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Amphiphilic and Biocompatible Properties of Poly (EA-MAA)

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ABSTRACT: The amphiphilic and biocompatible behaviors of Poly (acetate-methylacrylic acid) Poly(EA-MAA) were investigated in this study. The aggregation behavior of Poly(EA-MAA) in aqueous media was investigated by steady-state fluorescence spectroscopy and surface tensiometry. The critical aggregation concentration (cac) of Poly(EA-MAA) was determined to be ~0.03 mg/mL. Ultraviolet spectrum (UV) and fluorescence spectrum results indicated that there are interactions form between Poly(EA-MAA) and bovine serum albumin (BSA). And β-sheet can be changed into α-helix of BSA in Poly(EA-MAA) solution effectively revealed by Circular dichroism (CD), which is driven by the H-bond and hydrophobic interactions. The good cell-compatibility of Poly(EA-MAA) makes it great potential in the biomedical fields. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: poly(EA-MAA); amphiphilic; bovine serum albumin; biocompatibility

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INTRODUCTION

Amphiphilic copolymers composed of hydrophobic segment(s) and hydrophilic segment(s) undergo self-association to form various molecular assemblies in aqueous media.[1] Such amphiphilic copolymers are widely used in cosmetics and pharmaceuticals field including drug delivery and gene therapy owing to their self-assembling property[2–4] even in the case that the polymer surface is in direct contact with blood.[5]

Newly synthesized amphiphilic polyacrylamide, are able to incorporate into the liposomal membrane, and similar to poly (ethylene glycol) prolong liposome circulation in vivo and decrease liposome accumulation in the liver.[6] Many methods for immobilization of liposomes into gel matrices were reported, such as hydrophobic ligand adsorption,[7] covalent immobilization method.[8] For the immobilization of liposomes by hydrophobic interaction, hydrophobicity of the substrate polymer is an important factor to be controlled.[9]

The adsorption of serum proteins onto polymer microspheres is important in biomedical, such as biosensors,[10] immunomagnetic cell separation,[11] drug delivery systems,[12] artificial tissues and organs.[13] Adsorption behavior of polymer surface directly affects the biocompatibility of polymer. The biocompatibility is generally considered to have close relation with protein adsorption process, because the adsorbed proteins may trigger the coagulation sequence.[14] The polymer with hydrophobic surface will absorb different proteins due to an entropic driving force.[15]

Surface modification with protein or gels is an effective way to improve the biocompatibility and remain the bulk properties of biomaterials.[16–18] The covalent immobilization of proteins or gels on the surfaces of polymers is generally considered as a promising approach to enhance blood or tissue compatibility of biomaterials.[19] Albumin-conjugated PEG has also been used to increase the bioavailability, effectiveness and safety of PEG liposomal doxorubicin in rats.[20] Amphiphilic copolymer is considered to be a good candidate for gene transfection and expression because of its biodegradability, biocompatibility, mucoadhesive, and permeability enhancing properties. Gerrit Borchard demonstrated the ability of glutamate chitosan to enhance intestinal peptide drug delivery studied in an in vitro model (Caco-2 cell monolayers)[21] Chitosan hydrochloride has also been used to improve the bioavailability of buserelin in rats, as described by Lueβen et al.[22]

To enhance local drug delivery systems with biocompatibility and bio-penetrability, amphiphilic Poly(EA-MAA) was synthesized

Table I. Typical Recipe Used for the Preparation of P(EA-MAA)

Components	Weight (g)
EA	4.00
MAA	12.00
SDS	0.22
APS	0.11
Distilled water	51.00

and the cytocompatibility of Poly(EA-MAA) was investigated in this study. The self-assembly behaviors of Poly(EA-MAA) in dilute solution were examined using Steady-state fluorescence spectroscopy and Surface tensiometry. This hydrophobic domain of Poly(EA-MAA) aggregates will produce the interaction with BSA, resulting in an effective adsorption of BSA on Poly(EA-MAA). This adsorption of serum proteins onto polymeric aggregates is important for a good biocompatibility of Poly(EA-MAA), which makes it have great potential as a novel biocompatible matrix as a drug control release.

EXPERIMENTAL

Materials

Technical-grade monomers vinyl acetate (EA) with 10–20 ppm of hydroquinone monomethyl ether (MEHQ) and methylacrylic acid (MAA) (purity of 99%) were purchased from by Guangzhou Langri Chemical, China. EA was purified before use. The emulsifier sodium dodecyl sulfate (SDS) and ammonia persulfate (APS) were used as received. Doubly deionized water (DDI water) was used throughout the work. BSA was purchased from fraction V, Sigma-Aldrich.

P(EA-MAA) Synthesis

Semibatch emulsion polymerizations were carried out in a 500 mL jacketed glass reactor equipped with reflux condenser, thermocouple, stainless-steel stirrer at 250 rpm, nitrogen inlet, and three inlet tubes. Initial composition of the monomer mixture is given in Table I. EA, MAA, SDS, APS, 25 g water, and 0.15 g carbonic acid monosodium salt were mixed and stirred to emulsify for 60 min. Totally, 5% of the pre-emulsion, 26 g water, and 0.15 g carbonic acid monosodium salt were added into the reactor and stirred. The reactor was heated to 86°C, after polymerization for 45 min, the remaining pre-emulsion was continuously added into the reactor through inlet. After 1 h of addition the reactor was heated to 90–92°C and left for 20 min to allow for complete conversion. The reactor was cooled to 50°C. No coagulum was found on the reactor walls or on the surface of the stirrer.

CHARACTERIZATION

Surface Tensiometry

The Data physics DCAT|| tension meter equipped with a standard Du-Nouyring was used to detect the critical aggregation concentration (cac) of Poly(EA-MAA) in aqueous solution. The surface tensions at different polymer concentrations were recorded and analyzed by the SCAT program. Each experiment was repeated at least three times.

Steady-State Fluorescence Spectroscopy

Steady-state fluorescence spectra were recorded on a Shimadzu RF 5301 spectrometer equipped with a quartz fluorescence cell. Pyrene, from an acetone stock solution of 1×10^{-3} M, was used as a micropolarity sensitive probe in a final concentration of 6.0×10^{-7} M in water. The excitation wavelength was set to 335 nm. The changes in the intensity ratio of the first and the third vibronic band (I_1/I_3), namely 373 and 384 nm (I_1 and I_3, respectively) in the emission spectra were used to detect the shift of the hydrophobic microdomain.

Fluorescence and UV Spectroscopy

The intrinsic fluorescence properties of protein were studied on an F-4500 (Hitachi high-technologies corporation, Tokyo Japan) spectrometer with a 3 ml quartz cell with a 1 cm path length. The concentration of BSA in all the experiments was kept at 0.33 mg/mL. The concentration of Poly(EA-MAA) was varied from 0.575 to 4.6 mg/mL. The excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was set at 295 nm to selectively excite the tryptophan molecules, and the emission spectra were monitored in the wavelength range of 315–390 nm. The UV spectrum was measured by a UV-2501PC (Shimadzu corporation, Japan) spectrometer. All the solvent used in this study was phosphate buffered Saline (PBS) (pH = 7.4, 0.01M) unless other specified.

Circular Dichroism Measurements

Circular dichroism (CD) spectroscopy (J-810, JASCO corporation, Japan) was used to measure the conformation change of BSA after complex with Poly(EA-MAA) with respect to the native one. The BSA and BSA/Poly(EA-MAA) solutions were scanned over the wavelength range 200–260 nm, using a 5 mm quartz cylindrical cell. The secondary structures of native BSA and composite of BSA and Poly(EA-MAA) were evaluated by comparing the α-helix content, corresponding to the ellipticity of the bands at 208 nm. Since α-helices are one of the elements of secondary structure, the quantitative analysis of structural change of BSA could be evaluated by the content of the α-helix preserved. The α-helix content of proteins is estimated according to the following equation (Greenfield et al., 1969):

$$\%\,\alpha - \text{Helixcontent} = \frac{\text{mrd} - 4000}{33,000 - 4000}$$

where, θ_{mrd} is the mean molar ellipticity per residue at 208 nm (deg cm^2 dmol^{-1}). Usually the raw data from the experiment is expressed in terms of θ_d (the ellipticity in the unit of mdeg). However, it can be converted to mean molar ellipticity per residue, using the following equation:

$$_{\text{mrd}} = \frac{_dM}{10CLN_r}$$

where, M is the BSA molecular weight (Da), C is the BSA concentration (mg/ml), L is the sample cell path length (cm), and N_r is the number of amino residue.

Adsorption of BSA on Poly(EA-MAA)

Totally, 0.33 mg/mL of BSA phosphate buffer solution was added into Poly(EA-MAA) aqueous solutions (varied from

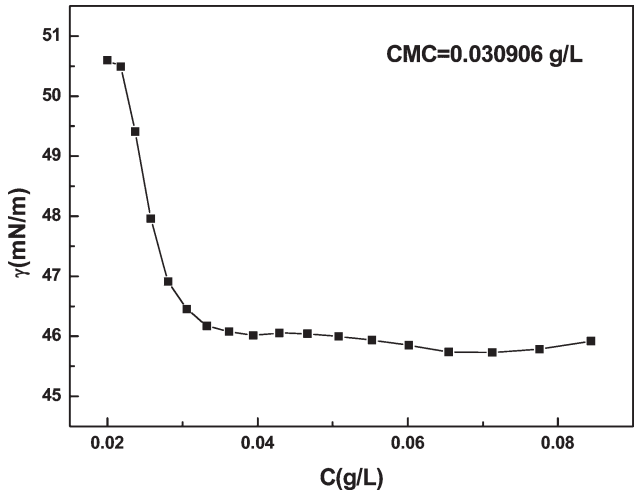

Figure 1. Concentration dependence of the surface tension for Poly(EA-MAA) in aqueous solution.

0.575 to 4.6 mg/mL). The mixture was stirred for 60 min to ensure the complete adsorption, the resulting suspension was precipitated with centrifugation (10,000 rpm for 30 min) to separate the complex of Poly(EA-MAA) and BSA. The upper solution was measured with fluorescence spectroscopy to detect and the unadsorbed BSA.

In Vitro Cell Toxicity

K562 cells were cultured in DMEM (Dulbecco's modified Eagle medium) containing 10% fetal bovine serum supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C under a 5% CO_2 containing atmosphere. Cells were seeded into 96-well plate at 5000/well and grown to 50% confluence. P(EA-MAA) solutions with 0.5, 0.25, 0.1, 0.05, and 0.01 mg/mL concentration were diluted with OPTI-MEMI (serum-reduced medium, Gibco). The growth medium was removed from the 96-well plate and 100 μL of above solution was added and incubated at 37°C under a 5% CO_2 atmosphere. Then 100 μL of growth medium containing 20% FBS was added to each well, and incubated at 37°C under a 5% CO_2 atmosphere. After incubation, 20 μL combined MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)/ PMS(phenazine methosulfate) solution (Promega) was added to each well. After incubating the plate for 2 h at 37°C in a humid, 5% CO_2 atmosphere, the absorbance of each well at 490 nm was recorded by using a 96-well plate reader (Bio-Rad Labs). Six samples were used, and this in-vitro experiment was performed in twice independently.

RESULTS AND DISCUSSION

Aggregation Behavior of Poly(EA-MAA)

The Surface Tension. The concentration dependence of the surface tension for Poly(EA-MAA) in aqueous solution is shown in Figure 1. From the figure, it is obvious that the surface tension dropped significantly with increasing polymer concentration at low Poly(EA-MAA) concentrations. After a transition concentration of about 0.03 mg/mL, the surface tension tended to become independent of the concentration. The transition point is attributed to the critical aggregation concentration (CAC) of Poly(EA-MAA) in aqueous solution. The dependence of surface tension on surfactant concentration has been well investigated; to decrease free energy, surfactant forms micelles in solution when the concentration is higher than the critical micelle concentration (cmc).[23] Analogously, aggregates should also be formed for amphiphilic polymers such as block copolymers in aqueous solution.[24] The experimental surface tension data indicates that the cac was about 0.03 mg/mL, which is much smaller than the cmc of common surfactants due to the high molecular weight of Poly(EA-MAA).

Fluorescence Spectroscopy. The aggregation behavior of Poly(EA-MAA) in aqueous solution was further examined by steady-state fluorescence spectroscopy using pyrene as a fluorescence probe. Figure 2 presents the fluorescence emission spectra of pyrene at 25°C and 335 nm excitation wavelength in different concentrations of Poly(EA-MAA) aqueous solution. The figure clearly shows that the emission intensity of pyrene increased with P(EA-MAA) concentration increasing. This relationship strongly indicates that in aqueous solution, hydrophobic Poly(EA-MAA) microdomains that could incorporate the hydrophobic pyrene were formed. Thus, when Poly(EA-MAA) concentration was raised, the pyrene concentration was increased in tandem, resulting in higher total emission intensity obtained. In addition, in fluorescence spectra, the intensity ratio of the first and the third vibrational bands (i.e., I_1/I_3) is sensitive to the environment of the pyrene molecules; this could be used as another measure of the critical aggregation concentration because transition in the intensity ratio would point to the threshold concentration for the aggregation of polymeric amphiphiles. Figure 3 shows I_1/I_3 dependence on Poly(EA-MAA) concentration. From this figure, it is obvious that the intensity ratio for low concentration of Poly(EA-MAA) is close to 1.83, which is the value for pyrene in water; and it drops significantly with the increase of Poly(EA-MAA) concentration to 0.03–0.06 mg/mL, after that this ratio tends to be independent

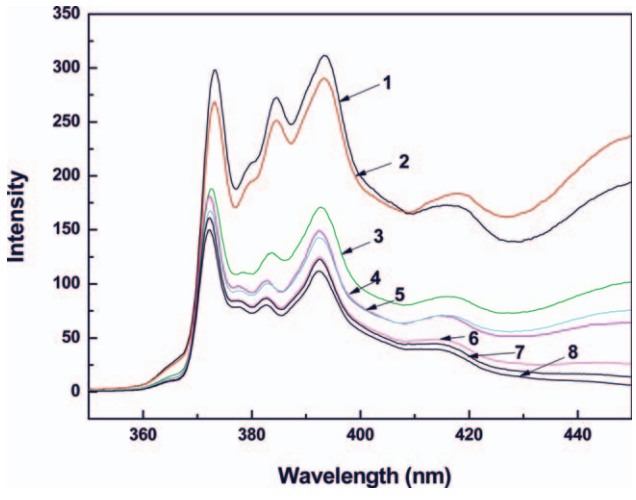

Figure 2. Effect polymer concentrations on the fluorescence emission spectra of pyrene (6.0 × 10–7 M) in aqueous solution. (1) 0.065; (2) 0.013; (3) 0.026; (4) 0.052; (5) 0.08; (6) 0.104; (7) 0.15; (8) 0.208; (9) 0.416 mg/mL of poly(EA-MAA). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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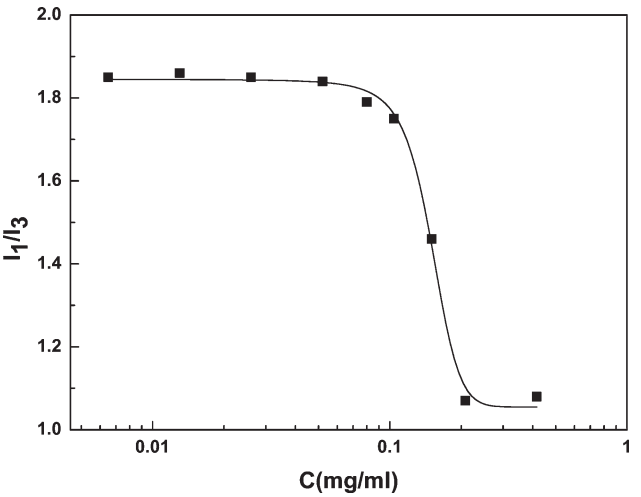

Figure 3. The concentration dependence of the intensity ratio I1/I3 of pyrene in Poly(EA-MAA) aqueous solutions.

of Poly(EA-MAA) concentration. This transition confirmed the cac value measured by surface tensionmetry.

The surface tension and fluorescence spectroscopy measurements revealed the amphiphilic property of Poly(EA-MAA). The aggregation mechanism should be the hydrophobic interactions, which are caused by the hydrophobic moieties such as CH_3COO-, -CH_2-CH_2 and -CH_3 in Poly(EA-MAA).

Interaction Between BSA and P(EA-MAA) Aggregates

UV Spectrum. Figure 4 shows the UV spectrum of BSA in different Poly(EA-MAA) concentrations. According to literature,[25] the large absorption peak at 275 nm appears due to phenyl group of tryptophan (Trp) residues and tyrosines. From Figure 4, it can be seen that all the peaks in different Poly(EA-MAA) concentrations do not show any shift, however, the intensity is strongly dependant on the Poly(EA-MAA) concentration. Because of the amphiphilic property of Poly(EA-MAA), it can form hydrophobic domains, in which, hydrophobic Trps and tyrosines residues are

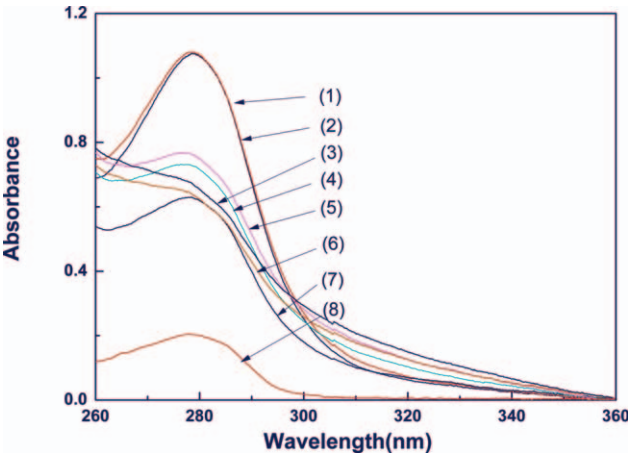

Figure 4. UV spectrum of BSA in different concentrations of Poly(EA-MAA) (BSA concentration was kept at 0.33 mg/mL). (1) 0.575; (2) 1.15; (3) 1.725; (4) 2.3; (5) 2.875; (6) 3.45; (7) 4.6; (8) 0 mg/mL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

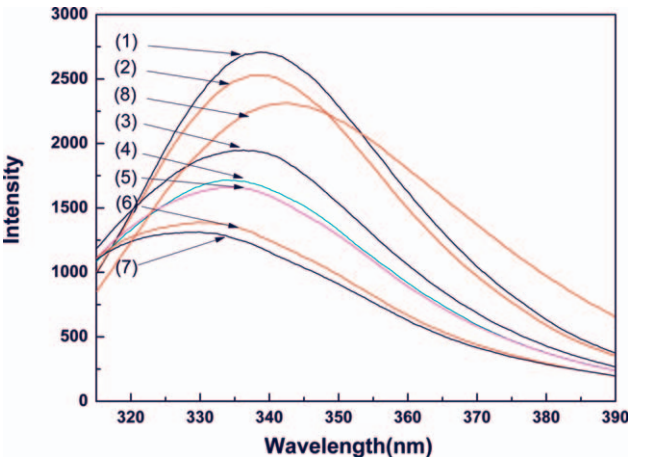

Figure 5. Fluorescence spectrum of BSA in different concentrations of Poly(EA-MAA) (BSA concentration was kept at 0.33 mg/mL). (1) 0.575; (2) 1.15; (3) 1.725; (4) 2.3; (5) 2.875; (6) 3.45; (7) 4.6; (8) 0 mg/mL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

easy to dissolve. As a result, the intensity of the UV peak for BSA increases significantly in all Poly(EA-MAA) solutions in comparison with that in single BSA aqueous solution. However, this enhancement does not increase monotonously with Poly(EA-MAA) concentration increasing. This result may be caused by the complex formed between BSA and Poly(EA-MAA) due to the interchain hydrophobic associations, causing the more hydrophilic microenvironment around the residues of Trps and tyrosines. The similar phenomenon is also found by Gao et al. that the complex interactions of modified CyDs with BSA lead to an altered solute-environment interaction and thus decrease the UV absorbance in the spectrum of BSA.[26]

Fluorescence Spectrum. Intrinsic fluorescence is used to assess the structural changes BSA with interacting with Poly(EA-MAA). The emission spectra of pure BSA and BSA in different concentration of Poly(EA-MAA) are depicted in Figure 5. In BSA, there is a tryptophan residue, which is one of the three natural occurring aromatic amino acid residues, fluorescing when excited with light. The fluorescence is usually dominated by the contribution of the Trp. Because both their absorbance at the excitation wavelength and their quantum yield emission are considerably greater than the respective values for tyrosine and phenylalanine. When a BSA molecule undergoes the conformation change, the Trp residue is exposed to the environment and therefore affects the fluorescence. The fluorescence of Trp in BSA varies with its conformational change and shows a blue shift of the emission maximum wavelength (λ_{em}).[27,28]

From Figure 5, the fluorescence peak at 330 nm of Trp is shifted slightly at low Poly(EA-MAA) concentrations and this shift enhances with further increasing the concentration of Poly(EA-MAA). The result may indicate that BSA can keep its conformation at low Poly(EA-MAA) solutions, while at high concentrations, Poly(EA-MAA) may disturb BSA conformation. However, the fluorescence intensity dependence on Poly(EA-MAA) concentrations is not always consistent with the case of UV spectrum. That is possible because the UV and fluorescence peaks are

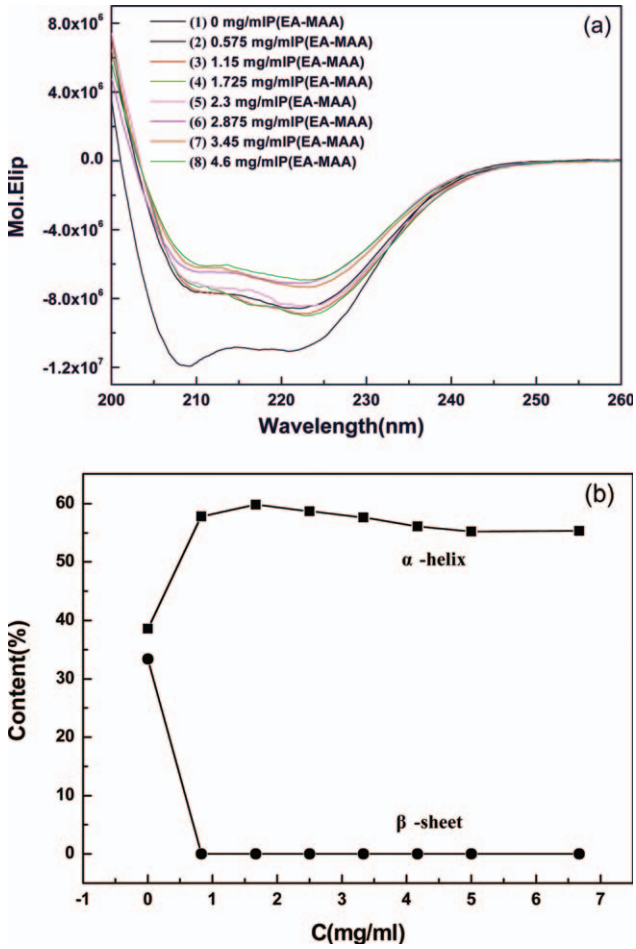

Figure 6. (a) Circular dichroism (CD) spectrum; (1) Pure BSA; (2) 0.33 mg/mL BSA+0.575 mg/mLP(EA-MAA); (3) 0.33 mg/mLBSA+1.15 mg/mLP(EA-MAA); (4) 0.33 mg/mLBSA+1.725 mg/mLP(EA-MAA); (5) 0.33 mg/mLBSA+2.3 mg/mLP(EA-MAA); (6) 0.33 mg/mLBSA+2.875 mg/mLP(EA-MAA); (7) 0.33 mg/mLBSA+3.45 mg/mLP(EA-MAA); (8) 0.33 mg/mLBSA+4.6 mg/mLP(EA-MAA); (b) α, β conformation content of BSA in different concentrations of Poly(EA-MAA), (BSA concentration was kept at 0.33 mg/mL). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

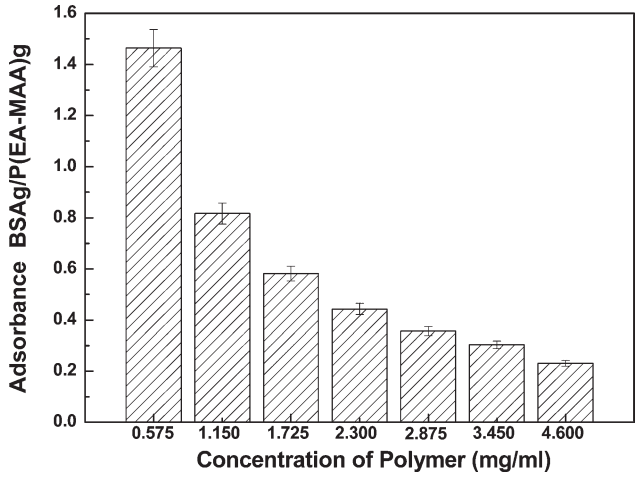

Figure 7. The BSA adsorbance dependent on Poly(EA-MAA) concentrations (BSA concentration was kept at 0.33 mg mL−1).

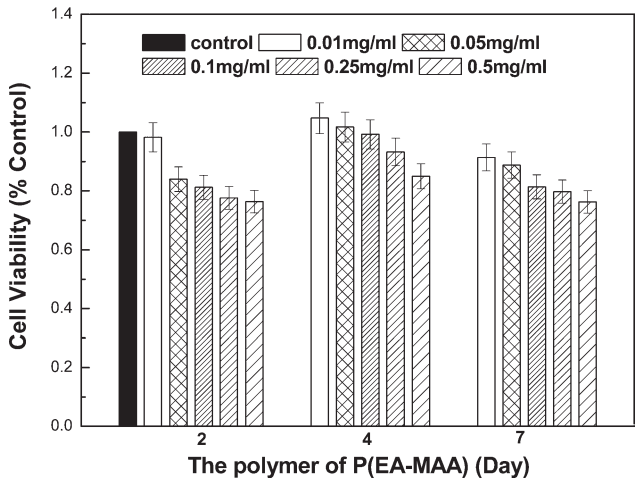

Figure 8. The dependence of the concentration of poly(EA-MAA) on the relative cell activity for a different period of cell culture.

produced by different contributions of residues of BSA, fluorescence peak is usually related to Trps, while UV peak is dependant not only on the two Trps but also 19 tyrosines. The intensity of the fluorescence peak for BSA increase with increase of the Poly(EA-MAA) concentration in the range of low concentrations (<1.15 mg/ml). By further increasing Poly(EA-MAA) concentration, the intensity decreases inversely in comparison with that of the pure BSA solution. This result may be caused by the complex formed between BSA and Poly (EA-MAA) due to the interchain hydrophobic associations, causing the more hydrophilic microenvironment around the residues of Trps.

Circular Dichroism. In addition to the fluorescence spectrum, CD technique has proved to be an alternative effective method to measure the conformation transition of a protein. It can provide useful information such as the content changes of the α-helix structure and secondary structure involving β-sheet components.[29–31] Figure 6 shows CD spectrum of BSA dependent on Poly(EA-MAA) concentrations. The characteristic peaks at 208 and 222 nm are ascribed to the α-helix structure. Proteins are organized by different levels of structure, such as primary, secondary, tertiary and quaternary structures. The primary structure is the chemical structure of the polypeptide chain or chains in a given protein, i.e., the number and sequence of amino acid residues linked together by peptide bonds. The secondary structure is such folding which is brought about by linking the carbonyl and amide groups of the backbone together by means of hydrogel bonds. There are three common secondary structures in proteins, namely α-helices, β-sheets, and turns. The estimated percentages of α-helix from CD spectrum are shown in Figure 6(b). As shown in Figure 6(b), the α-helix content increases significantly in the Poly(EA-MAA) solutions in comparison with that of pure BSA (38.6%). It is interesting to find that β-sheets of BSA disappear in all Poly(EA-MAA) solutions. The present result suggests

that Poly(EA-MAA) can make BSA to change the β-sheet into α-helix effectively. This finding is the first time to report.

BSA Adsorption on Poly(EA-MAA). Figure 7 shows the BSA adsorbance dependent on Poly(EA-MAA) concentrations. It is clear that, the BSA adsorbance per Poly(EA-MAA) deceases with increasing Poly(EA-MAA) concentration. This result may be related to the aggregation behavior of Poly(EA-MAA) having concentration dependence, which is very different from that of emulsifier of low molecular weight. The high BSA adsorbance (0.22–1.46 g/g) of Poly(EA-MAA) is caused by the hydrophobic interactions. It is well known that the immobilization of proteins on the surface of synthesized polymer is generally considered as a promising approach to enhance blood or tissue compatibility of biomaterials.[19] Moreover, there is functional carboxyl groups on Poly(EA-MAA) molecules, which provides great chance to link the bioactive molecules. After that, making use of the BSA adsorption on the other points of Poly(EA-MAA) molecules, the specific targeted matrix for drug controlled release can be designed.

Cytocompatibility of Poly(EA-MAA). The cytocompatibility should be carefully examined before being used as a new biomaterial. Figure 8 shows the dependence of the concentration of Poly(EA-MAA) on the relative cell viability for a different period of cell culture. From Figure 8, it can be found that in the Poly(EA-MAA) range of 0-0.5 mg/ml of Poly(EA-MAA), the relative cell viability decreases with increasing the Poly(EA-MAA) concentration. However, there is still more than 80% cell viability compared with the control for all the cases. These results may be related to the Poly(EA-MAA) adsorption on BSA. The in vitro cytocompatibility results indicate that the amphiphilic Poly(EA-MAA) is biocompatible polymeric materials.

CONCLUSIONS

In this study, the amphiphilic behavior of Poly(EA-MAA) was investigated. The aggregation mechanism is considered to be hydrophobic interaction. There are interactions formed between Poly(EA-MAA) and BSA, which make BSA to change the β-sheet into α-helix effectively and adsorb on Poly(EA-MAA) easily. This amphiphilic Poly(EA-MAA) is found to have high BSA adsorbance and good cytocompatibility and high has great potential in drug targeted and controlled release matrix.

ACKNOWLEDGMENTS

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