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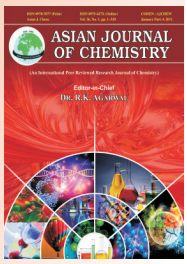

Characterization of Self-Assembly Complex Micelles Prepared from Casein and Keratin

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A novel amphiphilic complex micelles were developed utilizing self-assembly of casein and keratin in aqueous medium. The casein-keratin complex micelles were prepared in the pH ranges of 5.5-9.0 and became more stable and reinforced by the cross-linking effect of the transglutamianse. The formation of casein-keratin complex micelles was confirmed by dynamic light scattering, scanning electron microscope and atomic force microscopy. Results showed that the complex micelles near sphericity and there hydrodynamic diameters are 130-230 nm depending on the different mass ratio of casein to keratin and pH range. The structures of the complex micelles are more compact at low pH and looser at high pH. The complex micelles have the most compact structure at pH 7.5 with the mass ratio of casein to keratin is 2:1.

Keywords: Casein, Keratin, Self-assembly, Complex micelles.

INTRODUCTION

Molecular self-assembly is the spontaneous association of molecules under equilibrium conditions into stable, structurally well-defined aggregates joined by noncovalent bonds[1]. Proteins are versatile building blocks for fabricating materials and nature has already used them as scaffolds to produce a dizzying array of materials, including collagen, keratin, pearl, shell, coral and calcite mircolenses and optical waveguides[2]. People begin to exploit self-assembly for the synthesis of entirely novel synthetic materials by observing the processes by which biomacromolecular architectures are assembled in nature[3-5] Self-assembly of biomacromolecules is emerging as a new route to produce novel materials and to complement other materials, which can find many applications in biomedical technology and biomaterials technology.

Casein is the main component in mammalian milk. It is composed of four different phosphorylated proteins (αs_1-, αs_2-, β-, κ-casein) that are similar in structure and properties[6]. These phosphorylated proteins are linked together by colloidal calcium phosphate (CCP). Casein can be considered as amphiphilic block copolymers with amounts of hydrophobic and hydrophilic amino acid residues. Therefore exhibits strong tendency to self-assembly into casein micelles[7-9]. Casein micelles can be dissociated into smaller subunits called casein submicelles by removal of colloidal calcium phosphate. Chelating agents[10,11], acidification[12,13], or dialysis against Ca^{2+} -free buffers were used to dissolve the colloidal calcium phosphate and dissociated the micelles.

Recent studies revealed that casein micelles exhibited complicated aggregates with spherical and linear features in solution[14]. Casein micelles present a loosened conformation and have low level of secondary and tertiary structure[15], so they tend to undergo structural alterations in the presence of external factors. Madadlou *et al.*[16] studied the characteristics of re-assembled casein micelles over a broad pH range from 6.35 to 11.4 and found that re-assembled casein micelles swelled as pH increased. Liu and Guo[17] discovered that the casein molecules can self-assemble into casein micelles in the pH ranges 2 to 3 and 5.5 to 12 The structure of casein micelles was more compact at low pH and looser at high pH[17]. Chakraborty and Basak[18] investigated the effect of the oppositely charged surfactants (SDS and CTAB) on the structure of the intrinsically unstructured casein. Furthermore, the spontaneous self-assembly of keratin solutions had also been studied extensively both at the microscale[19,21] and macroscale levels[22].

Linear β-casein and globular lysozyme were used to fabricate nanoparticles and their properties has been studied by Pan *et al.*[23]. A bio-compatible materials suitable for film and fiber production through the self-assembly between keratin and poly (ethylene oxide) (PEO) were also obtained by Tonin *et al.*[24]. Proteins are made of amphiphilic peptides and have a strong tendency to self-assemble in solution[25]. Biological aggregates such as cellular membranes, micelles and vesicles utilize amphiphilic molecules as self-assembling building blocks[26-28]. Casein and keratin belong to different category of proteins and they can form aqueous solutions in specific

conditions. They have similar value of isoelectric point and when they were mixed together in solution usually did not result in precipitation and phase separation. Therefore, casein and keratin have a great possibility to self-assemble and interact each other to form complex micelles in solution.

A number of casein-based colloidal systems such as micelles are promising carriers for bioactive molecules. Cross-linked casein micelles were resistant to proteolytic enzymes and thus stable in the casein appears to be a promising carrier for the delivery of many orally administered drugs[29]. Keratin materials extracted from wool capable of forming self-assembled structures can be made into bio-based porous foams, scaffold, sponges, films, microfibers and other natural materials. These were used in tissue engineering, drug delivery and medical devices. Similarly, biomaterials such as drug carriers and biological films or scaffold with desired properties can be developed based on the casein-keratin self-assembly complex micelles.

EXPERIMENTAL

Casein (technical grade) from Sigma Chemical Co. Pyrene (98 %) was purchased from J&K. Casein was dispersed in deionized water under constant magnetic stirring at 50 °C and the dispersions stored at 4°C overnight. Then the pH was adjusted to desired values with 1 M NaOH and 1 M HCl. Millipore filters with 2 μm pore size were used to get rid of the impurities and microbial. Keratin powder (wool) was put into deionized water and NaOH was added under stirring until it completely dissolved at desired concentration, then the keratin solution was filtrated by filters mentioned above.

Preparation of stable casein-keratin complex micelles: Casein and keratin aqueous solutions were prepared separately at the concentration of 5 mg/mL. Casein and keratin solutions were mixed gently under different mass ratio (MR) and adjusted to the desired pH value in the range of 5-9, in that the trans-glutaminse was stable in this pH range. Then, the mixed solutions was stirred for 10 min and kept at 25 °C for at least 24 h to obtain casein-keratin complex micelles. Then, the complex micelles were crosslinked by transglutaminase for 1 h at 50 °C and the enzyme was inactivated at 80 °C for 10 min. The complex micelles could be fixed by enzymatic crosslinking effect at the surface and within the micelles through amido bond. The crosslinking effect led to form more stable and reinforced complex micelles.

Turbidity measurements: A Shimadzu UV1705 spectrometer was used for turbidity measurements. The casein-keratin complex micelles solution was injected into the quartz cuvettes and the turbidity was detected at 600 nm wavelength. Spectra were collected with a band width of 1 nm.

Dynamic light scattering (DLS) analysis: Hydrodynamic diameters of the complex micelles were measured by a ZetaSizer Nano ZS90 at a 90 degree scattering angle. The concentration of the complex micelles for DLS measurements was 5 mg/mL. The value of hydrodynamic diameter (Dh), polydispersity index (PDI) and the intensity for z-average was available in test.

Zeta-potential measurements: Zeta-potential was measured by laser doppler micro-electrophoresis and an electric field was applied to the complex micelles, which then moved with a velocity related to their ζ-potential. This velocity was measured using a patented laser interferometric technique called M3-PALS (Phase analysis Light Scattering). This enabled the calculation of electrophoretic mobility. The test was carried out by a ZetaSizer Nano ZS90 at 25 ± 0.1 °C. The electrophoresis mobility (UE) was used to calculate the potential (ζ) by the Henry equation UE = $2\varepsilon\zeta f(ka)/3\eta$ and ε, η, *f* (ka) represented the dielectric constant, the viscosity of the medium and the Henry's function.

Fluorescence analysis: 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 complex micelles solution for testing was 2×10^{-7} g/mL. Before measurement, the complex micelles solution with desired pH was kept still for 24 h at 4 °C after the pyrene was added. Excitation spectrum and emission spectrum scan resolution were 1 nm. The excitation and emission wavelength were recorded at the 335 nm and 390 nm wavelength.

Scanning electron microscope (SEM) observation: A Hitachi S-4800 field emission scanning electron microscope was used to observe the casein-keratin complex micelles. The 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) observation: Skiko atomic force microscopy (SPA400-SPI3800N) was used to acquire the images of the complex micelles in tapping mode. The complex micelles were fixed by 2 % glutaraldehyde on the freshly cleaved mica surface for at least 0.5 h, then washed with deionized water and dried in the room temperature before observation.

RESULTS AND DISCUSSION

Formation of the casein-keratin complex micelles: Casein and keratin solutions were blended together under different mass ratio and then the pH value of the mixed solution was adjusted by 1 M NaOH and 1 M HCl to the desired value. The solution was kept at 25 °C for at least 24 h. Complex micelles were crosslinked by transglutaminase for 1 h at 50 °C and the enzyme was inactivated at 80 °C for 10 min. Turbidity measurement was then carried out to observe the casein-keratin complex micelles solution. As shown in Fig. 1, complex micelles solution were pH sensitive. Turbidity of the complex micelles solution had a sharp increase at pH lower than 6 and a smooth decrease at pH higher than 6 under different casein-keratin mass ratio.

In order to obtain stable complex micelles, DLS technique was used to assist the experiment. The influence of different mass ratio of casein to keratin on complex micelles formation was investigated at neutral pH. The concentration was 5 mg/mL in each sample. These two kind of proteins molecules interacted each other and formed anionic polyamphoteric micelles with diameter about 130-230 nm at pH 7 (Table-1). We can

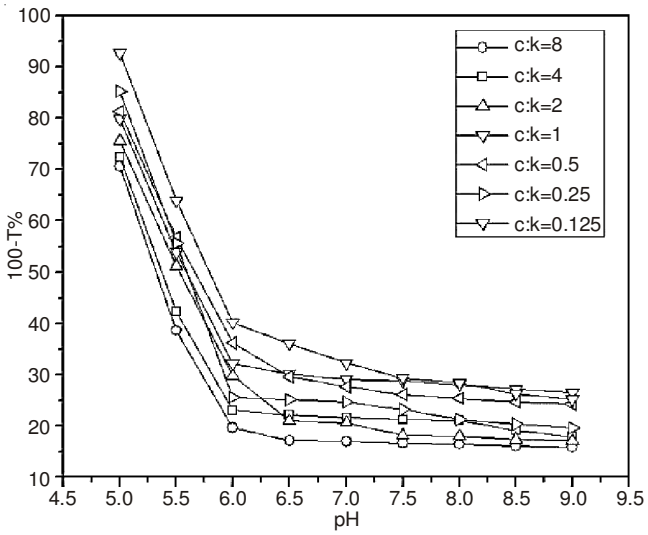

Fig. 1. Turbidity (100-T %) of the casein-keratin complex micelles solution varied (5 mg/mL) as a function of pH

found that any of the hydrodynamic diameter of the complex micelles at different mass ratio was small than the diameters of casein or keratin micelles individually. This can be hypothesis that casein micelles and keratin molecules were dissociated partly when these two proteins mixed together and then they undergo re-assembly procedure and new self-assembly complex micelles were formed through non-covalent bonds. Table-1 showed that the diameters of the complex micelles decreased when the content of the keratin in blended solutions increased. It can be explained that the colloidal calcium phosphate (CCP) in the casein micelles dissociated in the presence of keratin molecules and resulted in collision or close approaches between casein and keratin molecules, with lead either to linkage or to separation. As a consequence, the re-assembly process resulted in the formation of new complex micelles.

TABLE-1
INFLUENCE OF THE DIFFERENT MASS RATIO ON THE D_h, PDI, AND SCATTERING LIGHT INTENSITY FOR THE CASEIN-KERATIN COMPLEX MICELLES AT NEUTRAL pH

MR	D_h (nm)	PDI	Intensity (kcounts)
1:0	230.1	0.497	95.02
8:1	180.8	0.593	62.48
4:1	172.4	0.477	52.78
2:1	170.5	0.633	97.03
1:1	162.8	0.730	57.89
1:2	157.6	0.648	93.66
1:4	146.5	0.699	86.91
1:8	135.8	0.692	53.95
0:1	223.1	0.797	58.54

Note: MR represents the mass ratio of casein to keratin. D_h is the hydrodynamic diameters, PDI is the particle dispersion index

Table-1 showed that the complex micelles prepared at neutral pH with MR = 2:1 had the highest intensity and relatively lower PDI. For this reason, we prepared complex micelles with MR = 2:1. Fig. 2 showed the changes of the complex micelles diameter at pH 5-9 with MR = 2:1. It showed that the diameter of the complex micelles varied from 170 to 232 nm in pH 5.5-9 and increased dramatically when pH close to 5. The complex micelles had the smallest diameter at pH 7.5. Therefore, stable complex micelles can form at the pH 5.5-9.0. Furthermore, the introduction of keratin molecules into casein chains did not disrupt the structure of casein micelles completely. Calcium phosphate also played a crucial role in the maintenance of the stability of casein-keratin complex micelles.

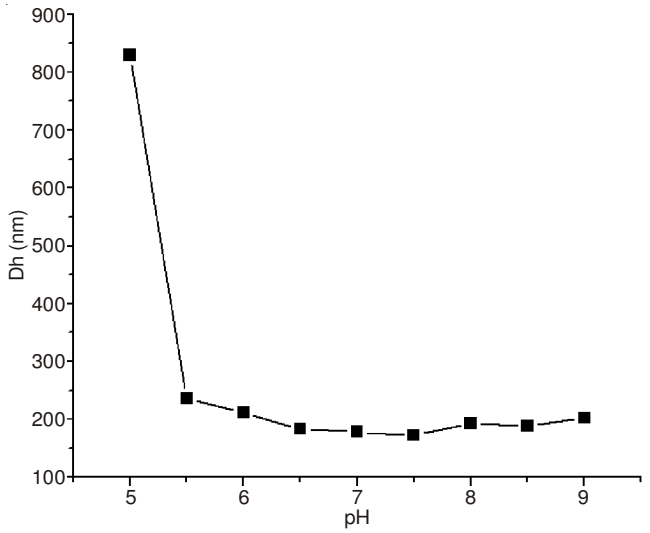

Fig. 2. Influence of the pH on diameter of the casein-keratin complex micelles in the range of pH 5-9. The complex micelles were prepared with MR = 2:1

Characterization of the casein-keratin complex micelles: ζ-potential relates to the net charge on the surface of the particles in solution. Fig. 3 showed the ζ-potential of the complex micelles in the range of pH 5-9 with MR = 2:1. As shown in Fig. 3, the absolute value of the ζ-potential for the complex micelles increased when the pH change from 5 to 9. It illustrated that the complex micelles became stabilized when pH increased from 5 to 9. Fig. 4 showed the ζ-potential of the complex micelles with different mass ratio at neutral pH. It is observed that the complex micelles absolute value of the ζ-potential increased with the content of the keratin enhanced in mixed solutions. It can be inferred that the introduction of keratin into casein chains stabilized the complex micelles dispersion system and enhanced the stability of the complex micelles.

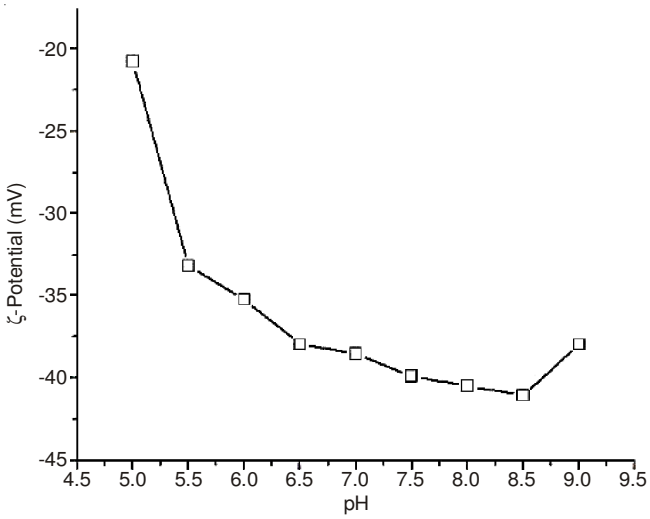

Fig. 3. ζ-Potential of the casein-keratin complex micelles prepared with MR = 2:1 in the range of pH 5 to 9

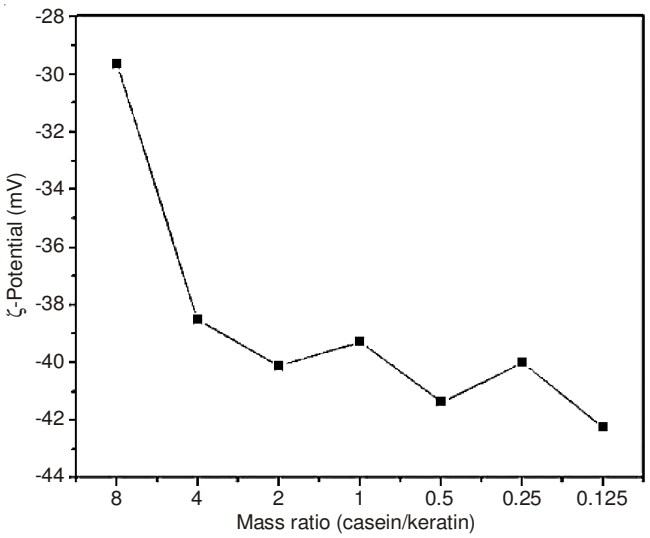

Fig. 4. ζ-Potential of the casein-keratin complex micelles prepared with different mass ratio at pH 7

Casein micelles, keratin particles and casein-keratin complex micelles were observed by SEM and AFM. In Fig. 5, A, B, C and D represent the casein micelles (× 40 k magnification), keratin particles (× 40 k magnification), casein-keratin complex micelles (× 40 k magnification) and casein-keratin complex micelles (× 80 k magnification) separately. The casein micelles exhibited spherical shape in A and it was similar to previously reports[30,31]. The keratin particles exhibited a specific shape in B and it was similar to the keratin images in solution (0.5 %) reported by Jiashen Li[32]. The image of casein-keratin complex micelles was shown in C and D near sphericity, which differed from casein micelles and keratin particles in shape.

The statistics on all of the micelles in A and B shown an average diameter of about 120 nm and 150 nm, which was much smaller than 230 nm and 223 nm measured by DLS in Table-1. This was attributed to the shrinkage of the micelles after water evaporation. Dynamic light scattering provided the data for the micelles swollen in solution, while SEM showed the image of dried micelles. The high shrinkage indicated that

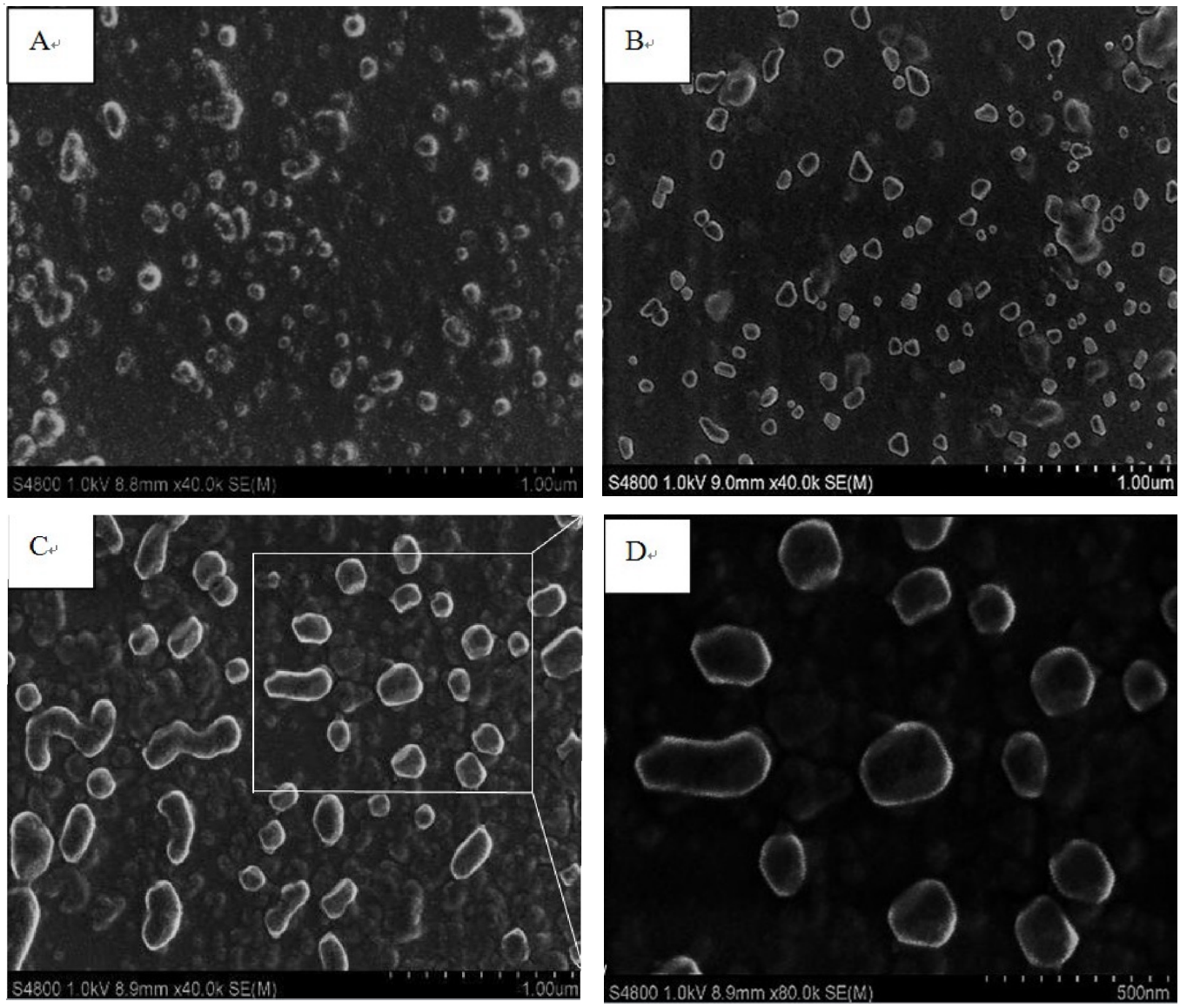

Fig. 5. SEM images of the casein micelles (A, × 40 k magnification), keratin particles (B, × 40 k magnification), casein-keratin complex micelles (C, × 40 k magnification; D, × 80 k magnification)

the casein micelles and keratin particles have a low-density structure and contain a large amount of water. Meanwhile, the average diameter of the complex micelles was about 160 nm in C and D, which basically conformed to the results observed by DLS. This may be due to the hydrophilic or hydrophobic property of complex micelles had a great difference from casein micelles or keratin particles in solution.

Atomic force microscopy was also used to investigate the morphology of the casein-keratin complex micelles. As shown in Fig. 6, the diameter of the complex micelles ranged from 100 to 300 nm and the results conform to the SEM observations. There were some micelles exhibited flatted shapes because of the evaporation of water.

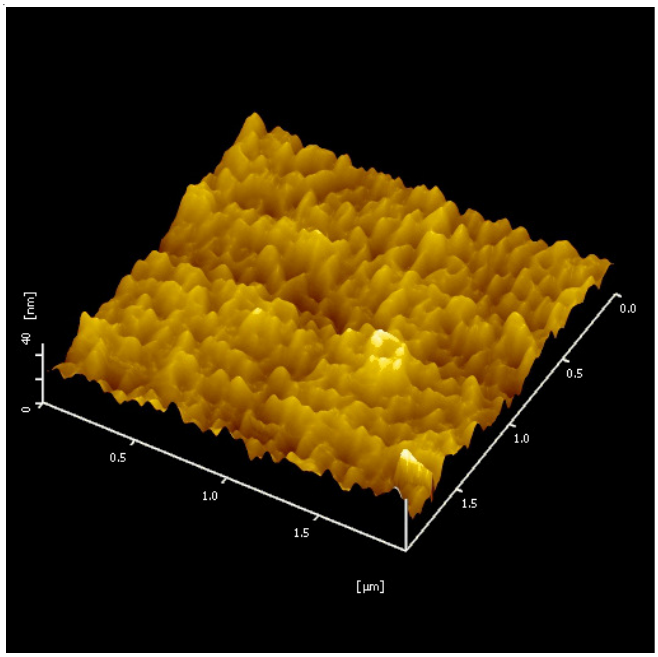

Fig. 6. AFM images of the casein-keratin complex micelles

Recrystallized pyrene was used as a probe to detect the hydrophobic and hydrophilic properties of casein-keratin complex micelles. Pyrene emission spectral profile and decay depend on the polarity of the medium in solutions. It forms excimers in organic solutions and cause near-violet fluorescence intensity changed. The pyrene is excited at wavelength of 338 nm and can produce five characteristics emission monomer fluorescence peak at wavelength of 350-550 nm. The intensity ratio of the first to the third peak (I_1/I_3) in the fluorescence spectrum can reflect the micro environmental polarity where the probe exists[33]. The greater the value of I_1/I_3, the weaker of the hydrophobic microenvironment for pyrene. Furthermore, an excited monomer of pyrene and a ground-state pyrene can meet and form an excimer in the appropriate configuration and produces a broad band at about 470 nm. The ratio of the maximum emission intensity of the excimer (I_e) to the monomer (I_1) can be used to evaluate the efficiency of excimer formation. The value of I_e/I_1 can provide further information for the micelles hydrophobic variation.

Fig. 7 showed the value of I_1/I_3 and I_e/I_1 for pyrene plotted as a function of concentration for casein-keratin complex micelles with MR = 2:1. The value of I_1/I_3 decreased gradually over a wide range of concentration from 0.05 to 10 mg/mL with a sharp breaking point at 2.5 mg/mL and this point can be considered as the critical micellar concentration (CMC) for complex micelles. Unlike the behaviour of the typical surfactant, the premicelles formed below concentration of 2.5 mg/mL for the complex micelles and sub-micelles aggregated to form stable micelles above concentration of 2.5 mg/mL. Fig. 7 also showed that the value of the I_e/I_1 was zero above the 7.5 mg/mL and increased gradually in the range of 7.5-2.5 mg/mL, then decreased rapidly below 2.5 mg/mL. This phenomenon can be explained that when the concentration of the complex micelles above the CMC, complex micelles became larger and separated the pyrene molecules, which hinder the excited monomer and ground-state pyrene meet to form the excimer and resulted the relatively lower value. When the concentration close to the CMC, complex micelles disassembles into amount of premicellar aggregates, which provide a space where the local concentration of pyrene is much higher than the bulk concentration and the pyrene molecules was close enough to form pyrene excimer.

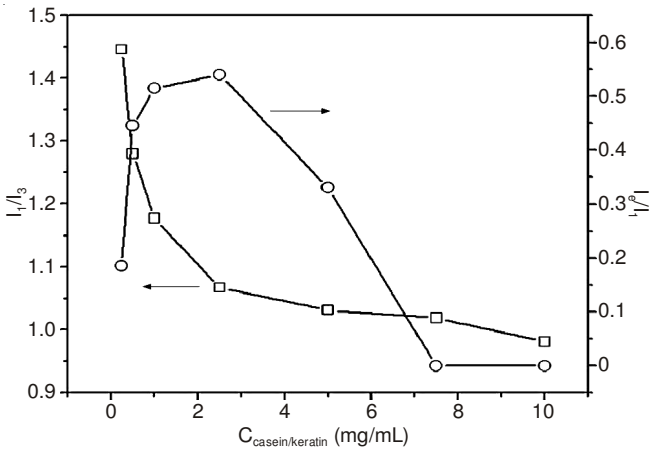

Fig. 7. Plots of I_1/I_3 and I_e/I_1 of pyrene for casein-keratin complex micelles as a function of concentration. The complex micelles were prepared at pH 7 with concentration of 5 mg/mL. The mass ratio of casein to keratin was 2:1

Fig. 8 showed that the value of I_1/I_3 and I_e/I_1 for the casein-keratin complex micelles were pH sensitive at pH 5-9. The values of I_1/I_3 of the complex micelles increased in the range of pH 5-9 with increasing pH. The complex micelles were more hydrophobic near the pI of the casein and keratin, which leads to the relative lower value of I_1/I_3. Meanwhile, the more hydrophobic structure of the micelles hampers the formation of excimer complexes and lead to a lower I_e/I_1. When the pH was increased from 5 to 9, complex micelles structure became loosen for the increase in electrostatic repulsion, the pyrene can penetrate and migrated into the deeper hydrophobic region, which led to the relative higher value of I_1/I_3. This variation in micelles structure diminished the space of the excited monomer and ground-state pyrene, resulted in the increasing value of I_e/I_1.

Fig. 9 showed that I_1/I_3 and I_e/I_1 for the casein-keratin complex micelles varied as a function of mass ratio. The value of I_1/I_3 of the complex micelles increased when the value of

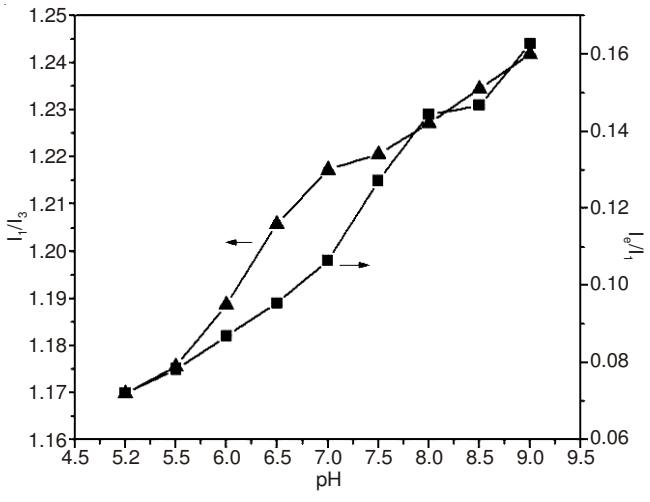

Fig. 8. Plots of I_1/I_3 and I_e/I_1 of pyrene for casein-keratin complex micelles as a function of pH in the range of 5-9. The complex micelles were prepared at the concentration of 5 mg/mL. The mass ratio of casein to keratin was 2:1

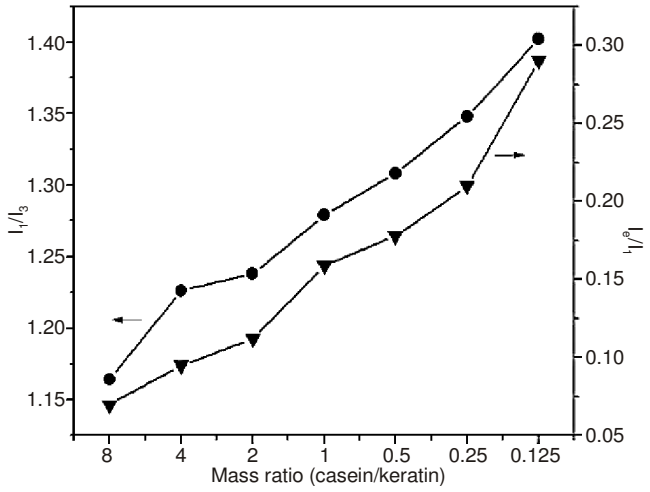

Fig. 9. Plots of I_1/I_3 and I_e/I_1 of pyrene for casein-keratin complex micelles as a function of mass ratio. The complex micelles were prepared at pH 7 with a concentration of 5 mg/mL

MR decreased. When keratin molecules were introduced, the casein micelles structure became loosen and disassociated partly, which led to the more hydrophilic structure of the complex micelles. As a result, the values of I_1/I_3 became increased. Meanwhile, the structure of the complex micelles became looser and promoted the accumulation of the excimer, which led to the higher value of I_e/I_1.

Stability of the casein-keratin complex micelles: Casein micelles (0.5 %) and complex micelles (0.5 %, MR = 2:1) with neutral pH were prepared separately. Stability of the micelles was investigated in 40 days storage time. The changes in diameter and PDI were showed in Figs. 10 and 11. Fig. 10 showed that size distributions of the complex micelles did not have significant change in 40 days storage time. On the contrary, the size distributions of the casein micelles changed greatly and coagula appeared in the storage time. Similar result was also acquired for the PDI of the casein micelles and complex micelles as showed in Fig. 11. These are valuable properties for practical use.

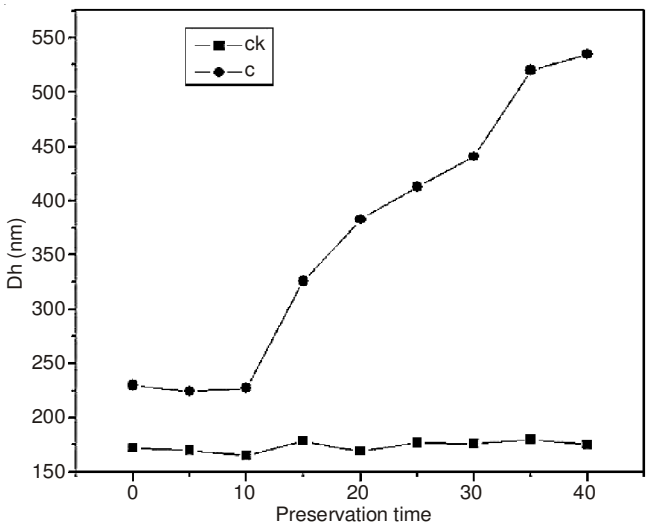

Fig. 10. Plots of the Dh of the casein-keratin complex micelles in the 40 days preservation time. The micelles solutions were prepared at pH 7 at concentration of 5 mg/mL

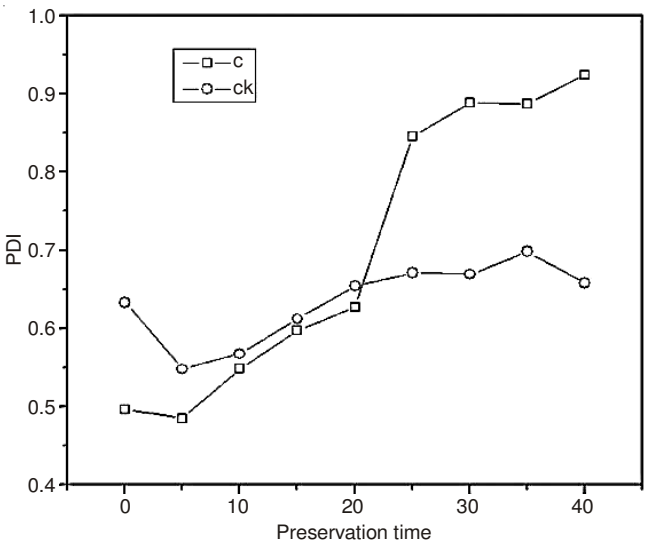

Fig. 11. Plots of PDI of the casein micelles in the 40 days preservation time. The micelles solutions were prepared at pH 7 at concentration of 5 mg/mL

Conclusion

Two types of proteins, keratin and unstructured casein, were used to fabricate complex micelles through self-assembly. In this process, no harmful reagents were used except alkali and acid. The two proteins formed stable polydisperse electrostatic complex micelles in range of pH 5.5-9.0 and MR 8-0.125. Transglutamianse was used to crosslink and form more stable and reinforced micelles. The complex micelles spherical shape and their size depend on the pH value and mass ratio of casein to keratin. The structure of the casein micelle is more compact at low pH and looser at high pH. The complex micelles had the most compact structure at pH 7.5 with the mass ratio of casein to keratin was 2:1.

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