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Synthesis and characterization of low molecular weight hyaluronic acid-based cationic micelles for efficient siRNA delivery

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ABSTRACT

The aim of this work was to design a new non-viral carrier for gene delivery. This carrier was equipped with low molecular weight hyaluronic acid (LMHA) as ligand, which was conjugated by covalent attachment of hydrophobic amines (fatty amines) with different chain lengths and spermine as cationic segments. Their chemical structure and self-association behavior of the LMHA conjugates were investigated using ^{1}H NMR, dynamic light scattering, fluorescence spectroscopy, and TEM. From the results, it was observed that polymeric micelles were spherical in shape. The micellar particle sizes and CMCs of the conjugates were significantly dependent on the degree of substitution of hydrophobic groups and the chain length. The CMCs of the conjugates were as low as 40–140 mg/L. The zeta potentials and agarose gel electrophoresis assays suggest that LMHA micelles encapsulating siRNA can be used as novel gene carriers for biomedical applications.

1. Introduction

Over the last two decades, considerable progresses have been made in the development of biocompatible/biodegradable polymers as drugs and/or genes carriers. An ideal drug carrier should satisfy a number of important requirements such as having favorable interaction with the drug/gene, high delivery capacity, suitable drug release profile, preservation of drug activity during delivery to the target site, and bio-inert (Allemann, Gurny, & Doelker, 1993; Jabr-Milane et al., 2008; Kreuter, 1991; Peppas, 1995).

Polymeric micelles that are supramolecular assemblies of block copolymers are useful nanocarriers for the systemic delivery of drugs and genes (Kataoka, Harada, & Nagasaki, 2001; Lavasanifar, Samuel, & Kwon, 2002). Amphiphilic di-blocks or graft copolymers composed of a hydrophilic and a hydrophobic segment can form micellar structures with either a hydrophobic compact inner core or a hydrophilic swollen outer shell or vice versa, depending on the solvent, which thermodynamically favors either of the two blocks. During the past decades, polymeric micelles have demonstrated their utility in delivering drugs and are currently recognized as promising formulations for enhancing the efficacy of drugs, such as paclitaxel and cisplatin (Matsumura et al., 2004; Nishiyama et al., 2003; Yokoyama et al., 1999). Recently, there has been a strong incentive to develop polymeric micelles with smart functions such as targetability to specific tissues (Lee, Na, & Bae, 2003; Nagasaki, Yasugi, Yamamoto, Harada, & Kataoka, 2001) and chemical (Bae, Fukushima, Harada, & Kataoka, 2003; Bae et al., 2005) or physical (Miyata et al., 2004) stimuli sensitivity.

Gene therapy has been recognized as a promising tool by delivery of genetic substances into targeting cells. RNA interference (RNAi) represents a promising gene silencing technology for functional genomics and a potential therapeutic strategy for a variety of genetic diseases (De Fougerolles, Vornlocher, Maraganore, & Lieberman, 2007; Iorns, Lord, Turner, & Ashworth, 2007; Wall & Shi, 2003). The use of small interference RNA (siRNA) in gene therapy research has surged over the past years following the discovery that the RNAi mechanism of gene-specific silencing can be exploited in human disease therapy (Aigner, 2007; Ryther, Flynt, Phillips, & Patton, 2005). However, due to the large molecular weight, negative charge of siRNA duplexes, and the susceptibility to enzymatic degradation, the effective cellular uptake and intracellular delivery of siRNA for clinical application represent a major challenge for the widespread use of RNAi as a therapeutic modality or even as an investigational tool in vivo (Aagaard & Rossi, 2007; Haupenthal, Baehr, Zeuzem, & Piiper, 2007; Kumar & Clarke, 2007).

Successful development of RNAi for clinical application is dependent on the discovery of safe and effective carriers (Gary, Puri, & Won, 2007; Itaka et al., 2004). In general, the ideal carrier for siRNA should be able to bind and condense siRNA, provide protection against degradation, specifically direct siRNA to target cells, facilitate its intracellular uptake, escape from endosome/lysosome into cytosol, and finally promote efficient gene silencing. Polymeric

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carriers have been of interest for siRNA delivery because they could be chemically engineered to meet all or some of these requirements simultaneously (Nagasaki et al., 2001; Peppas, 1995). Among different polymers designed for siRNA delivery, the micelles assembling block copolymers consisting of polyethylene glycol (PEG) and polycation segment such as polyethyleneimine (PEI) and poly(L-lysine) (PLL) have been emerging as promising carriers. These polymers display properties suitable for in vivo siRNA delivery, including siRNA binding and condensation, self-assembly into poly-ion complex (PIC) micelles with a diameter of around 100 nm, avoiding recognition by reticuloendothelial systems (RES), increasing nuclease resistance and tolerance under physiological conditions (Hwa Kim, Hoon Jeong, Chul Cho, Wan Kim, & Gwan Park, 2005; Lee, Kim, & Park, 2007). However, the safety profile of these polymers containing large polycationic segments and their non-biodegradable nature in some cases (e.g., PEI containing polymers) remain an obstacle for clinical application. In this regard, the development of siRNA carriers based on the biomaterials with a more proven safety record is desirable.

Polymeric micelles, especially the cationic micelles, are the ideal candidates to serve as nucleic acid carriers because they offer the versatility and ease of manipulation needed to address a wide range of gene therapy applications. As pointed out earlier, encapsulation generally provides much better protection of genes against serum degradation than chemical modification (Park, Jeong, & Kim, 2006). When block copolymers of this type are mixed with nucleic acid molecules in water, the cationic segments spontaneously complex with gene by electrostatic interaction and induce condensation of the gene into the core to form a compact structure.

Natural polysaccharides such as hyaluronic acid (HA, hyaluronan) have been extensively studied in medical applications, since they can provide intrinsic biological activity when used as the basis for biomaterials (Luo, Ziebell, & Prestwich, 2000; Mengher, Pandher, Bron, & Davey, 1986). HA is a naturally occurring, biocompatible, and biodegradable linear polysaccharide composed of unbranched repeating units of glucuronic acid and N-acetyl glucosamine linked by $\beta1 \rightarrow 3$ and $\beta1 \rightarrow 4$ glycosidic bonds with a wide molecular weight (MW) ranging from 1000 Da to 10,000,000 Da. The high MW HA degrades to small molecules through several metabolic pathways, maintaining the homeostasis. HA has been known to play different roles in the body depending on its molecular weight (Gupta, Vermani, & Garg, 2002). HA is responsible for various functions within the extracellular matrix such as cell growth, differentiation, and migration (Brekke & Thacker, 2005).

A wide range of activities can be explained by a large number of HA-binding receptors such as cell surface glycoprotein CD_{44}, receptor for hyaluronic acid-mediated motility (RHAMM), and several other receptors possessing HA-binding motifs, for example, transmembrane protein layilin, hyaluronic acid receptor for endocytosis (HARE), and lymphatic vessel endocytic receptor (LYVE-1) (Naor, Nedvetzki, & Golan, 2002; Naor, Sionov, & Ish-Shalom, 1997). It has been shown that the HA level is elevated in various cancer cells, such as epithelial, ovarian, colon, stomach, and acute leukemia (Ponta, Sherman, & Herrlich, 2003). Consequently, these tumor cells show enhanced binding and internalization of HA (Bourguignon, Zhu, & Zhou, 2001). CD_{44}-HA interactions play various important physiological roles, including mediation or promotion of macrophage aggregation (Faassen et al., 1992), cell migration (Henke, Roongta, Mickelson, Knutson, & McCarthy, 1996), chondrocyte pericellular matrix assembly (Knutson, Iida, Fields, & McCarthy, 1996), and leukocyte activation (Sleeman et al., 1996). It has been shown that the over expression of hyaluronic acid synthases increases the HA level, which leads to the acceleration of tumor growth and metastasis (Bartolazzi, Peach, Aruffo, & Stamenkovic, 1994; Huang, Shen, Wang, Wu, & Cheng, 2007; Sy, Guo, & Stamenkovic, 1991). On the other hand, exogenous oligomeric HA inhibits tumor progression most likely by competing with endogenous polymeric HA (Lesley & Tammi, 2000; Zeng, Toole, & Kinney, 1998). Although the mechanism of hyaluronan-CD_{44} binding is still not fully understood, it has been reported that the CD_{44} receptor contains the specific binding domain for HA, which consists of 160 amino acid residues (Sherman, Sleeman, Herrlich, & Ponta, 1994). The binding affinity of CD_{44} to HA was found to be dependent on the size of HA oligomers. For instance, hexamer (HA_6) and decamer (HA_{10}) are considered to be the minimum size oligosaccharides that bind to CD_{44}. Larger oligomers (HA_{20}) have higher binding affinity than smaller oligomers because of multiple interactions with more than one CD_{44} receptor simultaneously. Direct conjugations of a LMHA to cytotoxic drugs such as paclitaxel and doxorubicin have been reported (Eliaz, Nir, Marty, & Szoka, 2004; Yadav et al., 2007). It has been shown that these bioconjugates are internalized into cancer cells through receptor-mediated endocytosis, followed by intracellular release of active drugs, thus restoring their original cytotoxicity. The efficacy of bioconjugates depends on the level of cytotoxic agent loading to LMHA. LMHA was also used as a tumor-targeting moiety in drug and gene delivery system with polymers and liposomes (Lee, Mok, Lee, Oh, & Park, 2007; Rosato et al., 2006).

Hydrophobically modified polysaccharides can be used as efficient anti-tumor gene carriers (Hyung Park et al., 2006; Kim et al., 2005; Park et al., 2004, 2007). However, those carriers are not cancer targeting and these modified polysaccharides are negatively charged in physiological conditions which could not combine the nucleic acids effectively. In this article, we synthesized LMHA-grafted hydrophobic amines and spermine conjugates, which were self-aggregated in water to form polymeric micelles. Spermine was used as a cationic side chain, and then this cationic side chain can neutralize the negative charge of nucleic acids by electrostatic interactions and entrapped into the core of the LMHA polymeric micelles. The structural, chemical characteristics and the abilities of siRNA encapsulation of the polymeric micelles conjugates were studied.

2. Materials and methods

2.1. Materials

Sodium hyaluronates (molecular weights 9.6 kDa, 23 and 45 kDa) were purchased from Freda Biochem Co. Ltd. (Shandong, China). 1-Ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC), and N-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Medpep Co. Ltd. (Shanghai, China). 1-Octanamine (OA), 1-Laurylamine (LA) and 1-Octadecylamine (DA) were purchased from Fluka (Buchs, Switzerland). Spermine (SP) was purchased from Sigma. siRNA (5′-AAGTGCGATTGTACCCGGACA-3′) was purchased from Gene Pharma Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade and used as received.

2.2. Synthesis and characterization of hydrophobically modified low molecular hyaluronic acid-based cationic micelles

The alkylation reaction was performed under homogeneous aqueous conditions (Kim, Park, & Hahn, 2008). Briefly, LMHA (carboxyl group, 1.5 molar equivalent) was dissolved in 10 mL of distilled water containing EDC (2.5 molar equivalent) and sulfo-NHS (2.0 molar equivalent) to the form active esters of LMHA. During this period, the pH was remained at 6.0–6.5 for 2 h (Fig. 1-I). Then, OA (1.5 molar equivalent) and SP (1.0 molar equivalent) in 1-methyl-2-pyrrolidinone (30 mL) were added and the pH was adjusted to 8.0–8.5 by the addition of 0.1 N NaOH. After 24 h, the reaction mixture was extensively dialyzed against the excess

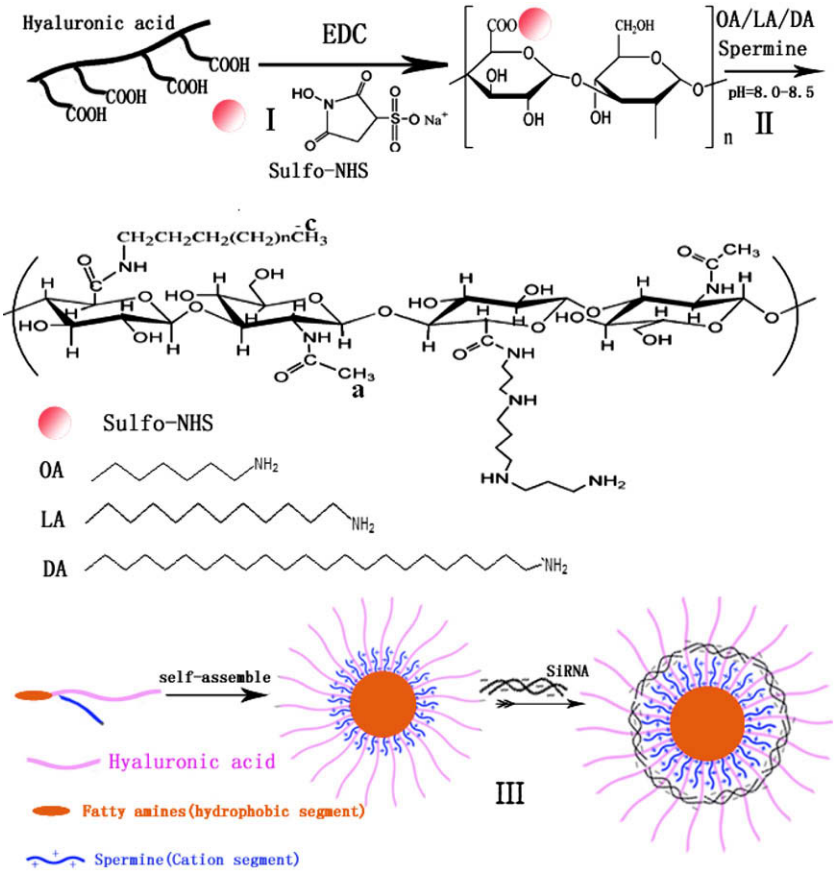

Fig. 1. Schematic representation for (I) the activation of the carboxyl terminal ligands of (HA) with EDC and sulfo-NHS, (II) the conjugation of alkyl chain to the active esters of HA and (III) the self-aggregates in the water.

amount of water/ethanol (1v/3v–1v/1v) and distilled water for 3 days, followed by lyophilization (Fig. 1-II) to form LMHA-OA-SP (HOP). The other alkylated LMHA derivatives were synthesized by the same procedure.

The degree of substitution of hydrophobic segment such as OA/LA/DA (DS_a) was determined using ^{1}H NMR (Bencherif et al., 2008) and calculated from the ratio of the relative peak integrations of the OA/LA/DA's methyl protons and the N-acetyl groups of LMHA to form LMHA-LA-SP (HLP) and LMHA-DA-SP (HDP).

2.3. Determination of substitution of spermine (DS_P)

Degree of substitution of spermine was determined by 2,4,6-trinitro-benzenesulfonic acid (TNBS) assay (Li, Guo, Wei, MacDiarmid, & Lelkes, 2006). In brief, about 2–4 mg of dried HOP/HLP/HDP micelles sample was treated with a reaction mixture of 1 ml of 4% (w/v) $NaHCO_3$ solution (pH 8–9) and 1 ml of 0.5% (v/v) TNBS solution prepared in deionized water. The reaction was allowed to take place for 2 h at 40 °C, at which temperature the TNBS reacts with free amine groups of spermine that was grafted onto the HA micelles and gives a brown color. The extent of color intensity depends upon the content of free amino groups in the test sample. Subsequently, 3 ml of 6 M HCl was added and incubated for 90 min, and temperature was raised to 60 °C. Absorbance of the resulting solution was measured at 345 nm after dilution using spectrophotometer (Thermo Spectronic, US). A control was prepared with the same procedure except that 6 M HCl was added before the addition of TNBS. The percentage degree of substitution was calculated as follows:

Degree of substitution % = The mole number of free amine groups/sample weight (per mg) * 100%. The data are listed in Table 1.

2.4. Preparation of siRNA cationic micelles

The siRNA/HOP cationic micelles were prepared according to the following process. Briefly, HOP micelles (10 mg) were dissolved in 3 mL water and stripped for 1 h. The alkylated HA derivatives were self-assembled to form "core-shell" structures due to their hydrophobic interactions of the alkylated chains (Fig. 1-III). One milliliter of blank HOP cationic micelles (1 mg/ml in water) was added dropwise to 1 ml of siRNA aqueous solution with 20 μg siRNA (DNA:HOP = 1:50, w/w) under stirring at a moderate speed. The resulting siRNA siRNA/HOP cationic micelles were allowed to remain at room temperature for 30 min to facilitate complexation. The polymeric micelles were obtained by filtration through 0.45 μm microporous filter (Wan Qing Sci-Tech Co., Ltd., Nanjing, China) to obtain a more homogeneously sized vesicles population. Experiments were performed to investigate whether HOP cationic micelles can entrap the siRNA. By the same procedure, the siRNA/HLP and siRNA/HDP cationic micelles were prepared immediately prior to the experiments.

2.5. Transmission electron microscopy (TEM) of HOP/HLP/HDP micelles

TEM observation of HOP/HLP/HDP micelles that are dispersed in double distilled water was photographed with a H-7000 transmission electron microscope (Hitachi, Japan) at an accelerating voltage

Table 1
Effect of the degree of substitution (DS)/size/zeta/CMCS of self-aggregates micelles of different Mns.

Sample	DS_P (μmol/mg)	DS_a (%)	Average yield (%)	Size (nm)	PI	Zeta (mV)	CMC (mg/l)
HLP_{9600}	2.63 ± 0.312	45.54 ± 4.67	92.1	125.6 ± 3.5	0.42 ± 0.12	+20.6 ± 2.4	40.97 ± 2.18
$HLP_{23,000}$	2.14 ± 0.296	40.50 ± 5.23	87.9	338.2 ± 5.2	0.35 ± 0.08	+19.4 ± 3.8	51.19 ± 3.25
$HLP_{45,000}$	2.53 ± 0.387	28.48 ± 4.76	85.3	439.9 ± 8.3	0.33 ± 0.05	+18.4 ± 3.3	139.4 ± 21.47
HOP_{9600}	2.85 ± 0.257	44.81 ± 6.25	90.3	555.1 ± 11.5	0.07 ± 0.01	+21.3 ± 2.6	117.66 ± 14.96
HDP_{9600}	3.12 ± 0.342	17.21 ± 3.27	84.6	275.1 ± 4.8	0.44 ± 0.06	+20.3 ± 1.9	73.15 ± 5.73

of 75 kV. A drop of the resultant conjugate dispersion was placed onto a carbon-coated copper grid, forming a thin liquid film. The films on the grid were negatively stained by adding immediately a drop of 2% (w/w) phosphotungstic acid, removing the excess solution using a filter paper followed by a thorough air-drying.

2.6. Particle size and zeta potential of polymeric micelles

Particle size of HOP/HLP/HDP micelles was analyzed by light scattering measurements using a Zetasizer 3000HS particle size analysis system (Malvern Instruments Ltd., Malvern, UK) at 20 °C. The zeta potential of micelles was measured with the laser Doppler method (heterodyne method) by using a Zetasizer 3000HS (Particle Sizing System, Malvern) at pH 7.4. The polydispersity index discloses the quality of the dispersion, from values lower than 0.5 for suitable measurements and good quality of the colloidal suspensions.

2.7. Determination of critical micelle concentration (CMC)

CMC is an important parameter, above which an amphiphilic copolymer forms core/shell structured micelles. The CMC values of HOP/HLP/HDP micelles in deionized (DI) water were estimated by fluorescence spectroscopy using pyrene as a probe, which was described previously (Zhang, Qiu, Jin, & Zhu, 2006). A pyrene solution (3.0×10^{-2} M in acetone), which had been stored at 4 °C, was added to the distilled water to give a pyrene concentration of 12.0×10^{-7} M, and acetone was removed using a rotary evaporator at 60 °C for 1 h. This solution was mixed with the solution of LMHA micelles to obtain a polymer concentration 1.0×10^{-4}–1.0 mg/mL, resulting in a pyrene concentration of 6.0×10^{-7} M. Then, these solutions were heated at 45 °C for 3 h to equilibrate pyrene with the micelles, and left to cool for 3 h at room temperature. Pyrene fluorescence spectra were obtained by using fluorescence spectrophotometer (F-5000, Hitachi). The excitation (λex) and emission (λem) wavelengths were 336 and 390 nm, respectively.

2.8. Surface tension of HOP/HLP/HDP micelles

The surface activity of all types of HOP/HLP/HDP micelles has been established by performing a systematic tensiometric study in aqueous solution. The critical micelle concentrations of the conjugates in aqueous solution were estimated as the inflexion point of plots of the surface tension versus conjugate concentration. All equipments were interfaced to a computer for both data collection and analysis.

2.9. siRNA binding ability of HLP micelles – Agarose gel electrophoresis (Jiang et al., 2008)

The siRNA binding ability of HLP micelles was analyzed by agarose gel electrophoresis. The siRNA/HLP complexes were prepared at various weight ratios. Six times siRNA loading buffer (50% sucrose solution, 5 μl) was added to the complex solutions. The mixtures were allowed to stay at room temperature for 45 min. Thereafter, the complexes were loaded into individual wells of 2% (w/w) agarose gel in TBE buffer solution (10.8 g/l Tris base, 5.5 g/l boric acid and 0.58 g/l EDTA) containing 0.5 mg/ml ethidium bromide, and were electrophoresed at 100 V for 90 min. The naked siRNA diluted with the same buffer without adding the micelles and the micelles without adding siRNA were used as the controls. The resulting siRNA migration patterns were revealed under UV irradiation (Bio Rad, USA), and photos were taken at times.

3. Results and discussion

3.1. Synthesis and characterization of HOP/HLP/HDP micelles

Different molecular weights of LMHA were used to synthesize HOP/HLP/HDP micelles. Direct carbodiimide-mediated coupling of amines to the carboxyl group of HA in an aqueous environment, e.g., with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), did not yield the predicted product, since the O-acyl isourea that is formed as a reactive intermediate rearranges rapidly to a stable N-acyl urea (Grayson, Doody, & Putnam, 2006a, 2006b). We found that the coupling of primary amines to HA was possible via the formation of a more hydrolysis resistant and non-rearrangeable active ester intermediate. So we formed the active esters of HA with N-hydroxysulfosuccinimide (sulfo-NHS) using the water-soluble carbodiimide EDC for coupling. Nucleophilic addition to the ester formed from sulfo-NHS requires the amine to be presented in an unprotonated form at neutral or slightly basic pH (about 7.0–8.5) and consequently yields products by reaction with simple primary amines.

The backbone of HA was hydrophobically modified by the chemical attachment of hydrophobic amines as shown in Fig. 1. The carboxylic acid group of HA was activated with equal amounts of EDC and sulfo-NHS to form amide linkage by the reaction with primary amino groups in hydrophobic amines. The detailed characteristics of hydrophobically modified LMHAs are summarized in Table 1. By varying the different lengths of alkyl chains to HA, five different self-associated nano-sized micelles were obtained. The amount of conjugated hydrophobic amines(c) in micelles was quantitatively characterized from the ^{1}H NMR spectra by using the integration method. The characteristic peaks (Fig. 2-1) of N-acetyl group of HA (marked a, (δ/ppm = 2, [t, 3H, —$COCH_3$—])) and hydrophobic amines (Fig. 2-2) (marked c, δ/ppm = 0.8–0.9 [t, 3H, CH_3—(CH_2)n—]; 1.3–1.4 [m, 22H, —CH_3(CH2)n—]) were used for integration (DS_a = c/a). The DS_a/DS_P values and yields are listed in Table 1.

As shown in Table 1, when the length of alkyl chains remains unchanged, the degree of substitution (DS_a) of the products declines as the molecular weight of the HA gets larger. This is because the larger the molecular weight of the HA is, the stronger the interaction of the intra or intermolecular hydrogen bonds will be, which makes it difficult for the reactions to proceed. On the other hand, larger molecular weight of the HA can cause higher viscosity of the macromolecular solution, and this will have a negative influence on diffusing steps for reacting reagents. So, the DS gets lower.

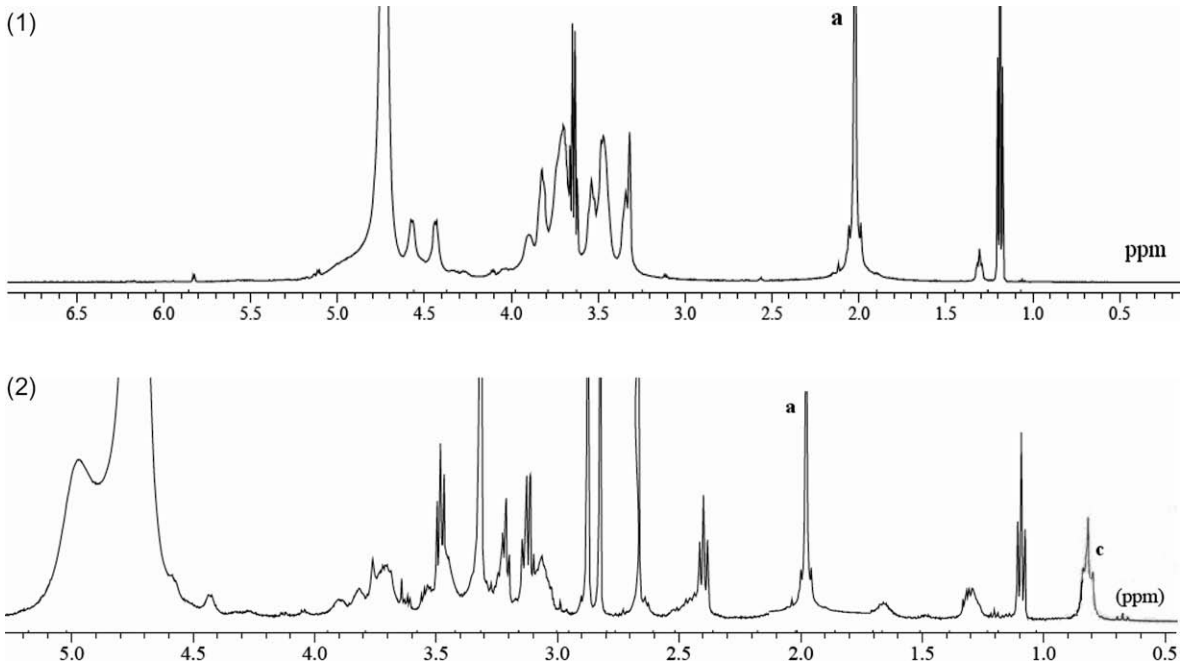

Fig. 2. (1) ^{1}H NMR spectra of native HA in CD_3OD:D_2O = 2:1 (1v/2v, A). (2) ^{1}H NMR spectra of HLP9600 in D_2O/CD_3OD (B, 1v/2v).

This phenomenon is significant when the molecular weight of the HA is beyond 2.3 kDa. When the molecular weight of the HA remains unchanged, the DS of the products gets smaller as the length of alkyl chains gets longer. This is because the longer length of alkyl chains may accrete stereo-specific blockades, which make the DS lower.

3.2. TEM determination

The TEM images of HOP/HLP/HDP micelles in double distilled water are shown in Fig. 3. After dissolving in double distilled water, HOP/HLP/HDP micelles formed spherical micelles of uniform size in the water. Hydrophilic substances such as LMHA can be dyed with phosphotungstic acid (PTA), while the hydrophobic inner core cannot. The shells of HAD micelles are hydrophilic which are dyed into black color. In these five pictures, the whole micelles were dyed and the hydrophobic inner core cannot be seen. This is because the hydrophobic chains are far shorter than the hydrophilic chains.

3.3. Particle size and zeta potential of HOP/HLP/HDP micelles

Hydrophilic outer shell of polymeric micelles plays an important part in the size of the micelles. So, when the length of alkyl chains remains consistent, the size of the micelles gets bigger as the molecular weight of the LMHA gets larger. Meanwhile, the DS_a and the length of alkyl chains also affect the size of the micelles: as the DS_a increases (i) the inner core of the aggregates with hydrophobic chain association gets more and more compact, (ii) more amphiphilic micelles get to the surface of the solution, and (iii) thereby smaller-sized micelles form in the solution. On the other hand, when the length of alkyl chains increases, the size turns smaller to bigger. The length of octyl-chains is short, which may weaken the intermolecular self-association. So, the size of the micelles formed by hydrogen bonds is larger than any other ones (Fig. 4-1).

The octadecyl-modifed micelles were bigger than lauryl-modified ones (Table 1). This is because the DS of octadecyl-modified micelles is lower than the lauryl-modified ones. So, we can conclude that DS may play a more important role in the size of the micelles than the length of alkyl chains.

As we all know, native HA bears negative charge when it is soluble in water and the surface charge was −14 mV, but hydrophobically modified HAD micelles bearing a positive surface charge ($|\xi| > 20$ mV) (Fig. 4-2) are believed to efficiently condense and then encapsulate the nucleic acids into a nanoparticle that can be internalized by cells and that can facilitate uptake by negatively charged cell membranes (Grayson et al., 2006a, 2006b), which is due to the spermine side chain condensation. One hundred and fifty nanometer is within the acceptable size limit for cellular uptake by non-specific endocytosis (Bishop, 1997), thus aggregates in excess of this size may be excluded from cellular internalization altogether (Oupicky, Konak, Ulbrich, Wolfert, & Seymour, 2000). So, we choose HLP_{9600} for gene delivery.

3.4. CMC determination of polymeric micelles

The amphiphilic polymers consisting of hydrophilic and hydrophobic segments can self-associate to form core/shell structure in aqueous solutions. The formation of core/shell micelles was examined by the detection of CMC values using a fluorescence technique, where pyrene was chosen as a fluorescent probe. The gradual shift of the third peak in the excitation spectra of pyrene from 333 to 338 nm was observed (data not shown), indicating the change in vibration structure of pyrene emission. Fig. 5 shows the intensity ratio I338/I333 of the pyrene excitation spectra versus the logarithm of the HOP/HLP/HDP micelles concentration. At low concentrations, the intensity ratio of I338/I333 changed slightly. However, as polymer concentration increased, the intensity ratio increased sharply, indicating the partitioning of pyrene into the hydrophobic core of the micelles.

As shown in Table 1, the CMC values of micelles with different lengths of hydrophobic chains in DI water were about 40–140 mg/L, respectively. The low CMC value indicates that the core/shell nanoparticles can be formed at low concentrations because of

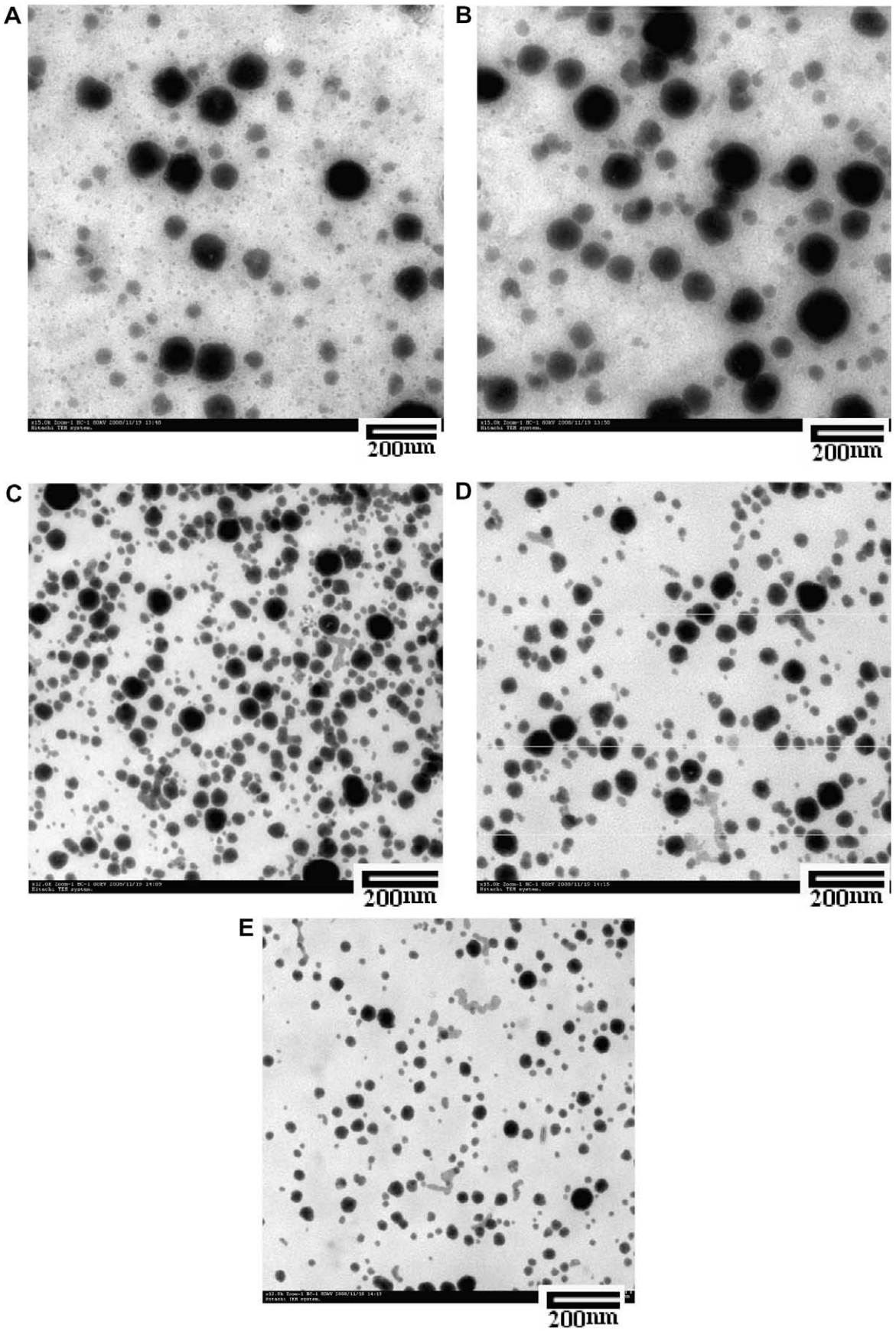

Fig. 3. The TEM image of blank micelles (A) HLP_{9600}; (B) $HLP_{23,000}$; (C) $HLP_{45,000}$; (D) HOP_{9600} and (E) HDP_{9600} in deionized water.

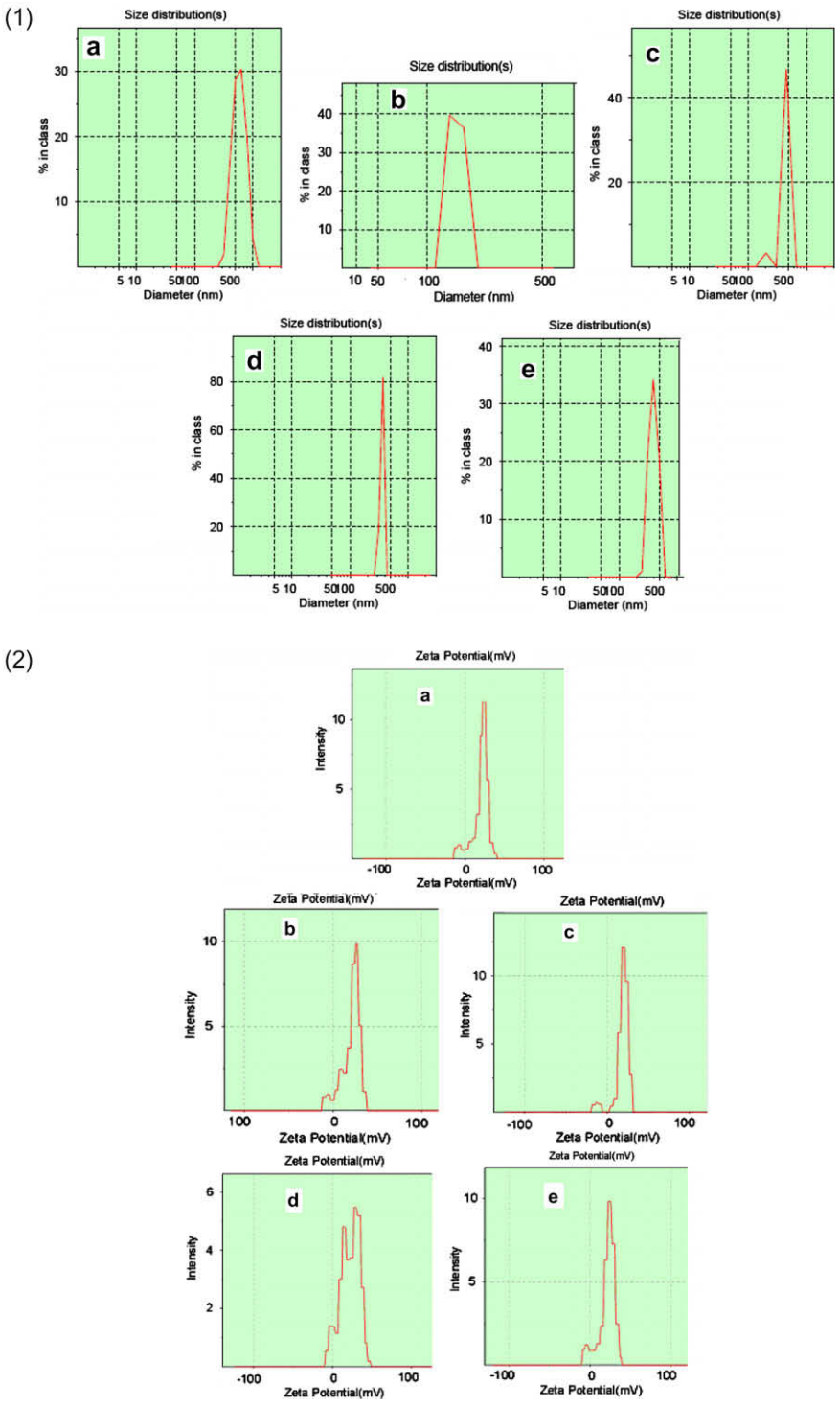

Fig. 4. (1) Size distribution of (a) HLP_{9600}; (b) $HLP_{23,000}$; (c) $HLP_{45,000}$; (d) HOP_{9600} and (e) HDP_{9600} measured by using dynamic light scattering. (2) Zeta potential of (a) HLP_{9600}; (b) $HLP_{23,000}$; (c) $HLP_{45,000}$; (d) HOP_{9600} and (e) HDP_{9600}.

the strong hydrophobicity of fatty amines allowing their use in very dilute media, such as body fluids.

3.5. Surface tension

Fig. 6 shows the surface tension of different conjugate dispersions over the concentration of 0–1.0 mg/ml. The ability to decrease the surface tension was brought about by the equilibrium of its surface-active analytes comprising both hydrophobic and hydrophilic structural regions. Thus, in aqueous solution, these species diffused towards the air/liquid interface and were preferentially adsorbed at the surface, thereby lowering the surface tension of the solutions (Yang, Zhu, Zheng, Ge, & Zhang, 2006). As it can be seen from the surface tension plateau values, the conjugates with different chains have exerted a surface activity with the critical micelle concentration (c.m.c.). As it can be seen from the sur-

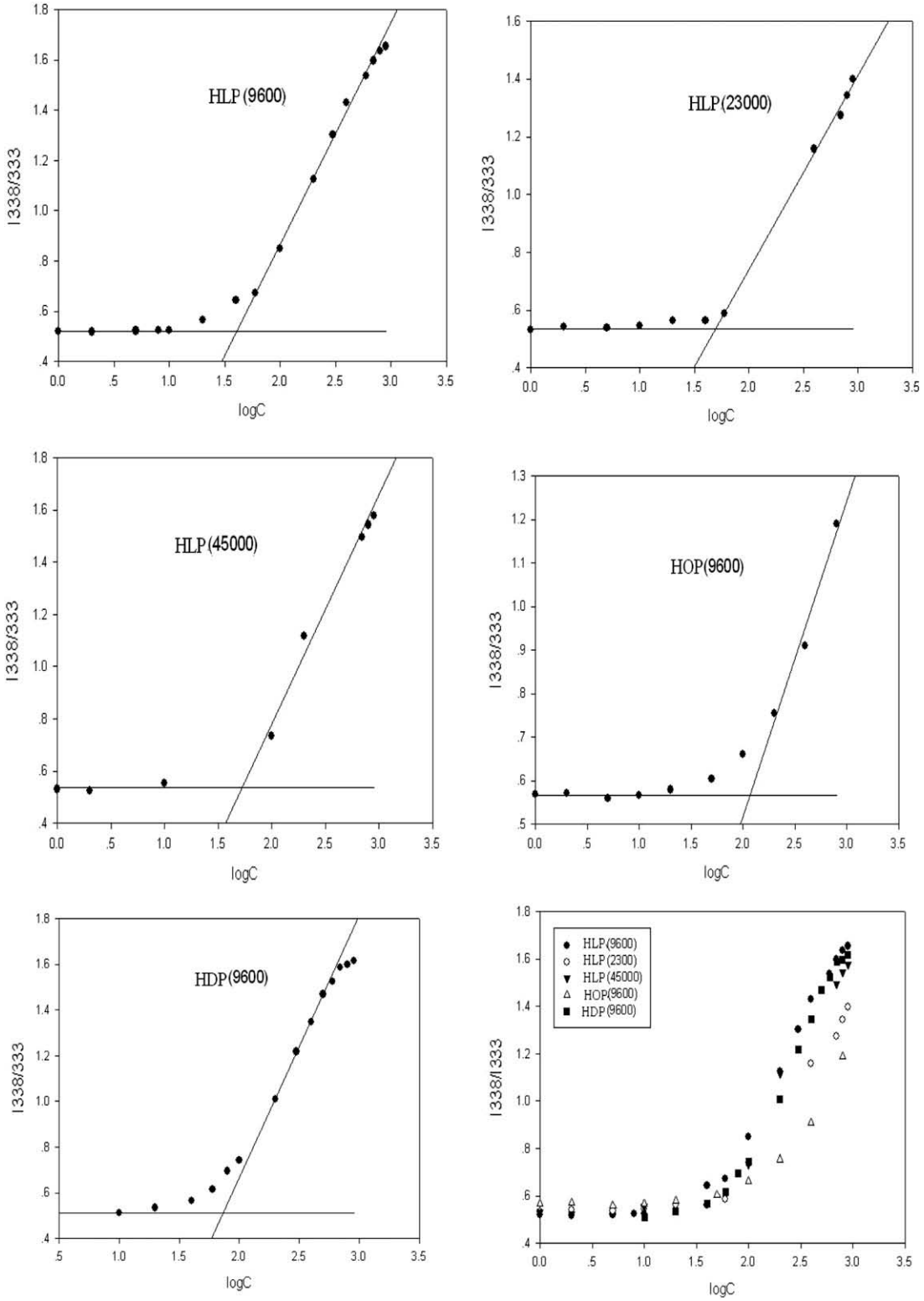

Fig. 5. Plots of CMCs as a function of the LMHA micelles concentration for (●) HLP_{9600}, (○) $HLP_{23,000}$, (▼) $HLP_{45,000}$, (△) HOP_{9600}, (■) HDP_{9600} at 25 °C.

face tension plateau values, the ability to decrease the surface tension for polymeric micelles has not much large variation to the different chain lengths, which is due to their high tension of chemical bonds and mutual repulsions of comparatively large hydrophilic groups. Thus, in aqueous solution, these hydrophobic chains cannot get close together, giving a difficulty to align on the water surface, so the variation to decrease surface tension was not distinguishing.

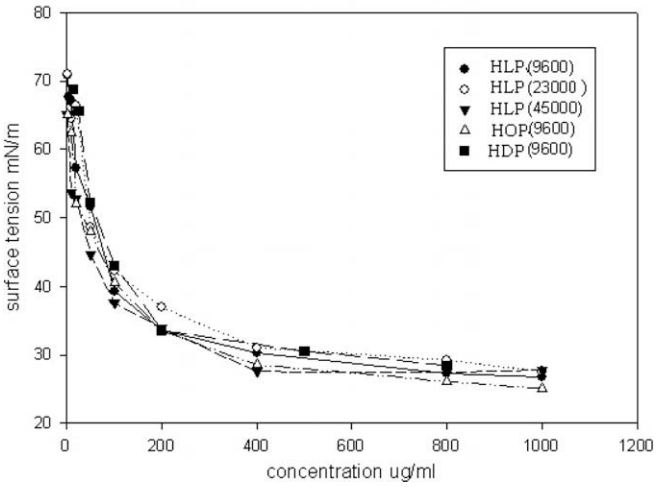

Fig. 6. Plots of surface tension as a function of the LMHA micelles concentration for (●) HLP_{9600}, (○) $HLP_{23,000}$, (▼) $HLP_{45,000}$, (△) HOP_{9600}, (■) HDP_{9600} at 20 °C.

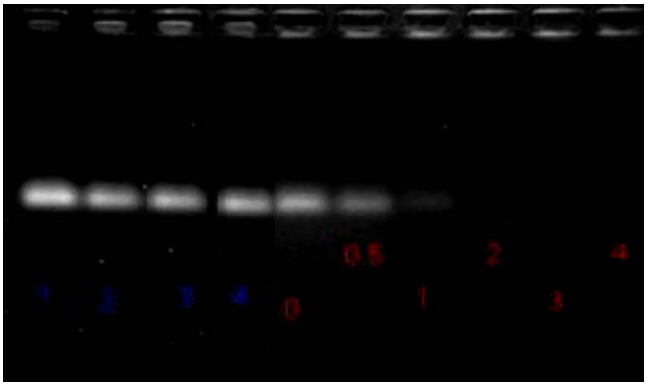

Fig. 7. Agarose gel electrophoresis of siRNA/HLP_{9600} micelles. (Lanes 1–4: the LMHA:siRNA = 1, 2, 3, 4; lanes 5–9: the HLP_{9600}:siRNA = 0, 0.5, 1, 2, 3, 4.)

3.6. Gel retardation assay

As shown in Fig. 7, native HA provided very weak siRNA binding ability (the blue marked lanes 1–4). The red marked lanes show the siRNA binding ability of HLP_{9600}. As it is shown, when weight ratio was 2:1 (w/w), the HLP_{9600} micelles could bind the siRNA effectively. Also, the HLP_{9600} micelles exhibited strong encapsulating ability which attributed to the spermine side chain condensation, thus cause complete retardation of siRNA, which was observed at siRNA/HLP_{9600} ratio of 2:1 (w/w).

Based on these results, it can be hypothesized that the HLP_{9600} micelles might be useful as a drug carrier because they can form self-assembled micelles bearing hydrophobic inner core which encapsulated the water-soluble genes such as siRNAs due to its strong gene binding and encapsulating abilities. Along this line of research, applications of HLP_{9600} micelles as a targeted gene carrier are currently under investigation.

4. Conclusion

A novel type of polymeric amphiphiles HOP/HLP/HDP micelles was prepared and characterized. The resulting modified conjugates could form self-assembled micelles with a size of 125–555 nm, which was significantly dependent on the DS_a and the chain length of the hydrophobic groups. The micelles were able to form at highly diluted concentrations as low as 40–140 mg/l, and the zeta potential of the HOP/HLP/HDP micelles was about +20 mV which might provide their potential for biomedical applications.

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