of fucoidan is obtained from the brown algae “*Focus vesiculosus*”. The structure of fucoidan is based mainly on L-fucose with mainly α (1→3) glycosidic bonds and sulfate groups at position four (11). Murakami et al. (2) prepared a composite hydrogel sheet composed of alginate,

chitin/chitosan and fucoidan as a wound dressing and reported its advantages in the repair of healing-impaired wounds. Xing et al. (7) used chitosan as extractant and studied on an extraction and separation of fucoidan from *Laminaria japonica*. The polyelectrolyte complex CFNs formed from polyelectrolyte complexation between chitosan and fucoidan in a chitosan/fucoidan mixed solution was originally reported by Lee et al. (8) at various pH and FCMRs. However, their investigation was performed without the concept of relative charge density (RCD) model and the related physico-chemical analysis thereof. Yu et al. (9) investigated CFN-induced tight junction opening for the transport of an anti-angiogenic sulfated polysaccharide across Caco-2 cell monolayers. Huang and Liu (12) prepared CFNs and chitosan/fucoidan/tripolyphosphate nanoparticles to control the release of stromal cell-derived factor-1 to regulate the mobilization of stem cells. Fredheim and Christensen (13) reported that the apparent irreversibility in the reaction between chitosan and lignosulfonate with increasing pH after complex formation was due to hysteresis, which is commonly observed for the precipitation/dissolution of other polyelectrolytes. 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.

This study proposes a relative charge density model to predict the extent of the ionic interactions according to the pH and mass

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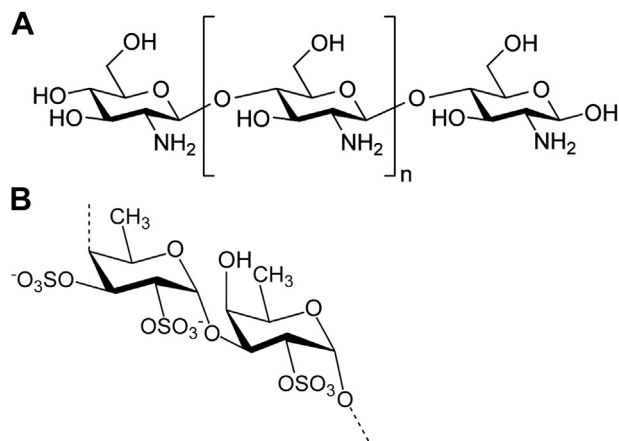


FIG. 1. Chemical structure of the basic unit of chitosan (A) and fucoidan (*Fucus vesiculosus*) (B).

ratio and explain qualitatively the stability of the CFN-suspension as well as the formation of CFNs ranked according to size. The relative charge density model ultimately provides a tool to control the size of the CFNs as well as the stoichiometric ratio of anionic sulfate ions to cationic amino groups loaded in prepared CFNs. The experimental characterization of the CFNs are explained by the predicted electrostatic interactions between cationic amino groups ($-\text{NH}_3^+$) of chitosan and anionic sulfate groups ($-\text{OSO}_3^-$) of fucoidan such as the crosslinking charge density as well as the free net charge density. The stoichiometry of the CFNs is examined by element analysis of the dried mass of the prepared CFNs. Using their yield and the result of element analysis, the stoichiometry of the element of CFNs is studied to obtain the separate yield-distribution of each chitosan and fucoidan loaded in the CFNs in terms of the pH and mass ratios. Then, in subsequent study (manuscript submitted), the distributions of both positive amino groups and negative sulfate ions loaded in the CFNs are predicted in terms of pH and their mass ratios and are correlated with the proposed relative charge density model. In particular, the proposed relative charge density model is quantitatively validated by correlating the stoichiometry of the amino groups and sulfate groups loaded in them with the relative charge density model to predict the size-distribution as well as zeta potential of the CFNs.

MATERIALS AND METHODS

Preparation of chitosan–fucoidan complex nanoparticles An one-tenth % w/v chitosan solution was prepared to make 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 a 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 a 0.1 %w/v chitosan solution (in 0.2 %w/v acetic acid) was adjusted to 2, 3, 5 and 6 with 1N NaOH or HCl (DC chemical Korea) to determine the effect of the pH of the chitosan solution. Accordingly, the chitosan solutions were prepared at a pH of 2, 3, 5 and 6 as well as at an unadjusted pH. Moreover, 100 mg of fucoidan from *Fucus vesiculosus* (Sigma–Aldrich, case no. F5631-1G) was dissolved in deionized water so that a 0.1 %w/v fucoidan solution was prepared to make 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 way, 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. Upon 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 \times g for 15 min. The pellets, if any, were re-dispersed in 3 ml water and transferred to empty glass tubes and freeze dried. Upon lyophilization in a freeze-dryer (FD 8512, Ilshin Lab Co. Ltd.), the yield of the dried mass 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, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0).

Measurement of pH Dispersions of the chitosan–fucoidan complex were prepared from a chitosan solution at various pH. The pH of the chitosan solution before and after adding the fucoidan solution was measured at room temperature using a digital pH meter (Istek-720P, Istek Inc.) and a glass electrode. The pH meter was calibrated to three buffer solutions (pH 4, 7 and 10) supplied by Istek Inc. 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 FCMR was 0.05. On the other hand, the pH increased slightly by between 0.1 and 0.2 when FCMR was changed from 0.1 to 1.0.

Measurement of absorbance of the suspension The turbidity of the dispersion was estimated by measuring the absorbance of the final dispersion before centrifugation at a wavelength of 550 nm using a double beam UV–Vis spectrophotometer (UV-1601 PC, Shimadzu). Deionized-water was run as a blank (zero absorbance) and the absorbance of the sample was measured at the same wavelength.

Calculation of the dried mass-yield Empty glass tubes with the identification labels pasted on them were dried and weighed. This weight was noted as the weight of the empty bottles before freeze drying. The redispersed pellets in the centrifugation tubes were transferred to the weighed empty glass tubes and freeze dried. After freeze drying, the tubes were weighed again, and the difference in the weight was taken as the weight of the dried mass. The yield of the dried mass was calculated gravimetrically as follows:

$$\text{Yield} = \frac{\text{Weight of dried mass (g)}}{\text{Sum of used chitosan and fucoidan mass (g)}} \times 100 \quad (1)$$

Element analysis on nitrogen, sulfur, carbon and hydrogen The composition of nitrogen and sulfur was analyzed using an element analyzer (Flash 2000, Thermo Fisher Scientific, Italy). One milligram was taken from each of various lyophilized particles of the chitosan–fucoidan complex prepared according to the aforementioned methodology. Subsequently, it was shaped as a pellet using a pellet press and used as the sample for the analysis of C, H, N and S.

Analysis of zeta-potential and mean particle size The zeta-potential and size of the suspended hydrogel particles of the chitosan–fucoidan complex prepared according to the aforementioned methodology were measured by dynamic light scattering (DLS), using Zetasizer Nano ZS (Malvern Instruments Ltd., UK) right after and a week after preparation of the suspended hydrogel particles.

Prediction of relative charge density and its validation Chitosan is often solubilized in aqueous acids because its solubility is dependent on the protonation of amino groups in the molecules (1). The protonation of chitosan results in it carrying positive charges. Therefore, the charge density of chitosan molecules depends on the pH of the solution (14). When dissolved fucoidan was added to the chitosan solution, molecular electrostatic attractions occurred between anionic sulfate groups of fucoidan and the cationic amino groups of chitosan. Consequently, the intermolecular chains of chitosan and fucoidan curled up. Subsequently, an insoluble chitosan–fucoidan complex formed. Therefore, the positively-charged chitosan may interact with the negatively-charged fucoidan. The pK_a of chitosan and fucoidan are approximately 6.5 (15) and 1–2.5 (16), respectively. Accordingly, their charge densities are affected by the pH of the chitosan solution.

The dissociation equilibrium models were established for both chitosan and fucoidan solutions. Protonated amino groups ($-\text{NH}_3^+$) are in equilibrium with the deacetylated amino groups ($-\text{NH}_2$) in the dissociation model for a chitosan solution. For a fucoidan solution, the hydrogenated sulfate groups ($-\text{OSO}_3\text{H}$) are in equilibrium with sulfate ions ($-\text{OSO}_3^-$). Under a pH of 2, 3, 3.69 (unadjusted), 5 and 6, the relative charge density of the protonated amino groups ($[-\text{NH}_3^+]/[\text{TN}]_0$) and sulfated ions ($[-\text{OSO}_3^-]/[-\text{OSO}_3\text{H}]_0$) can be predicted. Those predictions were performed in a mixed solution with a dilution effect before the occurrence of polyelectrolyte complexation. $[\text{TN}]_0$ and $[-\text{OSO}_3\text{H}]_0$ denote the sum of $[-\text{NHCOCH}_3]$ (acetylated amino groups) and $[-\text{NH}_2]_0$ (deacetylated amino groups), and the sum of $[-\text{OSO}_3^-]$ and $[-\text{OSO}_3\text{H}]$, respectively, before mixing. The relative charge density ($[-\text{OSO}_3^-]/[-\text{OSO}_3\text{H}]_0$) of sulfate ions was multiplied by $[-\text{OSO}_3\text{H}]_0/[\text{TN}]_0$ to allow a comparison with the relative charge density ($[-\text{NH}_3^+]/[\text{TN}]_0$) of the protonated amino groups. A degree of deacetylation of 0.8 was used to relate $[\text{TN}]_0$ with the deacetylated amino groups ($[-\text{NH}_2]_0$). A sulfate ratio of 0.33 was applied. As a result, the applied ratio of $[-\text{OSO}_3\text{H}]_0/[\text{TN}]_0$ was 0.63. Each relative charge density, when the chitosan solution was mixed with the fucoidan solution according to a given FCMR, was then predicted at each pH, 2, 3, 3.69 (unadjusted), 5, and 6. Then, a correlation was investigated between the stoichiometric ratio of the negative sulfate ions to the positive amino groups of the prepared CFNs and the ratio of negative sulfate ions to positive amino groups in a mixed solution prior to polyelectrolyte complexation. The predictions of those relative charge densities were used to validate the turbidity, pattern of aggregation, yields, stoichiometric ratios of the loaded protonated amino groups to the loaded negative sulfate ions, and the sizes of CFNs formed by polyelectrolyte complexation under various pH environments and chitosan–fucoidan mass ratios.

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