One-step Preparation of Cationic Sugar–Peptide Nanospheres Using the Water-soluble Chitosan-initiated Polymerization of L-Phenylalanine-*N*-carboxylic Anhydride

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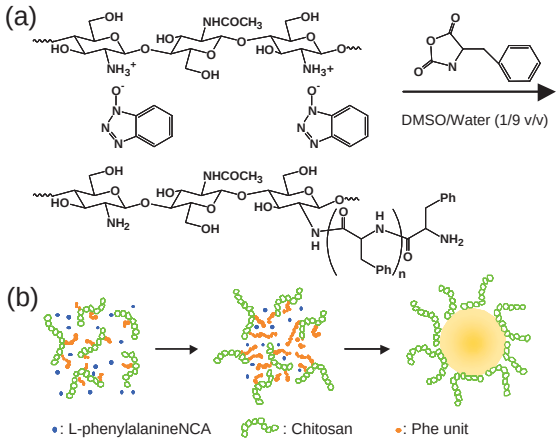
Biodegradable chitosan-based nanospheres were prepared by the one-step polymerization of L-phenylalanine-*N*-carboxylic anhydride initiated by water-soluble chitosan. The obtained chitosan-*g*-poly(phenylalanine) nanospheres with a diameter of 220–290 nm showed a strong positive charge, and thus it will be valuable as novel biodegradable cationic nanosphere.

Biodegradable and biocompatible nanospheres have attracted much attention in the biomedical field since they are promising carriers for drugs, genes, and proteins.[1] In particular, there has been growing interest in chitosan (CT)-based nanospheres because of their excellent properties, such as low toxicity, biocompatibility, biodegradability, low immunogenicity, and bioadhesive properties.[2] Various kinds of CT-based nanospheres have been prepared by the self-assembly of amphiphilic CT derivatives with hydrophobic groups such as deoxycholic acid,[3] palmitic acid,[4] linolenic acid,[5] and cholesterol[6] in aqueous media. These self-assembly methods of amphiphilic CT are suitable for biomedical applications, since toxic molecules such as soap and organic solvents are not necessary to form the nanospheres, unlike O/W emulsion methods. However, conventional self-assembly methods require at least a two-step operation, the hydrophobic modification of CT (first step), and the self-assembly process to obtain the nanospheres in water (second step). If a one-step self-assembly method to form CT nanospheres without any toxic components is developed, it would be valuable for drug delivery systems (DDS) or biomedical applications. To develop a one-step technique for CT nanospheres, the low solubility of CT has been a significant issue for chemical modification to control the hydrophilic–hydrophobic balance of CT derivatives.

Recently, we found that high-molecular weight CT was easily dissolved without any organic solvents or acids, and could be chemically modified in an aqueous solution by ion-complex formation with 1-hydroxybenzotriazole (HOBt).[7] The CT–HOBt aqueous solution was prepared by simply mixing the CT and HOBt in water, and provides an effective system to functionalize CT in an aqueous environment. Furthermore, we have also vigorously studied the development of one-step methods for preparing nanospheres composed of biodegradable polymers as well as synthetic polymers.[8,9] Our system enables the one-step synthesis of peptide nanospheres in a water/DMSO solution by the polymerization of L-phenylalanine-*N*-carboxylic anhydride (Phe-NCA) using hydrophilic PEG amine as a macroinitiator, exploiting the self-assembly process with the hydrophobic interactions of the Phe segments.[8] Accordingly, we speculated that novel cationic sugar–peptide nanospheres based on CT could be synthesized in one-step by the combination of the CT–HOBt system and the one-step NCA method. These cationic sugar–peptide nanospheres have great potential as biodegradable and low-immunogenicity cationic carriers for DDS or biomedical applications. In the present study, we report for the first time the one-step preparation of cationic CT–peptide nanospheres using a CT–HOBt aqueous solution in combination with the Phe-NCA method.



Scheme 1. (a) Synthesis of chitosan-*g*-poly(phenylalanine). (b) Schematic illustration of the self-assembly mechanism of the sugar–peptide nanospheres.

Phe-NCA was prepared from a reaction of L-phenylalanine with triphosgene, according to previously reported methods (yield: 77%).[10] Next, we performed the polymerization of Phe-NCA using CT (MW = 1.0×10^5, deacetylation degree = 95%) as a macroinitiator that was dissolved in water with HOBt (1.7 equiv per CT unit) at various feed molar ratios (Phe-NCA/CT unit = 16.0, 13.5, and 11.0) in a DMSO/water (1/9 v/v) mixed solvent for 24 h at 0 °C (Scheme 1a). During the reaction, the reaction solution turned to a milky color. The obtained reaction solution was dialyzed for 3 days against a 0.001 M HCl aqueous solution.

The polymerization conditions and the results of the graft copolymerization are summarized in Table 1. The yields of the copolymers were 50–51%. The solubility test showed that the obtained copolymers did not dissolve in ordinary organic solvents, such as DMSO, *N*,*N*-dimethylformamide, *N*-methylpyrrolidone, or chloroform. In contrast, they could dissolve in a trifluoroacetic acid/chloroform mixed solvent, which is a good solvent for Pphe, although the original CT did not dissolve in this

Table 1. Syntheses of chitosan-*g*-poly(phenylalanine)[a]

Run	Feed ratio NCA[b]/CT[c] unit	Yield /%	DP_{Pphe}[d]	d_m[e] /nm	C.V.[f] /%	ζ-potential[g] /mV
1	16.0	50	15.6	290	35	40
2	13.5	50	13.6	230	32	49
3	11.0	51	12.8	220	37	47

[a]Reaction time was 24 h, and temperature was 0 °C. Solvent was water/DMSO (9/1 v/v) mixed solvent. [b]NCA refers to L-phenylalanine-*N*-carboxylic anhydride. [c]CT refers to chitosan. [d]The average polymerization degree of Pphe was determined by ^{1}H NMR spectrum. [e]Mean diameter was measured by dynamic laser scattering (DLS). [f]Coefficient of variation (C.V.) is standard deviation/mean diameter. C.V. was calculated on the basis of DLS study. [g]ζ-potential was measured in water at pH 3.

solvent. In the FT-IR spectra of the copolymer, IR peaks assigned to the pyranose ring of CT (ν_{C-O}: 1050 cm^{-1}), and the amide I and amide II groups of Pphe ($\nu_{C=O}$: 1630 and δ_{N-H}: 1540 cm^{-1}) were observed (Figure S1),[11,12] and the relative intensity of the amide I peak to the pyranose ring peak decreased with a decreasing feed ratio of NCA/CT. These results support the successful synthesis of CT-*g*-Pphe via the CT initiated polymerization of Phe-NCA in aqueous media. The ^{1}H NMR spectra also confirm CT-*g*-Pphe formation in aqueous media (Figure S2).[12] We estimated the average polymerization degree of Pphe side chains (DP_{Pphe}) from the integration ratios of the methine peak of the terminal Pphe unit at 4.41 ppm to the total methine peaks at 4.66 ppm. The DP_{Pphe} values were in good agreement with the in feed NCA/CT units, and the values were 15.6, 13.6, and 12.8 for run 1, run 2, and for run 3, respectively. Furthermore, we confirmed the complete removal of HOBt after the reaction by the absence of its aromatic peaks in ^{1}H NMR measurements, indicating no effect of HOBt on the polymerization.

To confirm nanosphere formation, the obtained CT-*g*-Pphe dispersion was analyzed by dynamic laser scattering (DLS) measurements at the concentration of 0.1 mg/mL and 25 °C in water (pH 3) (Figure 1a). The DLS histogram of run 1 revealed that monodispersed nanospheres with an average size of 290 nm were formed. The diameter of the nanospheres decreased with decreasing NCA/CT units in the feed ratio from 290 to 220 nm. Figure 1 shows scanning electron microscopic (SEM) and transmission electron microscopic (TEM) images of the nanospheres from run 1.[13] These images show the monodispersity, highly spherical shape, and rough surface of the CT-*g*-Pphe nanospheres. Furthermore, the ζ-potentials of the nanospheres were +40, +49, and +47 mV for run 1, run 2, and run 3, respectively, strongly suggesting that the amino groups of CT were present on the surface of the CT-*g*-Pphe nanospheres in water. The SEM and DLS study confirmed that the CT-*g*-Pphe nanospheres were stably dispersed in water (pH 3) without considerable change of their diameter and morphology for more than two months. We reviewed the self-assembly mechanism of the CT-*g*-Pphe nanospheres on the basis of the above results (Scheme 1b). At the first stage, the Phe-NCA polymerization is initiated from some amino groups of CT to give rise to amphiphilic chitosan, thus inducing segregation between the hydrophilic CT backbone and the hydrophobic oligomeric Phe graft. Next, the Phe-NCA is polymerized in the hydrophobic domain to form a stable core. On the other hand, the electrostatic repulsions of the charged amino groups of CT impart the water-dispersion stability. Finally, surface cationic nanospheres composed of CT-*g*-Pphe are formed.

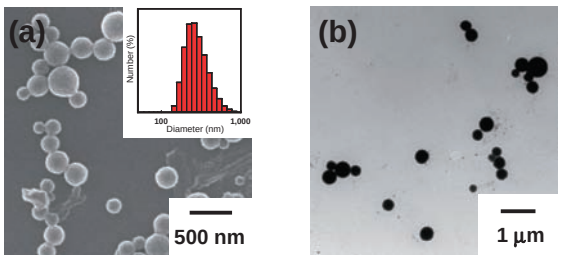


Figure 1. (a) SEM and (b) TEM images of CT-*g*-Pphe nanospheres (Table 1, run 1). The inset is a size distribution of CT-*g*-Pphe nanospheres (Table 1, run 1) measured by dynamic laser scattering.

In conclusion, we demonstrated that sugar–peptide nanospheres were successfully prepared by the one-step polymerization of Phe-NCA initiated from water-soluble CT–HOBt complex. The one-step preparation of sugar–peptide nanospheres is a universal methodology, which can be applied to other polysaccharides. These CT-*g*-Pphe nanospheres are expected to be useful as a novel cationic carrier for drugs, proteins, and genes.

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12 Supporting Information is also available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.
13 For TEM observations, the nanospheres were positively stained by 1% ammonium molybdate.

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