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# Polymeric Micelles Based on Hyaluronic Acid and Phospholipids: Design, Characterization, and Cytotoxicity

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**ABSTRACT:** Novel amphiphilic copolymers were synthesized and characterized by $^1$H NMR using Hyaluronic acid (HA) as a hydrophilic part and phosphatidylethanolamine (PE) including 1,2-dimiristoyl-sn-glycerol-3-phosphatidylethanolamine (DMPE) and 1,2-distearoyl-sn-glycerol-3-phosphatidylethanolamine (DSPE) as a hydrophobic segment. The newly developed HA-PE copolymers form a micelle in an aqueous media. The micellar properties, including critical micelle concentration (CMC) with pyrene as a fluorescence probe and micelle morphology, using transmission electron microscopy were assessed. It was found that the CMC values for HA-DMPE and HA-DSPE were 15.5 and 13.4 $\mu$g/mL, respectively. Also micelles were spherical in shape and within the size range of 162–214 nm. The solubility of cholesterol, a highly hydrophobic compound, was enhanced to 0.25 mg/mL which is much higher than it is in water (0.0001 mg/mL). *In vitro* cytotoxicity assay of HA-PE copolymers showed no toxicity on human breast cancer cell line (MCF-7). These results suggest that HA-PE micelles could be considered as a promising carrier for delivery of hydrophobic compounds. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40944.

**KEYWORDS:** biocompatibility; copolymers; micelles; self-assembly

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## INTRODUCTION

Amphiphilic polymers belong to a group of polymers which have both hydrophilic and hydrophobic parts and due to their architecture they can form different structures like dendrimers, hyperbranched polymers, star-shaped polymers and cyclic polymers.[1–4] These polymers have a unique property which enables them to form self-assembled structures in an aqueous media by self-aggregation, in order to reach their maximum thermodynamic stability.[5] Polymeric micelle (PM) forms a core-shell structure in a size range 10–300 nm. The hydrophobic part of these copolymers causes aggregation and induces self-assembly, as a result of the hydrophobic interactions between chains, while the hydrophilic part faces to the aqueous media. This self-assembled structure is formed above the critical micelle concentration (CMC) of such a copolymer which is an important factor in micelle stability both *in vitro* and *in vivo*.

Over the past decades, due to its relatively successful clinical outcomes, PM has been reported as a reliable delivery system, especially for hydrophobic drugs.[6] Various formulations based on PM are in the different phases of clinical trial for delivery of various cytotoxic drugs such as paclitaxel, doxorubicin and cisplatin.[7] PM has several advantages which make it an attractive delivery system, including its small size that facilitates efficient accumulation in tumor tissue via the enhanced penetration and retention effect (EPR), high loading capacity, long circulation time in blood stream, sustained release of payload into the plasma and acceptable stability.[8,9] Hyaluronic acid (HA) is a natural polysaccharide which is made by repeating disaccharide units composed of D-glucuronic acid and *N*-acetyl glucosamine linked by $\beta$ (1 $\rightarrow$ 4) and $\beta$ (1 $\rightarrow$ 3) glucosidic bands. HA is a biocompatible and biodegradable polymer which is derived from the umbilical cord or roster combs and it is also produced by streptococcus bacteria in large scale. It has a vast number of medical applications and has been used in various drug delivery systems.[10–12] HA has an important biological role in cell proliferation, differentiation, and also angiogenesis by binding to the cell specific receptors like CD44 and the receptor for HA-mediated motility (RHAMM) which are increasingly over expressed on the surface of tumor cells. Thus, tumor cells show high affinity to the uptake of HA and HA derivatives which make them attractive candidates to be used as targeted drug delivery systems.[13] Moreover, the sufficient amounts of functional groups like carboxylic acid (COOH) and hydroxyl (OH) in the structure of HA make it an interesting polymer for further chemical modifications. Recently, there has been a great interest in the architecture of different polymeric micelles by grafting either drugs or other hydrophobic polymers to HA

backbone for delivery of poorly soluble compounds. Although some of the research has shown suitable characteristic features of a polymeric micelle, the toxicity or non-biocompatibility of these polymers is still challenging because of their hydrophobic core.

Phospholipids are synthesized by most cells and have an important structural role in the body.[14] Due to their biocompatibility, phospholipids have been used interestingly in drug delivery systems such as liposomes,[15,16] solid lipid nanoparticles[17] and micro and nanoemulsions.[18] Regarding the hydrophobic characterization and also the biocompatibility of phospholipids, they could be considered as attractive candidates for the core part of polymeric micelles. PE has also been used in the architecture of PMs like PEG-PE. The PEG-PE micelle shows acceptable physicochemical characteristics and the solubility of hydrophobic compounds like paclitaxel is enhanced in an aqueous media by this copolymer. Gill et al. reported the sustained release of drug over a period of time due to the long fatty acyl chains of PEG-PE micelle which cause lower mobility in the entrapped drug.[19] Even though PEG-PE micelle has suitable characteristics, it has some major disadvantages as a drug carrier including lower hydrophilicity of polyethylene glycol relative to other natural polymers like dextran and hyaluronic acid. Another drawback of PEG-PE micelle is the lack of selectivity to the tumor cells due to absence of targeting moieties. Moreover, further chemical modification of PEG is also limited due to the lack of active functional groups in its structure.[20]

In this research, novel polymeric micelles based on HA and different phosphatidylethanolamines were synthesized and evaluated their physicochemical characteristics such as self-associative properties, morphology, size, surface charge and also optical contact angle. Furthermore, the *in vitro* cell cytotoxicity assay was also explored. Newly developed micelles enhanced the solubility of hydrophobic cholesterol several folded in water. Moreover, HA-PE copolymers showed no toxicity against MCF-7 cell line, which means they are safe and biocompatible carriers.

## EXPERIMENTAL

### Materials

Hyaluronic acid (MW = 10 KDa) was purchased from Freda Biochem (Shandong, China). 1,2-dimiristoylphosphatidylethanolamine (DMPE) and 1,2 distearoylphosphatidylethanolamine (DSPE) were purchased from lipoid (Germany). Cholesterol, *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and pyrene were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was purchased from Sigma (Germany), Roswell Park Memorial Institute-1640 medium (RPMI-1640), fetal bovine serum (FBS), and trypsin-EDTA 0.25% solution were from Gibco (Canada). All other reagents and solvents were of analytical grade and used as received.

### Synthesis of HA-PE Copolymers

HA-PE copolymers were synthesized by coupling primary amine group of PE with the carboxylic acid (—COOH) group of HA. First, 100 mg of HA was dissolved in 5 mL of deionized water. After complete dissolving of HA, two molar excess of EDC and NHS were added to this solution and stirred for 2 h in order to activate —COOH group of HA. Then 5 molar excess of PE (either DMPE or DSPE) was dissolved in 20 mL of tert-butanol/deionized water (90/10; V/V) containing 0.1 mol triethylamine, followed by heating to 50°C to form a clear solution. The activated HA was added to the PE solution dropwise, and the resultant mixture was sonicated for 30 min with probe sonicator. The mixture was stirred for 6 h at 50°C under nitrogen atmosphere, followed by stirring at room temperature for further 48 h. The resultant mixture was dialyzed using dialysis tube (Molecular-weight cutoff 3500 D) against excess volume of ethanol/water (70/30; V/V) for 48 h and against deionized water for further 24 h. Finally, the solution was filtered to remove any impurities, followed by lyophilization.

### Characterization of HA-PE Copolymers

**Nuclear Magnetic Resonance Spectroscopy (NMR).** $^1$H-NMR spectra of HA and HA-PE were performed via NMR spectrometer (500 Hz, Bruker, Germany). 2 mg of HA and HA-PE co polymers were dissolved in 2 mL of $D_2O$ and DMSO-$d_6$ respectively, and analyzed using NMR spectrometer.

**Critical Micelle Concentration (CMC) Measurement.** To ensure the formation of the core-shell structure of HA-PE conjugates, critical micelle concentration was measured by fluorescence spectroscopy using pyrene as a fluorescence probe.[21,22] Briefly, a stock solution of $6 \times 10^{-6}$ M of pyrene was prepared in acetone. 1 mL aliquot of the pyrene solution was added to series of 10 mL volumetric flasks, and acetone was evaporated completely on mild heating. To each flask, 10 mL of different concentration of HA-DMPE and HA-DSPE ranging from 0.5 $\mu$g mL$^{-1}$ to 1000 $\mu$g mL$^{-1}$ was added to obtain the final pyrene concentration of $6 \times 10^{-7}$ *M*. Each sample was sonicated for 30 min followed by stirring for 24 h at room temperature. The fluorescence spectra of each sample were analyzed by spectrofluorometer (RF-5000, Shimadzu, Japan) equipped with data recorder DR-3 at the excitation wave length of 336 nm and the emission spectra were monitored from 350 to 440 nm. The excitation and emission slit width was set at 3 nm. Upon the formation of micelles pyrene was solubilized in the core part and resulted in a decrease in the intensity ratio of $I_{373}/I_{386}$.

### Preparation of HA-PE Micelle

HA-PE micelles were prepared by dissolution and sonication method. 10 mg of each HA-PE copolymers were dispersed in 10 mL of deionized water and sonicated with probe sonicator (every 3 s with an output power of 180 watt) in ice bath for 10 min followed by stirring for 1 h. The resultant dispersion was filtered through a 0.45 $\mu$m filter followed by lyophilization and kept for further experiments.

### Evaluation of Solubilization Efficiency (SE %) of HA-PE Micelles

Cholesterol, a hydrophobic natural compound was selected to evaluate the solubilizing efficacy of HA-PE micelle. The cholesterol loaded micelle was prepared using the aforementioned method. Briefly, 3 mg of cholesterol was dissolved in 2 mL of methanol and added dropwise to 7 mL of either HA-DMPE or

HA-DSPE with a concentration of 1.0 mg $mL^{-1}$. The mixture was sonicated in ice bath for 10 min and stirred for a further 5 h at room temperature. The dispersion was centrifuged for 20 min at 4000 rpm to remove the unloaded Cholesterol, followed by lyophilization. 1 mL of cholesterol loaded micelle was dissolved in 10 mL of methanol and mixed vigorously for 15 min to dissolve the loaded cholesterol. The amount of cholesterol in the solution was determined spectrophotometrically in 205 nm and SE% was analyzed by the following equation:

$$\text{SE}\,(\%) = \frac{\text{Cholesterol amount in micelle}}{\text{Cholesterol amount in feed}}$$

### Characterization of the Cholesterol Loaded Micelles

**Particle Size and Zeta Potential Analysis.** The hydrodynamic mean diameter and the zeta potential of cholesterol loaded polymeric micelles were analyzed by dynamic light scattering (Zetasizer ZS, Malvern, UK). All dynamic light scattering measurements were analyzed with a wavelength of 633 nm at 25°C with an angle detection of 90°. Each sample was diluted with deionized water and sonicated prior to analysis.

**Morphology of the Cholesterol-Loaded Micelle.** For better understanding the core-shell structure of the HA-PE polymeric micelle, transmission electron microscopy (TEM, CM 30, Philips, Eindhoven, the Netherlands) was performed operating at 120 kV. 10 $\mu$L of micelle dispersion was placed on carbon coated grids. The sample was dried and fixed with gluteraldehyde 2% and stained negatively using uranyl acetate (1%; w/v). Any excess solution was removed with a filter paper before viewing on the TEM machine.

### Differential Scanning Calorimerty (DSC)

DSC analysis was performed using Metler-toledo (Greifensee; Switzerland). DSC was used to investigate the physical properties of HA-PE micelle, cholesterol, the physical mixture of HA-PE and cholesterol and also cholesterol loaded HA-PE micelle. The calorimetry study was performed at temperatures, ranging from 20°C to 300°C and the heating rate of 10°C $min^{-1}$.

### Optical Contact Angle Measurement

Sessile drop contact angles were measured for HA-DMPE, HA-DSPE with a concentration of 100 $\mu$g $mL^{-1}$ and deionized water as a control, using a contact angle goniometer (OCA 15 Plus, Dataphysics, Germany) equipped with a CCD camera and a SCA analyzing software. In all experiments the drop size was 4 $\mu$L and ten images of each sample were taken. Each sample drop was added separately onto the surface of the glass slide by a motor driven syringe at room temperature and the data was expressed as a mean of ten measurements.

### Cytotoxicity Study

**Cell Culture.** MCF-7 human breast cancer cell line was purchased from the national cell bank of Iran (NCBI) Pasture institute of Iran in 50 $cm^2$ flask. It was maintained in a standard medium consisting of RPMI-1640 with 10% (V/V) FBS (RPMI-1640-10% FBS) and 1% (V/V) antibiotic-antimycotic and incubated at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ prior to use. The medium was then replaced with fresh RPMI-1640-10% FBS. The cells were maintained by being sub cultured after arriving at an acceptable confluence. The cells with passage numbers between 40 and 50 were used in our experimental trials.

**Cell Viability Assay.** *In vitro* cytotoxicity assay was performed to evaluate the toxicity of different HA-PE copolymers using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The cells were removed from the culture flasks by means of Trypsin-EDTA 0.25% solution, resuspended in RPMI-1640-10% FBS, counted, and seeded into 96-well flat-bottomed cell culture plates at a concentration of $5 \times 10^3$ cells/mL. Cells were incubated overnight at standard condition to reach exponential growth prior to the assay test. The following day, the cells were treated with different concentration of HA-DMPE and HA-DSPE copolymers ranging from100 to 1000 $\mu$g $mL^{-1}$ for 24 h. At the end of the incubation time, the medium was removed and MTT reagent (0.5 mg $mL^{-1}$) was added to each well and left to incubate for 2–3 h at 37°C. The MTT reagent was replaced with 200 $\mu$L DMSO to dissolve the formazan crystals. The absorbance was measured at 570 nm using an ELISA plate reader (BioTek ELx800). Each assay was repeated in triplicates and MTT assay was performed in 4 replicates for each experiments. Cell viability was calculated using the following equation:

$$\text{Cell viability}\ (\%) = (\text{Abs}_s/\text{Abs}_{\text{control}}) \times 100$$

where $\text{Abs}_s$ is the fluorescence absorbance of the cells incubated with the polymer suspension, and $\text{Abs}_{\text{control}}$ is the fluorescence absorbance of the cells incubated only with the culture medium.

## RESULTS AND DISCUSSION

### Synthesis and Characterization of HA-PE Copolymers

HA-PE conjugates were successfully synthesized by grafting the carboxylic acid group of HA with amine group of PE. In this synthesis, the amine group of PE was conjugated with carboxylic acid group of HA which was activated by NHS and EDC in presence of triethylamine as illustrated in Figure 1(a).

The chemical structure of HA, HA-PE conjugate and also degree of substitution (DS) of PE on the backbone of HA was determined by $^1$H NMR analysis. As shown in $^1$H NMR spectrum of HA [Figure 2(a)], *N*-acetyl (—$NHCOCH_3$) peak is identified at $\delta$ 1.9 ppm along with glucosidic H at $\delta$ 3.0 to 4.0 ppm.[23] The $^1$H NMR spectrum of HA-PE conjugate [(Figure 2(b)] showed a new emerged peak at $\delta$ 0.87 ppm which is attributed to the protons of terminal methyl (—$CH_3$) group and a sharp peak at $\delta$ 1.2 ppm which is corresponds to the protons of methylenes (—$CH_2$—) group of PE. All the other new absorption peaks are attributed to protons of PE moiety.[24] The results confirmed successful grafting of PE onto the HA backbone.

DS was defined as the number of PE molecules which were grafted to 100 sugar residue of HA polymer and was calculated by the relative integration ratio of methylene group of PE to the *N*-acetyl peak of HA in $^1$H NMR spectrum of HA-PE. The DS of different HA-PE conjugates are summarized in Table I.

### CMC Evaluation of the HA-PE Micelles

The CMC value is an important factor which not only determines the solubilization efficiency of micelle but also

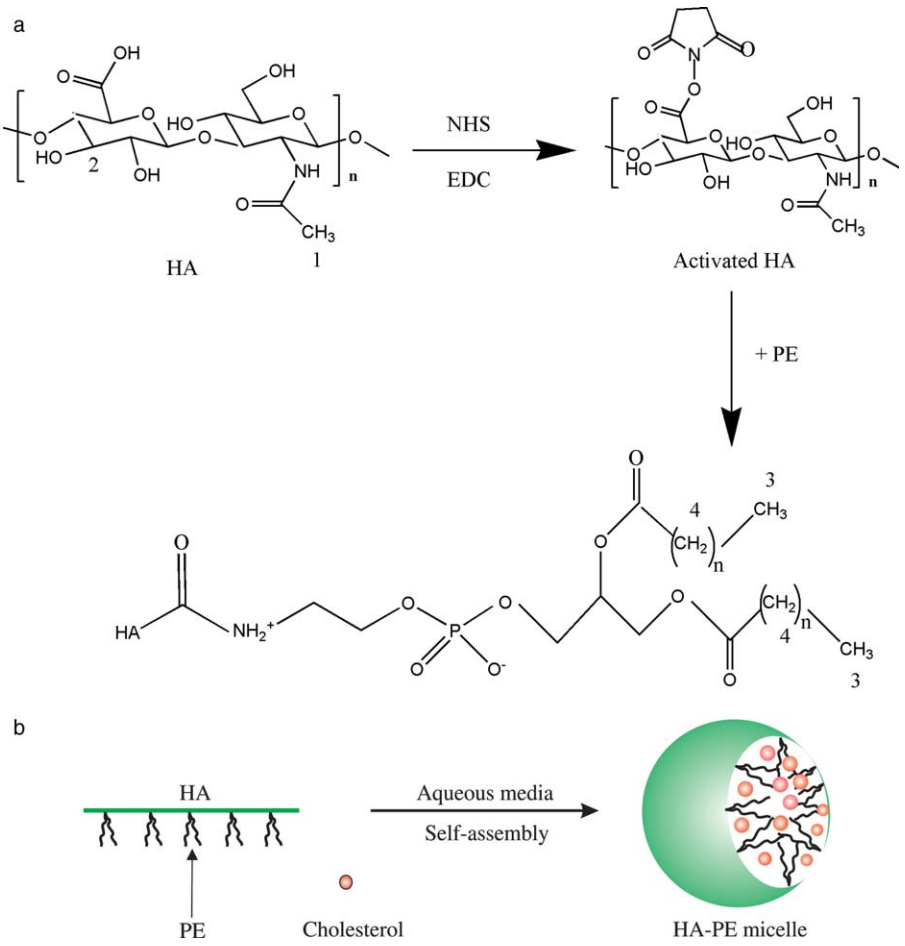


**Figure 1.** (a) Schematic representation for activation of HA carboxylic acid groups with NHS and EDC and the conjugation of PE to the HA backbone. (b) Scheme of HA-PE self-aggregation in an aqueous media. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

demonstrates its stability both *in vitro* and *in vivo*. From a biological point of view micelle dispersion is diluted in blood stream and is expected to be disassembled. So, the lower the CMC value, the more stable the micelle.[5]

The CMC value of HA-PE conjugates were determined spectruflurimetrically using pyrene as a hydrophobic florescence probe. The emission spectra of pyrene is highly depended on its microenvironment. In hydrophilic media the intensity of its first emission peak ($I_1 = 373$ nm) is higher than that of the third one ($I_3 = 384$ nm) and in hydrophobic media due to solubilization of pyrene in the micelle core, the intensity of the third peak grows higher than that of the first peak.[25] So, the CMC value of HA-PE micelle can be determined by calculation of the relative emission intensity peaks ($I_1/I_3$). Figure 3(a) and (b) shows the pyrene emission intensity ratio ($I_1/I_3$) versus log concentrations of HA-DMPE and HA-DSPE, respectively. The intensity ratio of $I_1/I_3$ at lower HA-PE copolymers concentrations was high and nearly unchanged; meanwhile, at higher concentrations this ratio decreased due to the aggregation of HA-PE copolymers. Using this method, the CMC values of HA-DMPE and HA-DSPE were calculated to be 16.5 $\mu$g mL$^{-1}$ and 13.4 $\mu$g mL$^{-1}$, respectively. The CMC value of HA-DSPE was found to be lower than that of HA-DMPE, which is attributed to more hydrophobic characteristics of DSPE compared to DMPE so HA-DSPE forms a micelle at a lower concentration. These findings are in agreement with previously reported hydrophobized HA derivatives. For instance, it has been shown that using fluorescence spectroscopy with Nile red as a probe, HA- (21,000 Da) modified octenyl succinic anhydride with DS of 18% had the CMC value of 15 $\mu$g mL$^{-1}$.[26] A similar finding also reported that HA (300,000 Da) modified with alkyl chains containing 10 carbon atoms ($C_{10}$-HA, DS of 5%) and 12 carbon atoms ($C_{12}$-HA, DS of 4%) have CMC values between 10 to 100 $\mu$g mL$^{-1}$.[27] The obtained results in this study could not be compared with aforementioned results due to the fact that different molecular weights of HA were used. Moreover, different hydrophobic micelle cores and various degrees of HA substitutions make direct comparison between obtained CMC values less possible. The CMC values of HA-PE micelles were found to be much lower than traditional surfactants or copolymers like Pluronic ($1.97 \times 10^{-5}$ $M$), which indicates higher HA-PE micelle stability in an aqueous media.[28]

In summary, this result suggests acceptable HA-PE micelle stability under extreme dilution. This means that the integrity of HA-PE micelles would be preserved even after intravenous administration into the blood stream. Moreover, HA-PE micelle

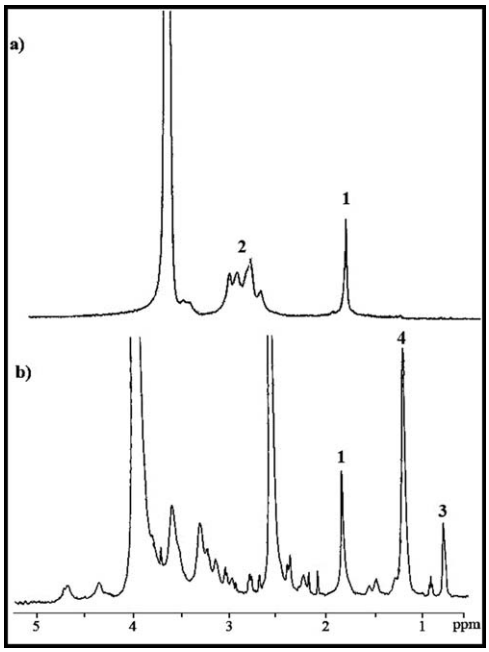


**Figure 2.** $^1$H NMR spectrum of (a) HA in $D_2O$ and (b) HA-PE copolymer in DMSO-$d_6$.

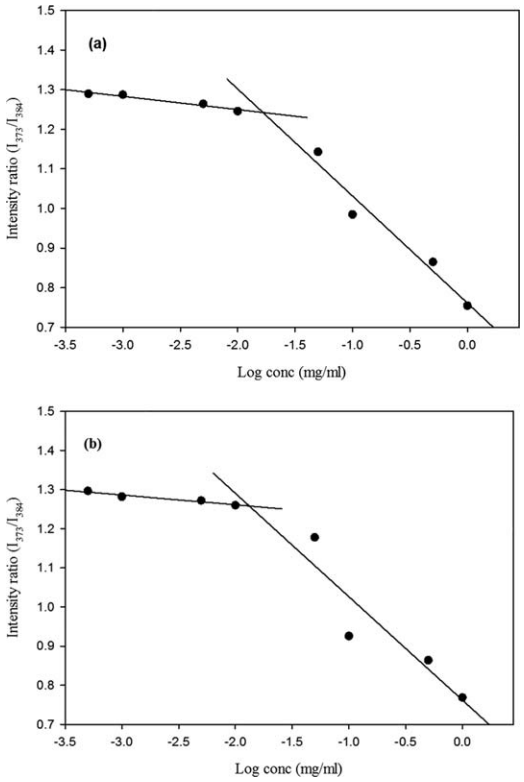


**Figure 3.** Intensity ratio plots of pyrene ($I_{373}/I_{384}$) versus log concentrations of (a) HA-DMPE and (b) HA-DSPE copolymer at 25°C in water.

seems to have a great potential to act as a drug carrier because of their tendency to form self-assembled micelles which can enhance solubility of hydrophobic compounds such as pyrene.

### Preparation of the HA-PE Micelles

Amphiphilic self-assembled micelles are prepared by various methods including sonication, dialysis and emulsification according to their physicochemical properties.[29] In this study, HA-PE micelles were prepared by sonication method in which no emulsifiers or other additives are needed. Amphiphilic polymers form self-assembled structures to reach maximum stability and in such structures hydrophilic parts face water while the hydrophobic segments face the opposite direction. From the thermodynamic point of view, the hydrophobic interactions in micelle core and also the tendency of the hydrophilic segment of copolymer towards water are all driving forces to form such a self-assembled structure in an aqueous media. The hydrophobic interactions between grafted PE chains form core-shell nano structures in which the core part was composed of a network of PE chains and the shell part is from hydrophilic HA. The core part of micelle serves as a cargo, while the hydrophilic corona provides a steric protection layer. This layer shields micelle against the phagocytic system and lets it circulate more in blood stream.[30] Figure 1(b) shows the schematic self-assembled structure of HA-PE micelle in an aqueous media.

### Size, Zeta Potential, and Morphology of the Cholesterol Loaded Micelle

Cholesterol is a hydrophobic compound which is practically insoluble in water. In the present study cholesterol was physically entrapped in the HA-PE micelle which enhanced its solubility more than 300 times in water. The physicochemical characteristics of cholesterol loaded micelle are summarized in Table II. The results indicate that SE (%) of HA-DSPE was higher than that of HA-DMPE, which may be attributed to the more hydrophobic nature of HA-DSPE that provides a stronger binding core relative to HA-DMPE and thus incorporates a large amount of cholesterol. It is important to mention that this micellar system is a polymer–lipid conjugate whereas liposomes are phospholipid bilayer system in which cholesterol has been used to stabilize them in small amounts (usually 0.1 molar ratio of lipid content).[15,31] However, in this newly developed micellar system almost 60% of feed cholesterol was entrapped in the lipid core which shows the high ability of HA-PE micelles to enhance the solubilization of hydrophobic compounds such as cholesterol, in an aqueous media.

The mean particle size and zeta potential of plain HA-PE micelle and cholesterol loaded micelle are tabulated in Tables I

**Table I.** Physicochemical Characterization of the Plain HA-PE Micelle (Mean ± SD, $n = 3$)

| Sample | DS (%) | CMC ($\mu$g/mL) | Size (nm) | PI | Zeta (mV) |
|---|---|---|---|---|---|
| HA-DMPE | 10.2 | 16.5 | 165.66 ± 11.67 | 0.55 ± 0.06 | −21.9 ± 2.3 |
| HA-DSPE | 8.1 | 13.4 | 214.33 ± 8.73 | 0.48 ± 0.04 | −29.1 ± 3.6 |

**Table II.** Physicochemical Characterization of the Cholesterol-Loaded HA-PE Micelle (Mean ± SD, $n = 3$)

| Sample | Size (nm) | PI | Zeta (mV) | SE (%) |
|---|---|---|---|---|
| HA-DMPE | 208.3 ± 13.5 | 0.40 ± 0.05 | −18.4 ± 1.2 | 53.0 |
| HA-DSPE | 291.6 ± 12.6 | 0.38 ± 0.03 | −23.9 ± 4.1 | 60.3 |

and II, respectively. The mean size of plain HA-DSPE micelle was bigger than that of HA-DMPE due to the large cargo part of HA-DSPE micelle. Similar findings were observed for cholesterol loaded HA-PE micelle. The mean size of cholesterol loaded micelle was bigger than HA-PE plain micelle which could be explained by different amounts of cholesterol in micelle cores, which lead to the expansion of the hydrophobic cargo. The micelle size was also analyzed by means of TEM imaging system (Figure 4) and found it smaller than that reported by DLS method. The difference between these two methods comes from sample preparation which micelles undergo vacuum, and lose their water content by TEM imaging method. Therefore micelles shrunk and the particle size was reduced. When samples were analyzed by DLS method, micelle size was calculated indirectly by diffusion in solution using the Stokes–Einstein equation in which the sample passes no preparation except being suspended in the solution. Eenschooten et al.[26] performed a statistical analysis to determine micelle size by TEM micrographs parallel with DLS measurements. They showed that the values of hydrodynamic diameter from DLS were about twice larger than those from the TEM data when swelling coefficient ($\alpha$) assumed to be zero, but better correlation between DLS and TEM was obtained in case of micelle hydration ($\alpha = 12$ g of water/g of polymer). These results indicate the possibility of micelle hydration, especially due to HA part, which is swollen up to several folded of its original weight in aqueous media. Apart from size measurement techniques, micelle size is an important factor from a biological point of view. It has been found that most of human tumors have vasculature pore sizes ranging between 200 to 600 nm which facilitate passive targeting of micelles due to EPR effect.[32,33]

The zeta potential of plain and cholesterol loaded micelles were determined by measuring the electrophoretic mobility in deionized water at 25°C. The micelle surface charge was found to be in the range −14 to −30 mV. The zeta potential of HA-DSPE is slightly higher than that of HA-DMPE because of less DS. The negative zeta potential is a result of carboxylic acid groups of HA which exist on the surface of the particles. This negative charge enhanced micelle stability and retention time in blood circulation.[34] The negative zeta potential of HA based micelle exhibits some limitations for the cell uptake of such systems due to the repulsion of micelles by the cell surface negative charge. Lee et al.[35] reported a reduction in the nonselective uptake of HA-paclitaxel conjugate micelle by HA receptor-deficient cells like NIH-3T3. The cellular uptake of HA based micelles is highly increased by cancerous cell lines like MCF-7 and HCT-116 due to their over expressed HA recognizable CD44 receptors on the cell membrane. However, repulsion of these systems by other cell lines which express no receptors for HA on their surface is still challenging.[36]

TEM image of the cholesterol loaded HA-PE micelle is presented in Figure 4. This figure shows spherical shaped nanoparticles with a core-shell structure, composed of hydrophobic inner core containing PE molecules and a hydrophilic HA corona.

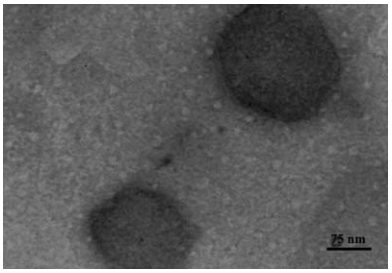


**Figure 4.** TEM image of cholesterol loaded HA-PE micelle. Sample was prepared in PBS (pH 7.4) prior to deposition onto the carbon coated grid.

### Differential Scanning Calorimetry (DSC)

DSC analysis is performed to investigate the physical state of molecules and also the possible inter and intramolecular interactions.[37] Figure 5 illustrates the DSC thermograms of (from

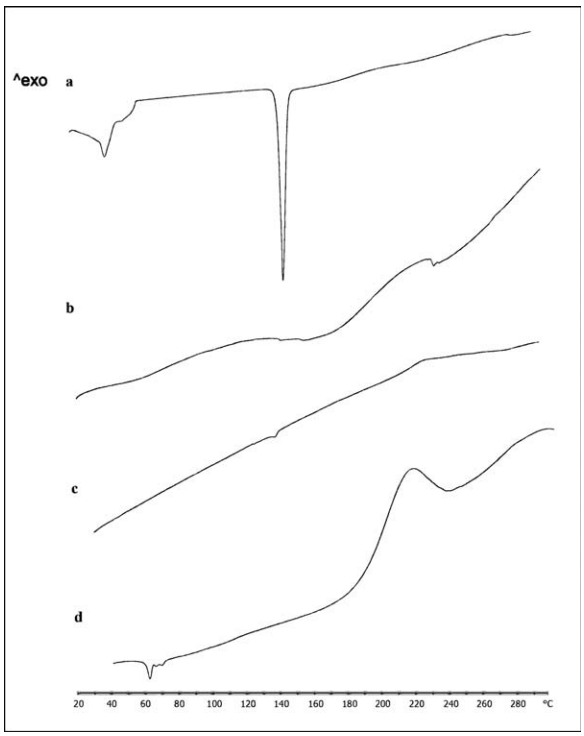


**Figure 5.** DSC thermograms of (a) Cholesterol, (b) physical mixture of Cholesterol, and HA-PE, (c) freezed dried cholesterol leaded HA-PE micelle, and (d) HA-PE copolymer.

**Table III.** Optical Contact Angle of HA-PE Micelle and Water (Mean ± SD, $n = 10$)

| Sample | Contact angle ($\theta$) |
|---|---|
| Water | 86° ± 2.1° |
| HA-DMPE | 61° ± 1.9° |
| HA-DSPE | 60° ± 2.3° |

top to bottom) cholesterol, physical mixture of cholesterol and HA-PE, freeze dried cholesterol loaded HA-PE micelle and HA-PE copolymer. Cholesterol monohydrate exhibits two endothermic peaks at 43°C and 146°C which are attributed to water loss and melting point, respectively. HA-PE copolymer showed a melting endothermic peak at 59°C, followed by an exothermic peak at 243°C which are most probably related to PE molecules phase transition and HA-PE polymer degradation, respectively.[19,38] As seen in the thermogram of HA-PE loaded micelle the PE endothermic peak surprisingly disappeared by loading the cholesterol in the micelle core. This phenomenon is seen along with cholesterol sharp peak abolishing at 140°C. Several studies on the thermal analysis of the cholesterol/phospholipids mixture reported a decrease in the PE phase transition temperature ($T_m$) due to the miscibility of cholesterol in liquid-crystalline phase and so the formation of $L_c$ crystalline phase is not observed during DSC analysis. For example McMullen et al. reported that the transition temperature and enthalpy of the PE/cholesterol mixture decrease in a monotonic manner because cholesterol is fully miscible in PE molecules.[39] Moreover, they found that cholesterol always decreases the $L_\beta/L_\alpha$ phase transition temperature, regardless of the PE hydrocarbon chain length. It was reported that the increasing amount of cholesterol relative to the PE molecules resulted in effective abolishment of the PE endothermic peak.[40] The physical mixture of cholesterol and HA-PE also showed a fusion of cholesterol molecules within PE acyl chains and as a result no crystalline peak of cholesterol molecules is seen. Regarding the cholesterol loaded micelle thermogram, it can be concluded that the cholesterol was successfully incorporated in the PE micelle core and so not only the PE endothermic peak disappeared, but also the sharp cholesterol endothermic peak was abolished due to solubilization in micelle core.

### Optical Contact Angle Measurements

The contact angle is an index which defines the spreading or wetting of a solid surface by a liquid. A low contact angle shows good spreading of the liquid on the surface and well wetting the solid. Meanwhile, a high contact angle indicates the poor wetting of solid by means of liquid. The smaller the contact angle is than 90°, the wetter the solid gets by the liquid. Whenever the angles are larger than 90°, the spreading of the liquid on the solid surface is poor.[41] The role of HA-PE micelle in solubility enhancement is further investigated by evaluating its wetting effect by measuring the contact angle. As illustrated in Table III the contact angles ($\theta$) of HA-DMPE and HA-DSPE conjugates were evaluated to be 61° ± 1.9° and 60° ± 2.3°, respectively, while the contact angle of water was 86° ± 2.1°. The ($\theta$) values of HA-PE micelles were less than that of water which suggested better spreading and wetting properties of HA-PE micelle on solid hydrophobic surfaces relative to water. This finding supported the role of HA-PE micelle in solubility enhancement of hydrophobic substances like cholesterol.

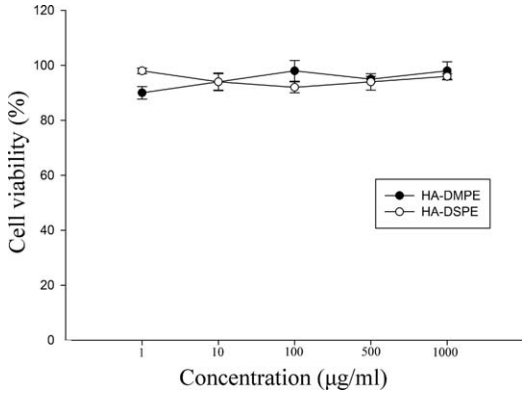


**Figure 6.** Cell viability of HA-PE copolymers against MCF-7 cell line after incubation for 24 h at 37°C. Data represent as mean ± SD ($n = 3$).

### Cytotoxicity Study

The cell viability data of HA-PE conjugates against MCF-7 cell line are illustrated in Figure 6. The viability data suggested no evidence of HA-PE copolymers toxicity to MCF-7 cells after 24 h of incubation, even at high concentration relative to the control group. Unlike the commercial surfactants and emulsifiers like Cremophore EL, which have potentially toxic effects both *in vitro* and *in vivo*,[35] HA-PE conjugates showed no toxic effect. This result suggests that HA-PE micelles can be used as a safe and efficient vehicle for hydrophobic drug delivery in pharmaceutical research.

## CONCLUSION

Novel polymeric micelles were synthesized using HA and PE successfully and evaluated for their physicochemical properties. HA-PE micelles were in the size range of around 200 nm and had negative zeta potential. HA-PE micelles exhibited low CMC values relative to common surfactants, which indicate their stability upon dilution. The newly developed micelle showed excellent solubilizing properties by which the solubility of cholesterol was improved significantly in aqueous media. Calorimetric studies also reveal the disappearance of cholesterol endothermic peak, which shows the solubility of cholesterol molecules in the micelle lipid core. Based on the present study, HA-PE micelles have low contact angles relative to water on the hydrophobic surface, which shows the wettability property of these copolymers. Moreover, HA-PE micelles exhibit no toxicity on MCF-7 cell line. Therefore, the present study suggests that the HA-PE micelles could be considered as a promising delivery system for poorly soluble drugs not only because of their attractive physicochemical characteristics, but also due to their safety and biocompatibility.

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## REFERENCES


1. Paleos, C. M.; Tsiourvas, D.; Sideratou, Z.; Tziveleka, L.-A. *Expert Opin. Drug Deliv.* **2010**, *7*, 1387.
2. Lavasanifar, A.; Samuel, J.; Kwon, G. S. *Adv. Drug Deliv. Rev.* **2002**, *54*, 169.
3. Jiang, X.; Li, L.; Liu, J.; Hennink, W. E.; Zhuo, R. *Macromol. Biosci.* **2012**, *12*, 703.
4. Wang, Q.; Zhu, L.; Li, G.; Tu, C.; Pang, Y.; Jin, C.; Zhu, B.; Zhu, X.; Liu, Y. *Macromol. Biosci.* **2011**, *11*, 1553.
5. Owen, S. C.; Chan, D. P.; Shoichet, M. S. *Nano Today* **2012**, *7*, 53.
6. Matsumura, Y. *Adv. Drug Deliv. Rev.* **2008**, *60*, 899.
7. Nishiyama, N.; Kataoka, K. *Pharmacol. Ther.* **2006**, *112*, 630.
8. Torchilin, V. *Pharm. Res.* **2007**, *24*, 1.
9. Maeda, H.; Bharate, G.; Daruwalla, J. *Eur. J. Pharm. Biopharm.* **2009**, *71*, 409.
10. Schanté, C. E.; Zuber, G.; Herlin, C.; Vandamme, T. F. *Carbohydr. Polym.* **2011**, *85*, 469.
11. Nakai, T.; Hirakura, T.; Sakurai, Y.; Shimoboji, T.; Ishigai, M.; Akiyoshi, K. *Macromol. Biosci.* **2012**, *12*, 475.
12. Akiyoshi, K.; Sunamoto, J. *Supramol. Sci.* **1996**, *3*, 157.
13. Platt, V. M.; Szoka Jr, F. C. *Mol. Pharm.* **2008**, *5*, 474.
14. Dowhan, W. *Adv Lipobiol* **1997**, *2*, 79.
15. Mohammed, A.; Weston, N.; Coombes, A.; Fitzgerald, M.; Perrie, Y. *Int. J. Pharm.* **2004**, *285*, 23.
16. Lee, S.; Lee, S.-Y.; Park, S.; Ryu, J. H.; Na, J. H.; Koo, H.; Lee, K. E.; Jeon, H.; Kwon, I. C.; Kim, K.; Jeong, S. Y. *Macromol. Biosci.* **2012**, *12*, 849.
17. Müller, R. H.; Mäder, K.; Gohla, S. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 161.
18. Ruan, J.; Liu, J.; Zhu, D.; Gong, T.; Yang, F.; Hao, X.; Zhang, Z. *Int. J. Pharm.* **2010**, *386*, 282.
19. Gill, K. K.; Nazzal, S.; Kaddoumi, A. *Eur. J. Pharm. Biopharm.* **2011**, *79*, 276.
20. Wang, H.; Han, S.; Sun, J.; Fan, T.; Tian, C.; Wu, Y. *Carbohydr. Polym.* **2011**, *83*, 1408.
21. Wolszczak, M.; Miller, J. *J. Photochem. Photobiol. A* **2002**, *147*, 45.
22. Kim, K.-H.; Lee, J.-C.; Lee, J. *Macromol. Biosci.* **2008**, *8*, 339.
23. Pouyani, T.; Prestwich, G. D. *Bioconjugate Chem.* **1994**, *5*, 339.
24. Percot, A.; Briane, D.; Coudert, R.; Reynier, P.; Bouchemal, N.; Lievre, N.; Hantz, E.; Salzmann, J.; Cao, A. *Int. J. Pharm.* **2004**, *278*, 143.
25. Fattahi, A.; Golozar, M.-A.; Varshosaz, J.; Sadeghi, H. M.; Fathi, M. *Carbohydr. Polym.* **2012**, *87*, 1176.
26. Eenschooten, C.; Vaccaro, A.; Delie, F.; Guillaumie, F.; Tømmeraas, K.; Kontogeorgis, G. M.; Schwach-Abdellaoui, K.; Borkovec, M.; Gurny, R. *Carbohydr. Polym.* **2012**, *87*, 444.
27. Creuzet, C.; Kadi, S.; Rinaudo, M.; Auzély-Velty, R. *Polymer* **2006**, *47*, 2706.
28. Zhao, L.; Du, J.; Duan, Y.; Zang, Y. n.; Zhang, H.; Yang, C.; Cao, F.; Zhai, G. *Colloids Surf. B* **2012**, *97*, 101.
29. Liu, Y.; Sun, J.; Cao, W.; Yang, J.; Lian, H.; Li, X.; Sun, Y.; Wang, Y.; Wang, S.; He, Z. *Int. J. Pharm.* **2011**, *421*, 160.
30. Gong, J.; Chen, M.; Zheng, Y.; Wang, S.; Wang, Y. *J. Controlled Release* **2012**, *159*, 312.
31. Immordino, M. L.; Brusa, P.; Arpicco, S.; Stella, B.; Dosio, F.; Cattel, L. *J. Controlled Release* **2003**, *91*, 417.
32. Miller, T.; Breyer, S.; van Colen, G.; Mier, W.; Haberkorn, U.; Geissler, S.; Goepferich, A. *Int. J. Pharm.* **2013**, *445*, 117.
33. Cheng, Y.; Yu, S.; Wang, J.; Qian, H.; Wu, W.; Jiang, X. *Macromol. Biosci.* **2012**, *12*, 1326.
34. Yamamoto, Y.; Nagasaki, Y.; Kato, Y.; Sugiyama, Y.; Kataoka, K. J. *Controlled Release* **2001**, *77*, 27.
35. Lee, H.; Lee, K.; Park, T. G. *Bioconjugate chem.* **2008**, *19*, 1319.
36. Choi, K. Y.; Chung, H.; Min, K. H.; Yoon, H. Y.; Kim, K.; Park, J. H.; Kwon, I. C.; Jeong, S. Y. *Biomaterials* **2010**, *31*, 106.
37. Mu, L.; Feng, S. *J. Controlled Release* **2001**, *76*, 239.
38. Luo, Y.; Kirker, K. R.; Prestwich, G. D. *J. Controlled Release* **2000**, *69*, 169.
39. McMullen, T.; Lewis, R.; McElhaney, R. N. *Biochim. Biophys. Acta* **1999**, *1416*, 119.
40. Cullis, P.; Van Dijck, P.; De Kruijff, B.; De Gier, *J. Biochim. Biophys. Acta* **1978**, *513*, 21.
41. Abdelkader, H.; Wu, Z.; Al-Kassas, R.; Alany, R. G. *Int. J. Pharm.* **2012**, *433*, 142.