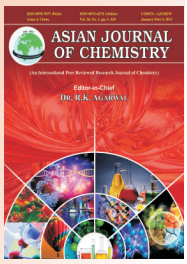
Asian Journal of Chemistry; Vol. 26, No. 12 (2014), 3553-3558

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.16321



Stable Nano-Micelles Made of Casein and Keratin Through Self-Assembly

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Received: 10 September 2013; *Accepted*: 2 January 2014; *Published online*: 5 June 2014; AJC-15293

Nano-micelles made of casein and keratin were produced through self-assembly, the complex micelles were fixed by transglutaminase and became stable. The method for preparing the complex nano-micelles proved to be harmless and green. The nano-micelles behaviour and properties has been investigated in the pH ranges of 5-9. Turbidity measurements, dynamic light scattering, fluorescence measurement, transmission electron and atomic force microscopy studies revealed that the complex nano-micelles have a unique morphology. Their hydrodynamic diameters depend on their behavior under different pH value and mass ratio. The complex nano-micelles had a more stable feature than the casein micelles and can be used to develop environmentally friendly coatings or other biomass materials.

Keywords: Casein, Keratin, Complex nano-micelles.

INTRODUCTION

Molecular self-assembly is the process by which molecules adopt a defined arrangement without guidance or management from an outside source through non-covalent binding. There was more attentions focus on the natural biological macromolecules self-assembling because many new biomaterials can developed from them.

Casein is the main component of the mammalian milk and made up of four separate casein molecules (αs1, αs2, β and κ-casein) that are hold together by colloidal calcium phosphate (CCP)[1]. It is considered as amphiphilic block copolymers which have amounts of hydrophobic and hydrophilic amino acid residues and associate to form casein micelles. Casein is different from globular and fibrous proteins and regarded as the intrinsically unstructured protein[2-5]. Casein micelles can be dissociated into smaller subunits called casein submicelles by removal of colloidal calcium phosphate. Polyphosphate[6], organic acids[7] Chelating agents[8], acidification[9], adialysis against Ca^{2+} free buffers were used to dissolve the colloidal calcium phosphate and dissociate the micelles.

Casein and keratin are heterologous proteins with the isoelectric points of 4.6 and 4.8. The interactions between casein and keratin usually did not result in precipitation and phase separation in the preliminary experiment. Casein and keratin both have a strong tendency to self-assemble spontaneously in solutions. Madadlou *et al.*[10] studied the re-assembly process of casein micelles in the pH range of 6.35-11.4 and found that the turbidity of micelles solutions reduced and the structure became loosen with the increasing pH value. Moreover, they found the micelles still maintained the three-dimensional conformation integrity even the pH reached 12.6. Liu and Guo[11] discovered that the casein micelles can formed in the pH ranges of 2-3 and 5.5-12 and the micelles display the most compact structure at the pH 5.5. Pan *et al.*[12] prepared a nano-gel by self-assembling of β-casein and lysozyme and studied its characteristics. Tonin *et al.*[13] explored that self-assembly can occur between keratin and poly (ethylene oxide) (PEO) and resulted in a poly (ethylene oxide) (PEO) and keratin blended films with improved structural properties. Furthermore, the spontaneous self-assembly of keratin solutions has been studied extensively both at the microscale[14-16] and macroscale levels[17]. Recent studies revealed that casein exhibits complicated aggregates micelles with spherical and linear features in solution. It presents a loosened conformation and has no typical secondary and tertiary structure. It tends to undergo structural alterations in the presence of external factors. All these conditions implied that casein and keratin has a great possibility to self-assemble and interact each other to form new micelles.

The polymer micelles were characterized by its good drug loading capacity, high structural stability, excellent water solubility, non-accumulation and tiny particle size (100 nm or smaller). Based on these viewpoints, our studies intended for structural stability and nano-particle have been conducted. Biomaterials such as ecological coating or cosmetic ingredients with desired properties can be developed based on the complex nano-micelles without toxicity.

EXPERIMENTAL

Casein, technical grade (Sigma), was dispersed in ultra-pure water by magnetic stirring at 50 °C, then stored at 4 °C at least for 10 days. The casein solution was prepared and adjusted to pH 7 with 1 M NaOH and 1 M HCl solution. Millipore filters with 2 μm pore size were used to get rid of the impurities and microbial. Keratin powder was put into ultra-pure water and solid NaOH was added until it completely dissolved at desired concentration, then the keratin solution was filtrated by filters mentioned above. Sodium citrate as a dispersed material was used to disassociate the casein micelles. All the other reagents used were of analytical grade.

Complex nano-micelles formation and fixation: The aqueous casein and keratin solutions with desired concentrations were prepared separately. Then the casein and keratin solution were dispersed with sodium citrate at the mass of 5:1 (dry matter). Casein and keratin solutions were then mixed under different mass ratio and adjusted to the desired pH at the range of 5-9, in that the transglutaminse are stable in this range[18]. Then, the mixed solutions was shaked for 10 min and kept at 4 °C for at least 24 h to allow the casein and keratin to self-assemble completely. The complex micelles were then fixed by transglutaminase for 1 h at 50 °C and the enzyme was inactivated at 80 °C for 10 min.

Turbidity measurements: A Shimadzu UV1705 spectrometer was used for turbidity measurements. Solution was injected into the Quartz cuvettes and the turbidity was detected at 600 nm wavelength. Bands width: 1 nm, resolution: 0.1 nm.

Dynamic light scattering (DLS): Hydrodynamic diameter of the self-assembly particles was measured by a Zeta Sizer Nano ZS90 and at a scattering angle of 90° at 25 ± 0.1 °C. The instrument equipped with He-Ne laser at 633 nm, max 5 mW. The concentration of the self-assembly micelles for DLS measurement was 5 mg/mL. The z-average diameter (D_h), polydispersity index (PDI) and the intensity for z-average were available for test.

Zeta-potential: The zeta-potential was measured by laser doppler micro-electrophoresis and an electric field is applied to a self-assembly dispersion of self-assembly complex micelles, which then move with a velocity related to their zeta-potential. This velocity is measured using a patented laser interferometric technique called M3-PALS (phase analysis light scattering). This enables the calculation of electrophoretic mobility. The test was carried out by a Zeta Sizer Nano ZS90 at 25.0 ± 0.1 °C. The electrophoresis mobility (UE) was used to calculate the zeta-potential (ζ) by the Henry equation $U_E = 2\varepsilon\zeta f(ka)/3\eta$ and ε, ζ, f (ka) represented the dielectric constant, the viscosity of the medium and the Henry's function.

Fluorescence measurement: The steady-state fluorescence test was carried out on a fluorescence spectrophotometer Hitachi F-7000. Recrystallized pyrene was dissolved in acetone to prepare a concentration of 2×10^{-5} g/mL stock solution and its final concentration in the micelles solution for testing was 2×10^{-7} g/mL. Before measurement, the nano-micelles solutions with desired pH value was kept still for 24 h after the pyrene was added. Excitation spectrum and emission spectrum scan resolution were 1 nm. The excitation and emission wavelength were recorded at the 338 nm, 381 nm and 373 nm wavelength.

Scanning electron microscopy (SEM): Hitachi S-4800 field emission scanning electron microscope was used to observe the nano-micelles at 1 kV. The nano-micelles were fixed by 2 % glutaraldehyde on the silicon surface for at least 0.5 h in order to keep the micelles primitive form, then washed with deionized water and dried at the room temperature. Before observation, the specimens were coated with gold.

Atomic force microscopy (AFM): Skiko atomic force microscopy SPA400-SPI3800N was used to analyze the micelles in tapping mode. The nano-micelles were fixed by 2 % glutaraldehyde on the freshly cleaved mica surface for at least 30min, then washed with deionized water and dried in the room temperature.

RESULTS AND DISCUSSION

Casein and keratin are heterologous proteins with the isoelectric points of 4.6 and 4.8. The interactions between casein and keratin in solution usually did not result in precipitation and phase separation in the preliminary experiment. Casein solution prepared at pH 7 and keratin solution prepared at strong alkali condition are transparent before mixing.

When these two protein solutions were blended together under different mass ratio, the electrostatic complex micelles were produced and then the mixed solution's pH was adjusted by 1 M NaOH and 1 M HCl to the desired value. The solutions were mixed evenly and kept at 25 °C for at least 24 h, complex micelles were then fixed by transglutaminase for 1 h at 50 °C and the enzyme was inactivated at 80 °C for 10 min.

In order to obtain stable self-assembly complex nano-micelles, dynamic light scattering technique was used to assist the research. The influence of mass ratio of casein to keratin on complex nano-micelles formation was investigated at neutral pH. The protein concentration was 5 mg/mL in each sample. The 2 proteins particles interacted each other and formed anionic polyamphoteric micelles with diameter about 42-71 nm at pH 7 (Table-1). We can found that any of the diameter of casein/keratin self-assembly nano-micelles at different mass ratios was less than the diameter of casein and keratin separately. This phenomena can be explained that casein micelles and keratin particles was dissociated partly when these 2 proteins mixed, then they undergo re-assembly procedure and new self-assembly complex micelles formed by non-covalent bond. From (Table-1) we can see the diameter of the self-assembly micelles decreased with the increasing keratin content. This phenomenon was similar to the effect observed on the effect of SDS on casein micelles reported by Lefebvre-Cases *et al.*[19]. This can be hypothesis that the keratin molecules compete with casein molecules and bind to the interior sites of the casein micelles and lead to the dissociation of the colloidal calcium phosphate (CCP). So, casein/keratin self-assembly complex nano-micelles can exist stably at different mass ratio at neutral pH. Table-1 shows that the nano-micelles prepared at neutral pH with mass ratio of casein to keratin 2:1 have the highest intensity and relatively lower PDI. For this reason, we chose mass ratio 2:1 to prepare the complex nano-micelles at different pH in the range of 5-9. Turbidity

measurement and dynamic light scattering were then carried out to detect the self-assembly complex nano-micelles in the different pH value. As shown in Fig. 1, the complex nano-micelles are pH sensitive. The turbidity for the complex nano-micelles declined with the increasing pH value in the range of 5-9. The diameter of the complex nano-micelles became bigger when the pH reach the protein isoelectric point and had the smallest diameter at pH 7. At the pH 7-9 the complex nano-micelles' grew larger but still under 100 nm. This result was similar to turbidity measurement made by Liu and Guo[11] and Madadlou *et al.*[10]. From above, the smallest and stable nano-micelles can acquired at the neutral pH.

TABLE-1
INFLUENCE OF THE MASS RATIO OF CASEIN TO KERATIN ON THE D_h PDI, AND SCATTERING LIGHT INTENSITY OF THE CASEIN/KERATIN COMPLEX NANO-MICELLES

C/K	D_h (nm)	PDI	Intensity (k counts)
1:0	70.85	0.591	82.47
8:1	65.22	0.552	72.26
4:1	60.24	0.425	96.53
2:1	64.55	0.613	92.31
1:1	50.48	0.652	66.89
1:2	46.57	0.611	75.25
1:4	42.15	0.654	89.95
1:8	48.52	0.687	63.25
0:1	120.23	0.712	60.54

Note: C/K represents the casein/keratin complex nano-micelles. D_h is the hydrodynamic diameters, PDI is the particle dispersion index

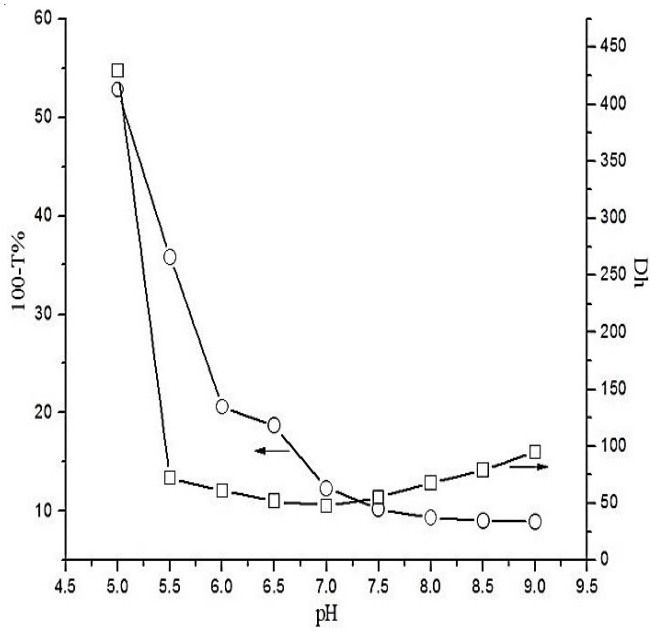

Fig. 1. Turbidity and diameters of the casein/keratin complex nano-micelles (5 mg/mL) as a result of pH value at the mass ratio of 2:1

Characterization of the casein/keratin complex nano-micelles: ζ-Potential relates to the net charges on the surface of the particles in solution. Fig. 2 shows that the absolute value of the ζ-potential of the nano-micelles increased in the range of pH 5-9 when the mass ratio of casein to keratin 2:1. This illustrated that the micelles became stabilized when pH of the casein/keratin complex nano-micelles solution increased from 5 to 9.

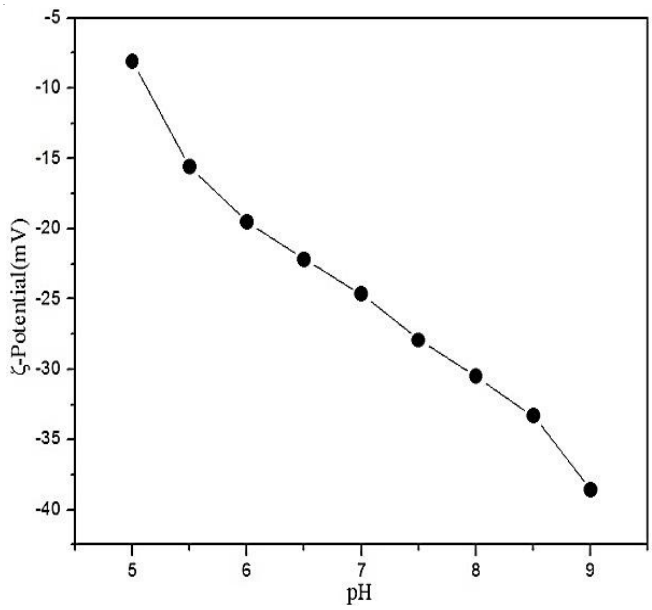

Fig. 2. ζ-Potential of the casein/keratin complex nano-micelles prepared at the mass ratio of 2:1 and at pH 5-9

Fig. 3 shows the ζ-potential of the different mass ratio of casein to keratin on complex micelles solution at pH neutral. We can see the complex micelles absolute value of the ζ-potential increased with the content of the keratin increased. It can be inferred that the adding of the keratin increased the stability of the complex nano-micelles. So, the introduction of keratin into casein chain stabilized the complex micelles dispersion system.

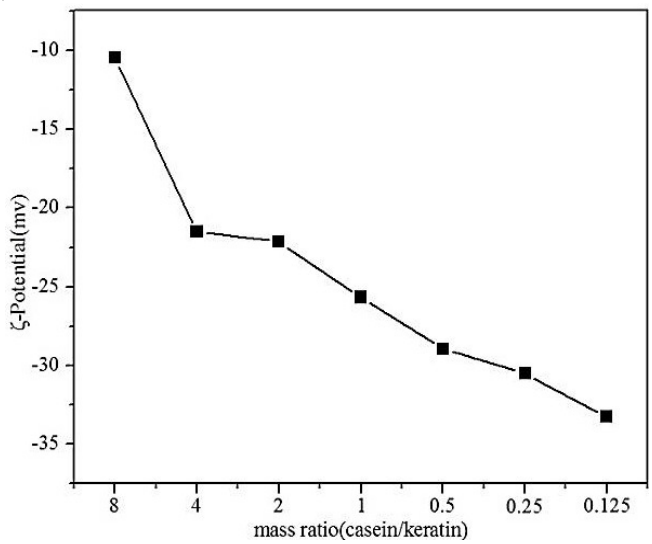

Fig. 3. ζ-Potential of the casein/keratin complex nano-micelles prepared at pH 7 at different mass ratio

The morphology of the casein/keratin complex nano-micelles were observed by TEM and AFM. Fig. 4a represented the nano-micelles micelles at magnification of 20 k and Fig. 4b represented the nano-micelles micelles at magnification of 100 k. The micelles made of casein and keratin *via* self-assembly had a diameter under 100 nm universally. B was the amplified image for the part of the A. From this image, it is clear that the casein/keratin complex nano-micelles had a

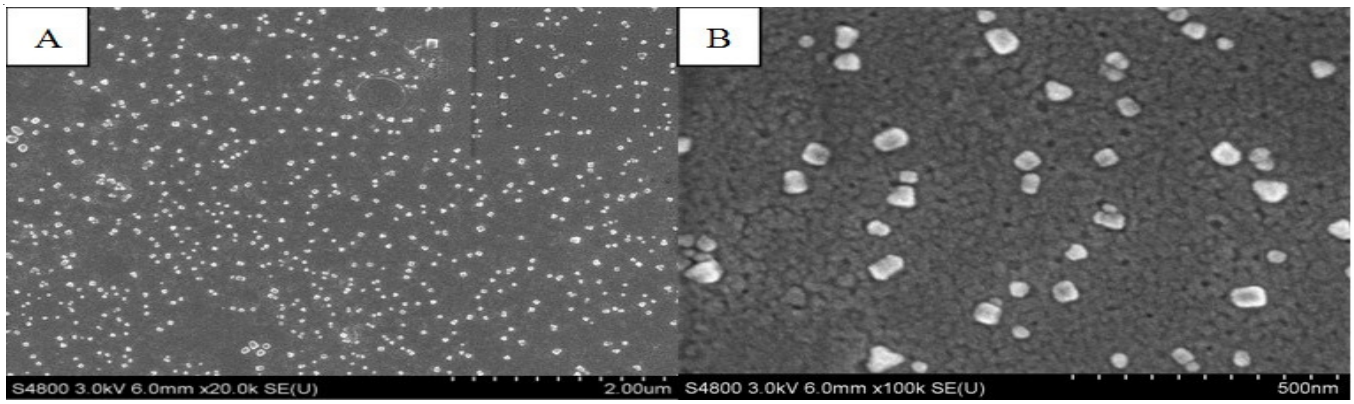

Fig. 4. TEM images of the casein/keratin complex nano-micelles (A. 20 k magnification), (B.100 k magnification)

unique morphology and most of the micelles like a cube sugar and had four upright side. Fig. 5 showed the morphology of the complex nano-micelles by the atomic force microscope. The diameter was about 40-80 nm and accord with resulted measure by dynamic light scattering.

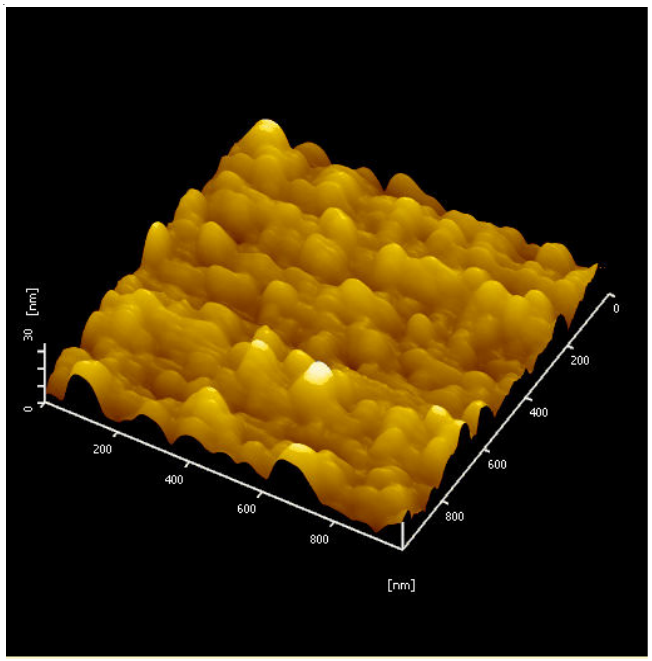

Fig. 5. AFM images of the casein/keratin complex self-assembly micelles

Recrystallized pyrene was used as a probe to detect the hydrophobic and hydrophilic properties of casein/keratin complex nano-micelles. Pyrene emission spectral profile and decay depend on the polarity of the medium in solutions. The pyrene was excitated at wavelength of 338 nm and can produce 5 characteristics emission monomer fluorescence peak at wavelength of 350-550 nm. The intensity ratio of the 1 to the 3 peak (I_1/I_3) in the fluorescence spectrum can reflect the micro-environmental polarity where the probe exists[20]. The greater the value of I_1/I_3, the weaker of the hydrophobic micro-environment for pyrene was.

Fig. 6 showed the value of I_1/I_3 for the casein/keratin complex nano-micelles were pH sensitive at pH 5-9. The value of I_1/I_3 of the self-assembly micelles increased in the pH range of 5-9. The casein/keratin complex nano-micelle was more

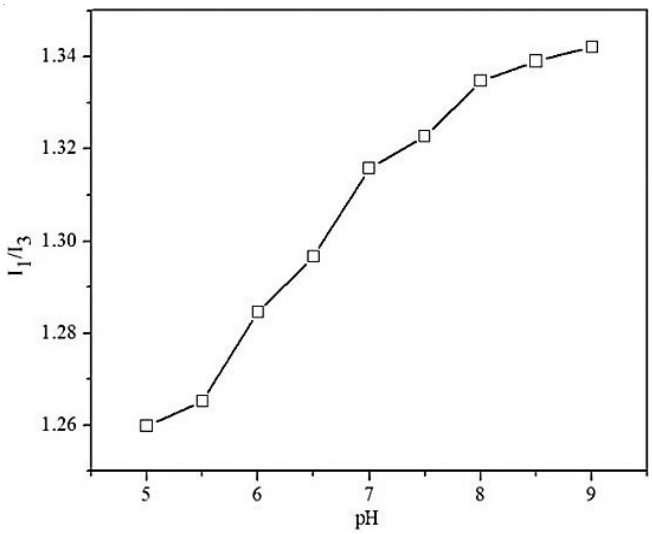

Fig. 6. Plots of I_1/I_3 ratio of pyrene fluorescence in casein/keratin complex nano-micelles solution as function of pH in the range of 5-9. The micelles solutions were prepared at pH 7 with a protein concentration of 5 mg/mL

hydrophobic near the pI of the casein and keratin, which leads to the relative lower value of I_1/I_3. When the pH was increased from 5 to 9, the micelles' structure became loosen because of the increase in electrostatic repulsion and more hydrophilic group stretch out the micelles, which leads to the relative higher value of I_1/I_3.

Fig. 7 showed that the value of I_1/I_3 for the casein/keratin complex nano-micelles varied as a function of the mass ratio. The value of I_1/I_3 of the micelles increased when the value of mass ratio for casein/keratin decreased. When the keratin was added gradually, the micelles dissociate partly and the structure became loosen and the micelles got more hydrophilic relatively.

Stability of the casein/keratin complex nano-micelles: Casein micelles (0.5%) and casein/keratin complex nano-micelles (0.5 %, mass ratio = 2:1) with neutral pH were prepared separately and their stability was compared when storage in the room temperature in 40 days. The changes in diameters and PDI were showed in Figs. 8 and 9. Fig. 8 showed that the value of the diameters for the complex nano-micelles almost constant in the forty days storage time. On the contrary, casein micelles' diameters increased greatly with the days passing by. The result of the PDI also exhibited the similar result in Fig. 9.

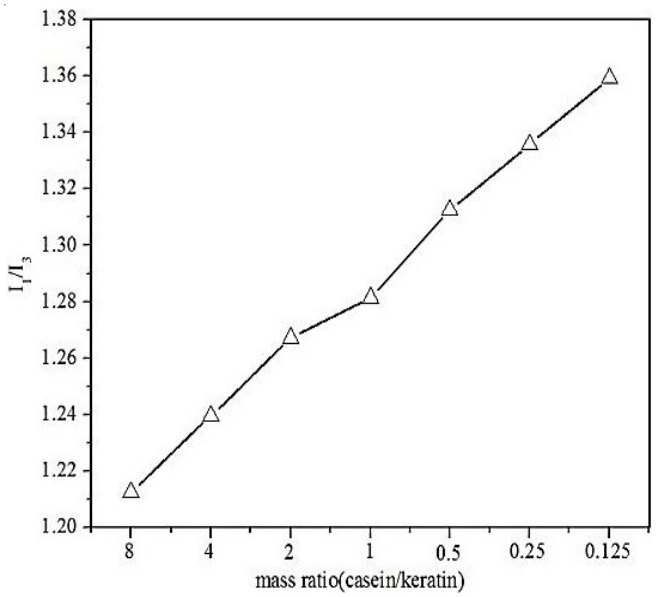

Fig. 7. Plots of I_1/I_3 ratio of pyrene fluorescence in casein/keratin complex nano-micelles solution as functions of mass ratio. The micelles solutions were prepared at pH 7 with a protein concentration of 5 mg/mL

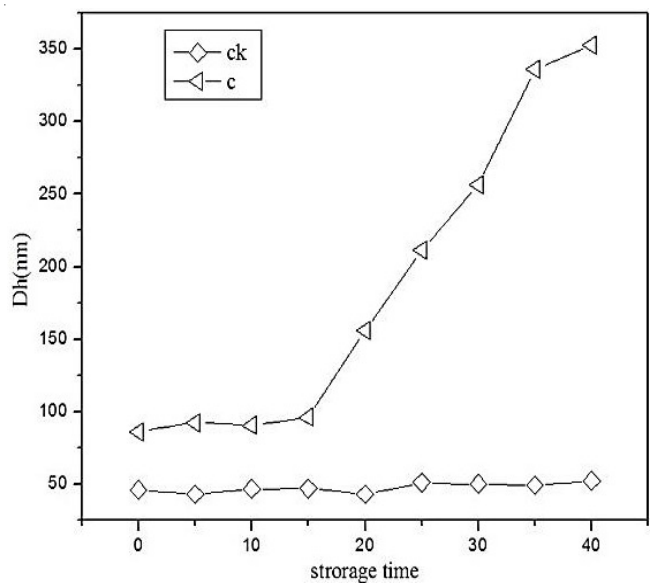

Fig. 8. Plots of D_h variations for the casein micelles and the casein/keratin complex nano-micelles in the 40 days preservation time. The micelles solutions were prepared at pH 7 with a protein concentration of 5 mg/mL

Keratin is a kind of biopolymer with many physical and chemical properties. It is highly stable due to their inter-molecular bonding of disulfide cystine amino acid and inter- and intra-molecular bonding of polar and non-polar amino acids[21]. Treatment of wool in the presence of excess alkali produces the transparent keratin solution with low molecular weight and their secondary structures are not changed. Casein/keratin complex nano-micelles through self-assembling became stably due to the more stably keratin was added.

Casein can be used in leather finishing agent and packing materials, but casein belongs to amorphous protein and cannot exist stably. Moreover, it easy to go mold. At present, the chemical modifications of casein are widely used, but these

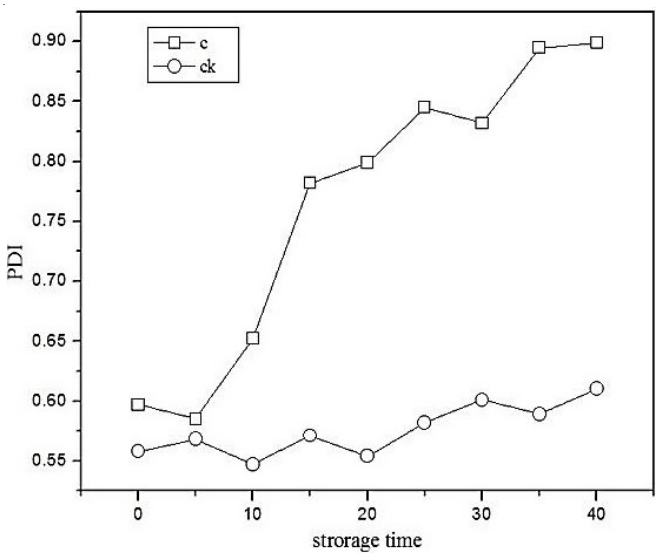

Fig. 9. Plots of PDI variations for the casein micelles and the casein/keratin complex nano-micelles in the 40 days preservation time. The micelles solutions were prepared at pH 7 with a protein concentration of 5 mg/mL

methods also bring about pollution and toxicity problems. The products with improved performance made from the casein/keratin complex nano-micelles can be used to replace the previous materials and don't have to worry about their defects.

Conclusion

Casein and keratin were firstly used to fabricate complex nano-micelles through self-assembling. In this process, no harmful reagents were used. Casein and keratin formed stable polydisperse electrostatic complex nano-micelles with negative charge in the pH range of 5-9. The diameters of the complex micelles are about 42-70 nm due to the different mass ratio and the value of pH. The morphology of the complex nano-micelles exhibited unique appearance due to the disassociated of the casein micelles and re-assembly of these two proteins. The structure of the complex nano-micelles got more compact with the increasing content of keratin and the pH value. The complex nano-micelles are more stable than the casein micelles and this feature can be used in the development of the new products.

ACKNOWLEDGEMENTS

The material in this work was supported by Jiangsu Yiming Bio-products Co.

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