Doxorubicin-loaded microgels composed of cinnamic acid–gelatin conjugate and cinnamic acid–Pluronic F127 conjugate

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Abstract

Microgels were prepared by cinnamic acid–gelatin (type B) conjugate (CA-GelB) and cinnamic acid–Pluronic F127 conjugate (CA-Plur). 1H NMR confirmed that CA was conjugated to gelatin and the gelatin to CA residue molar ratio was estimated to be 1:4.7 by a colorimetric method. CA-Plur of which the CA residue to Plur molar ratio was 1.2:1 was used as a thermo-sensitive polymer. The CA residues of CA-Plur/CA-GelB mixture were readily photo-dimerized to form microgels by UV irradiation. The isoelectric point of the microgel was found to be pH 5.8 and the hydrodynamic diameter decreased when the suspension temperature increased. The microgel could hardly retard the release of doxorubicin (DOX) at pH 3.0 and pH 5.0, but it could suppress and control the release at pH 7.4 possibly due to electrostatic attraction. Meanwhile, the release of DOX at pH 7.4 was less suppressed when the medium temperature was higher, possibly because of thermal thinning of Pluronic chain layer.

Introduction

Several kinds of self-assemblies have been developed by covalently conjugating cinnamic acid (CA, 3-phenylprop-2-enonic acid) to water-soluble polymers and using the conjugates as a building block. CA is found in cinnamon, balsam and shea butter and it can be chemically synthesized. CA and its derivatives are used in flavors, in pharmaceuticals and in perfume industries. CA undergoes (2 + 2) cycloaddition to be dimerized under the irradiation of a UV light and the dimers are subjected to photo-cleavage by a shorter UV light. It can be readily attached to various compounds covalently due to its reactive functional group, carboxylic acid. If CA is conjugated to a water-soluble polymer, the conjugate is likely to be amphiphilic because it has a phenyl group which is non-polar. Accordingly, CA/water-soluble polymer conjugate can be self-assembled in an aqueous phase. The mixture of cinnamoyl alginate and cinnamoyl Pluronic F127 was assembled into nanogels in an aqueous phase. In this study, microgel composed of CA/gelatin (type B) conjugate (CA-GelB) and CA/Pluronic F127 conjugate (CA-Plur) were prepared by dissolving them in an aqueous solution. Owing to the inter/intra-molecular hydrophobic interaction among CA residues, the mixture of CA-GelB and CA-Plur will be assembled into microgels. When the pH value of medium is greater than the isolectric point of CA-GelB, doxorubicin (DOX, positively charged anti-cancer agent) can be readily loaded in the CA-GelB/CA-Plur microgel by electrostatic interaction because CA-GelB will be negatively charged, and it would hardly be released from the microgel. When the pH value of medium is less than the isolectric point, DOX would be readily released from the microgel because CA-GelB will be positively charged. Accordingly, the release of DOX will depend on the pH value of medium. Meanwhile, Pluronic F127 solution becomes gel at a temperature when the solution is heated, and the gelation temperature is around 29°C when the concentration is low (e.g. less than 20%). Below the gelation temperature of Pluronic F127, the Pluronic chains will be fully hydrated and take an expanded form. Thus, they would form stagnant layer around the microgel and hinder the release of DOX. When the polymer solution is heated to a temperature above the gelation temperature, the Pluronic chains will be collapsed to form a thinner stagnant layer so the release of DOX from the microgel would be less suppressed. Accordingly, the release of DOX will also depend on the temperature of medium.

Experimental

Materials

Gelatin (type B), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), cinnamic acid (CA), doxorubicin (DOX) and phosphotungstic acid (PTA) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Dialysis membrane (MWCO 50 000, MWCO 3500) was purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA) CA-Pluronic F127 conjugate (CA-Plur) was provided from Institute of Bioscience and Biotechnology, Kangwon National University (Chunchon, Korea) (the molar ratio of cinnamic acid residue to Pluronic F127 is 1.2:1).

Preparation of CA-gelatin conjugate

CA-gelatin (type B) conjugate (CA-GelB) was prepared by a condensation reaction. Twelve milliliters of EDC solution (1.7% (w/v)) were put into 4 ml of CA solution (1.98% (w/v)), in sodium
hydroxide solution (1 M), pH 8.0) contained in a 50 ml beaker, and it was stirred around 4 °C for 30 min. Ten milliliters of gelatin solution (1% (w/v), in PBS (30 mM, pH 7.4)) was put into the CA/EDC mixture solution and it was stirred at 30 °C for 24 h. In order to remove unreacted chemicals, the reaction mixture was put in a bag of dialysis membrane (MWCO 3500) and dialyzed for 48 h in distilled water (500 ml). During the dialysis, the dialysis medium was exchanged three times. The purified reaction product (CA-GelB) was freeze-dried for further use.

\[ \text{Determination of extinction coefficient and content of CA residue of CA-GelB} \]

The UV spectra of gelatin solution (0.0156–1 mg/ml) and those of CA-GelB solution (in the same concentration range) were recorded on a UV spectrophotometer (6505 UV/Vis. Spectrophotometer, JENWAY, UK). In order to determine the concentrations of CA residue in CA-GelB solutions, the contribution of gelatin to the absorbance at 270 nm of CA-GelB was removed at all the CA-GelB concentrations (0.0156–1 mg/ml) by subtracting the absorbance of gelatin solution from the absorbance of CA-GelB solution of the same concentration. Then, the concentrations of CA residue of CA-GelB at all the CA-GelB concentrations were calculated using the calibration curve of CA and the corrected CA residue absorbance obtained above. The calibration curve of CA was obtained by plotting the absorbance at 270 nm of CA versus its concentration (0–0.015 mg/ml). Then, the corrected absorbance values were plotted versus the calculated concentration of CA residue in mM. The extinction coefficient of CA and CA residue was obtained from the slope of calibration curve of CA and from that of the plot of corrected absorbance versus calculated concentration of CA residue, respectively. On the other hand, the gelatin to CA residue molar ratio of CA-GelB was calculated at all the CA-GelB concentrations by using the calculated concentration of CA residue and assuming that the average molecular weight of gelatin is 50 000. The average value of the gelatin to CA residue molar ratio was reported.

\[ \text{Measurement of dimerization degree} \]

CA, CA-Plur, CA-GelB and the mixture of CA-Plur/GelB (1/0.83, w/w) were dissolved in distilled water so that the concentration of CA or CA residue was 0.016% (w/v). A UV light (365 nm, 400 W) was irradiated to the solutions for 60 min, and the dimerization degree was calculated by the following equation:

\[ \text{Dimerization degree } (\%) = \left(1 - \frac{A_t}{A_0}\right) \times 100\% \]

where \(A_0\) and \(A_t\) are the absorbance at 270 nm of the solutions before and after UV being irradiated, respectively.

\[ \text{Preparation of microgels} \]

CA-GelB (54.5 mg) was dissolved in 5 ml of distilled water contained in a 20 ml vial so that the CA residue concentration was 1 mM. In parallel, 45.5 mg of CA-Plur was dissolved in 5 ml of distilled water so that the CA residue concentration was 0.86 mM. The CA-GelB solution was mixed with the CA-Plur solution in a 20 ml vial. Then the mixture solution was subjected to UV irradiation (400 W, 365 nm) for 1 h (The pH value of microgel suspension was 5.6). Then 0.5 ml of microgel suspension was mixed with 0.5 ml of DOX solution (200 μg/ml in distilled water) contained in a 10 ml vial. Then 3 ml of concentrated buffer solution (×1.33) was added to the mixture of microgel suspension and DOX solution. McIlvaine buffer was used for pH 3.0 and pH 5.0, PBS buffer was used for pH 7.4 and carbonate–bicarbonate buffer was used for pH 9.0 and pH 10.0. For the examination of release of DOX, the microgel suspensions containing DOX (pH 3.0–10.0) were used as they were.

\[ \text{Characterization of microgels} \]

The transmission electron microscope (TEM) photos of microgels were taken using a negative staining technique. The microgel was stained by mixing the suspension and tungstophosphoric acid solution (2% (w/v) in distilled water) in 1:1 (v/v) ratio and being allowed to stand at room temperature for 3 h. The suspension was transferred to a formvar carbon/copper-coated grid (200 mesh) and air-dried for 24 h. The size of microgel suspended in distilled water, with pH value 5.6, was measured on a DLS equipment (Plus 90; Brookhaven, Holtsville, NY) with the suspension temperature being changed from 10 to 50 °C. The zeta potential of microgel was determined on the same machine at 25 °C with the pH value of the suspension being changed from 3.0 to 10.0.

\[ \text{Release of DOX from microgel suspension} \]

For release experiment, 4 ml of microgel suspension containing DOX (pH 3.0–10.0) was put in a dialysis bag (MWCO 50 0000) and it was dialyzed in 146 ml of the corresponding buffer solution contained in a 200 ml beaker. Dialysis medium (0.5 ml) was taken at a given time for 8 h. And the fluorescence intensity of DOX was determined on a fluorescence spectrophotometer at 556 nm with excitation wavelength of 485 nm. The release amount was calculated using a calibration curve of DOX and the fluorescence intensity of the dialysis medium. The calibration curve was established at each pH value of the dialysis medium because the fluorescence intensity is pH-sensitive.

\[ \text{Results and discussion} \]

\[ \text{1H NMR spectroscopy} \]

Figure 1 shows the 1H NMR spectrum of CA-GelB. The signal around 0.98 ppm is assigned to the methyl proton of amino acid residues, the signal around 3.01 ppm is due to the amino proton of lysine residues, and the signal in 7.05–7.25 ppm is ascribed to the phenyl proton of phenyl alanine residues, and the signal in 7.25–7.50 ppm is attributed to the cinnamoyl proton, indicating that CA was successfully conjugated to gelatin.

\[ \text{Determination of extinction coefficient and gelatin to CA residue ratio of CA-GelB} \]

Figure 2 shows the UV spectra of gelatin (type B) and CA-GelB. The signal around 0.98 ppm is assigned to the methyl proton of amino acid residues, the signal around 3.01 ppm is due to the amino proton of lysine residues, and the signal in 7.05–7.25 ppm is assigned to the phenyl proton of phenyl alanine residues, and the signal in 7.25–7.50 ppm is attributed to the cinnamoyl proton, indicating that CA was successfully conjugated to gelatin.
the equations, the molar extinction coefficient of CA and CA residue was estimated to be 15.9 and 14.9 mM$^{-1}$cm$^{-1}$, respectively. Since the extinction coefficients were not markedly different from each other, it can be said that the photochromic property of CA remained almost intact after it was covalently attached to gelatin. On the other hand, by taking advantage of the CA residue concentrations calculated at all the CA-GelB concentrations, the gelatin to CA residue molar ratio of CA-GelB was estimated to be 1:4.7 on average.

**Measurement of dimerization degree**

Figure 3 shows the dimerization degree of CA and the dimerization degree of the CA residue of CA-Plur, CA-GelB
and CA-Plur/CA-GelB mixture. The dimerization took place rapidly for the first 5 min of UV irradiation and then slowly during the rest period. CA is known to be dimerized by a UV irradiation and the dimer can also be cleaved to be monomer by the UV irradiation of the same wavelength. This would account for the reason the dimerization rate is fast in the early stage of UV irradiation and it decreased with the irradiation time. On the other hand, the dimerization degree of the CA residue of CA-Plur, CA-GelB and CA-Plur/CA-GelB mixture was lower than CA throughout the irradiation period. Since CA residue is bound to gelatin, its mobility depends on that of the macromolecule thus the collision frequency among CA residues would be lower than that of free CA. In addition, the collision of the CA residue could be sterically hindered due to the bulky macromolecule it is bound to. These may be a reason why the dimerization degree of CA was higher than that of the CA residues.

TEM photos of microgels

Figure 4 shows the TEM photo of CA-Plur/CA-GelB microgel after being exposed to UV light for 1 h. CA-Plur/CA-GelB microgels were observed as nearly circular and white objects and the diameter were hundreds of nanometers. CA-Plur would be associated with CA-GelB through the hydrophobic interaction among CA residues, and they could be assembled to microgels. PTA, a staining agent used in the presents study, will be hardly captured by CA-Plur/CA-GelB microgels, because PTA is negatively charged and CA-GelB also have the same charge at pH 6.8 where microgels were stained with PTA (the isoelectric point of CA-GelB was found to be around pH 5.8 in Figure 5). As a result, electron beam could penetrate through the microgel and it would scattered back on the PTA-stained area where no microgel was because electron beam can hardly penetrate though heavy metal ions. This could explain why CA-Plur/CA-GelB microgels were found as white objects.

pH-dependent zeta potential and temperature-dependent size of microgels

Figure 5 shows the pH-dependent zeta potential of CA-GelB and CA-Plur/CA-GelB microgels. The zeta potential of CA-GelB decreased from +26.9 to −15.6 mV, when the pH value increased from pH 3.0 to pH 10.0. As the pH value increases, the carboxylic group of CA-GelB will be ionized to be negative carboxylate and the positive protonated amine group will be deprotonated to be neutral amine group, thus the zeta potential will change from positive value to negative one. The isoelectric point of CA-GelB was found to be around pH 5.8 and the value is close to the isoelectric point of gelatin type B (pH 4.8–pH 5.5). According to the result of colorimetric analysis described previously, one molecule of gelatin was estimated to have 4.7 CA residues. It means that the number of amine group disappeared upon the reaction was 4.7 per one molecule of gelatin. 4.7 amino groups can be thought to be a small portion of total amine groups since the number of basic amino acid residues (e.g. histidine, arginine and lysine) per one molecule of gelatin is 28.2 on average. This may explain why the isoelectric point of CA-GelB did not deviated significantly from that of gelatin. On the other hand, the zeta potential of CA-Plur/CA-GelB microgels decreased from +5.6 to −4.6 mV when the pH value increased from pH 3.0 to pH 10.0. The absolute value of the zeta potential was less than that of...
CA-GelB in the full range of pH value tested. But the isoelectric point of the microgel was almost the same as that of CA-GelB. Microgel is composed of CA-Plur and CA-GelB, where CA-Plur is thought to associate with CA-GelB through the hydrophobic interaction and the dimerization among CA residues. So the attachment of CA-Plur to CA-GelB will have no effect on the number of positive charge point and the number of negative charge point. This could explain why the isoelectric point of the microgel was almost the same as that of CA-GelB. When CA-Plur is associated with CA-GelB, the chains of Pluronic F127 could surround the CA-GelB and they will be the major component of the microgel surface. In this circumstance, the surface potential of the microgel will be less than that of CA-GelB because the chain of Pluronic F127 is non-ionic. The hydrodynamic diameter of microgel decreased from 285 to 220 nm when the suspension temperature increased from 10 to 50 °C (Supplementary data). Pluronic F127 is a temperature-sensitive polymer exhibiting gelation temperature. When the solution temperature increased across gelation temperature, the polymer chains become dehydrated to be condensed. This could be a reason why the hydrodynamic diameter decreased with increasing the temperature.

Release of DOX from microgel suspension

Figure 6 shows the release profiles of DOX in solution and in CA-Plur/CA-GelB microgel suspension at 25 °C when the pH value of release medium was pH 3.0 (○, ●), pH 5.0 (△, ▽) and pH 7.4 (□, ■). That is, it could also hardly control the release of DOX as it could at pH 3.0. This is possibly because the pH value of release medium, pH 5.0, was still lower than the isoelectric point of microgel. Meanwhile, when the pH value was 7.4, the release degree was much lower than that obtained with DOX solution throughout the release experiment period. At the same time, the release degree at pH 7.4 was lower than the release degree observed at pH 3.0 and pH 5.0. For example, the release degree in 24 h was 72.2% at pH 7.4 and the value was lower than the release degree at pH 3.0, 95.3%, and the release degree at pH 5.0, 105.1%. The microgel will be negatively charged at pH 7.4, because the pH value is greater than the isoelectric point of microgel (pH 5.8). Thus, the microgel will electrostatically attract with DOX and it will retard and control the release of the positively charged drug. At pH 9.0 and pH 10.0, DOX precipitated out in dialysis bag and formed claret-colored sediment, and it could hardly be detected in release medium.

Figure 7 shows the release profiles of DOX in solution and in CA-Plur/CA-GelB microgel suspension at pH 7.4 when the temperature of release medium was pH 3.0, 5.0, 7.4, 9.0, and 10.0. When the temperature was 25 °C, the maximum release degree of DOX from microgel was around 72.2%. When the temperature was 35 and 45 °C, the maximum release degree was 92.7% and 99.5%, respectively. When the medium temperature is above gelation temperature, Pluronic chains associated with CA-GelB will collapse to form a condensed layer. Accordingly, the thickness of the polymer layer around CA-GelB molecules will be thinner at a higher temperature. Since the Pluronic chain layer can act as stagnant layer because the polymer chains can imbibe water, it would provide CA-Plur/CA-GelB microgel with a diffusion barrier. Thus, the thinner Pluronic chain layer at a higher temperature would result in a lower diffusion resistance and a higher degree of release.

Conclusion

Microgel composed of CA-Plur (1.2/1, mol/mol) and CA-GelB (4.7/1, mol/mol) could be prepared utilizing the self-assembling property of the conjugates and the photo-dimerizable property of
CA residue. The molar extinction coefficient of CA residue of CA-GelB was 14.9 mM$^{-1}$cm$^{-1}$, and the value was close to that of CA, 15.9 mM$^{-1}$cm$^{-1}$, indicating that the photochromic property of CA remained almost unchanged after its conjugation with gelatin. Although the dimerization degree of the CA residue of CA-Plur/CA-GelB mixture was lower than free CA throughout the UV irradiation period, the CA residue could readily be photodimerized. The microgels were observed as nearly circular objects and the diameter were hundreds of nanometers on the TEM photos. The hydrophobic interaction among CA residues seemed to allow CA-Plur/CA-GelB mixture to be assembled into microgels. The isoelectric point of the microgel was found to be around pH 5.8. And the absolute value of the zeta potential of the CA-Plur/CA-GelB microgel was less than that of CA-GelB, possibly due to the non-ionic Pluronic chain layer of the microgel. The microgel decreased from 285 to 220 nm in diameter when the medium was heated from 10 to 50°C, possibly due to the thermally-induced contraction of Pluronic chain. The microgel could not sustain the release of DOX at acidic condition (e.g. pH 3.0 and 5.0), however it could sustain the release at physiological condition (e.g. pH 7.4) possibly due to the electrostatic interaction between the microgel and DOX. On the other hand, the release of DOX was less sustained when the medium temperature was higher, possibly because the thermally-induced collapse of Pluronic chain would serve thinner stagnant layer.

Declarations of interest

The authors report no conflicts of interest.

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