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Synthesis and characterization of novel water-soluble polyamide based on spermine and aspartic acid as a potential gene delivery vehicle

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Abstract. We developed a novel and convenient method for the synthesis of a potentially safe non-viral gene delivery vehicle based on the cationic block copolymer of spermine and aspartic acid (ASSP) and coupled it with polyethylene glycol (PEG). The copolymer ASSP was prepared by direct polycondensation in the ionic liquid, butylmethylimidazolium hexafluorophosphate, using triphenyl phosphite as the condensing agent under mild reaction conditions. The highly hydrophobic ASSP was transformed into a water soluble hydrophilic micelle by coupling ASSP with polyethylene glycol (PEG) using the same ionic liquid and 1,1-carbonyl diimidazole as the condensing agent without harsh conditions. The polycationic ASSP-PEG was then used to condense calf thymus and plasmid deoxyribonuclceic acids (DNAs) in Tris-HCl buffer (pH 7.4) to get a series of block ionomer complexes with various charge ratios. The physicochemical properties of the copolymer micelle and the DNA polyplexes were studied using fourier transform-infrared (FTIR), nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy, matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), differential scanning calorimetry (DSC), transmission electron microscopy (TEM) and particle size measurements. It was observed that the DNA was condensed to compact particles by its interaction with the copolymer. Since DNA condensation to nano/micrometer sized particles is essential for gene delivery, our results indicate a potential use of the copolymer for gene delivery applications.

Keywords: *nanomaterials, gene delivery vector, water-soluble polyamide, DNA polyplexes, synthesis*

1. Introduction

The basic concept of gene therapy involves the treatment of human diseases by transferring genetic material to specific cell types in order to correct or supplement defective genes responsible for disease development [1]. Progress in the clinical development of this approach has been hampered by the inefficient transport of plasmid DNA/oligonucleotides through the cell membrane. Therefore, the success of gene therapy is largely dependent on the development of efficient gene delivery vehicles. There are two types of carriers used in experimental gene therapy protocols, viral and non-viral vectors, both of which present specific advantages and disadvantages [2]. The search for non-viral vectors began when viral vectors met with serious draw-

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backs such as high risk of mutagenicity, immunogenicity, low production yield, and limited ability to carry long gene sequences [3]. Several approaches have been tested in order to circumvent problems associated with each type of non-viral gene delivery vehicles [4, 5]. The use of polymeric materials as delivery vehicles has been well established and widely used to improve therapeutic potential of peptides, proteins, small molecules and oligonucleotides [6–19].

The cationic molecules which are currently under development as gene delivery vehicles are organic cations, including cationic lipids, polyamine-based polymers, chitosan based polymers, dendrimers, and polyethyleneimine (PEI) [1, 3, 4, 7–10, 13, 16, 20–24]. The spontaneous formation of polyplexes by the interaction of negatively charged phosphate groups of DNA/oligonucleotides and positively charged polymers under physiological salt conditions and the successful transport of these polyplexes to cells has been demonstrated [25–33]. Several investigators have reported encouraging results on spermine and its chemical analogues DNA condensing agents for gene therapy applications [34–38]. Since DNA molecules condensed with low-molecular weight cations are susceptible to aggregation under physiological conditions [39], advanced polymeric gene delivery systems employ macromolecules, with high cationic charge density, that can protect the DNA from degradation [40]. So, this has necessitated attempts towards modification of spermine with a view to developing high molecular weight copolymers [40–43].

Jere *et al.* have reported synthesis of a poly(β-amino ester) of spermine and poly(ethylene glycol) (PEG), which showed higher degree of safety and transfection efficiency in comparison to polyethyleneimine, when studied in 293T human kidney carcinoma cells [40]. On the other hand, Vinogradov *et al.* [41] reported that poly(ethylene glycol)-spermine complexes are less stable in the presence of low molecular weight electrolytes compared to the PEG-PEI complexes. Domb and coworkers [42, 43] showed that dextran grafted spermine improved cell transfection compared to the unmodified one. Kanatani *et al.* [44] showed that pullulan-spermine-mediated transfection of plasmid DNA resulted in greatly reduced cytotoxicity and a 10-fold increase in the level of gene expression when compared to lipofectamine 2000, a commercially available cationic lipid. A copolymer of spermine with amino acids such as aspartic acid and glutamic acid might present a number of advantages, including biocompatibility and biodegradability. The loss of charge density on copolymerization might be compensated by the presence of the additional amino group which could be protected during polymerization. Coupling the copolymer with hydrophilic compounds, such as PEG, might reduce non-specific interaction of the copolymer with blood components as well as make it water soluble. The goal of the present work was to synthesize and characterize a novel cationic polymer based on the naturally occurring polyamine, spermine and aspartic acid, using an ionic liquid and triphenyl phosphite as solvent and condensing agent, respectively. Since ionic liquids have negligible vapor pressure, they are attractive agents for use as 'green' reaction media and can potentially replace conventional organic solvents in synthesis and other applications.

2. Experimental

2.1. Materials

Plasmid DNA, spermine, methyl imidazole HPF6 and chlorobutane (Sigma Aldrich) N-tert-butyloxycarbonyl (Boc)-L-aspartic acid, triphenyl phosphate, 1,1-carbonyl diimidazole, polyethylene glycol metyl ether (MW = 2000) (Aldrich) were used as received. Calf thymus DNA (molecular weight = $6 \cdot 10^6$) was obtained from Worthington Biochemical Corporation, USA. Plasmid pEGFP (3.4 kilo base pair length), which has the early promoter of CMV and enhanced green fluorescence protein (EGFP) gene was obtained from Clontech.

2.2. Preparation of ionic liquids

The ionic liquid butylmethylimidazolium hexafluorophosphate were synthesized according to the literature [45].

2.3. Copolymerization of Boc-Aspartic acid and spermine (Boc-ASSP)

Boc-ASSP was prepared by stirring N-Boc-L-aspartic acid (0.001 mol), spermine 4HCl (0.001 mol), and 3.2 g of ionic liquid at room temperature for 10 min, and 0.52 ml (0.00225 mol) of triphenyl

phosphite was added. The temperature was gradually elevated to 100°C under an inert gas atmosphere, the reaction mixture was held at this temperature, and stirring for 2.5 h. Boc-ASSP was isolated by precipitation in excess methanol. Polymer was thoroughly washed with methanol and dried in vacuum at 70°C.

2.4. Deprotection of N-BOC fron BOC-ASSP

Manual removal of the N-terminal Boc group can be accomplished by placing the resin in a RB flask and washing with 50% [v/v] trifluoroacetic acid (TFA)/dichloromethane (DCM) for 15 min at room temperature with constant stirring. After 15 min, the mixture was extracted with ethyl acetate and washed with sodium bicarbonate solution and then with saturated sodium chloride solution. The product was the precipitated with methanol.

2.5. Synthesis of PEG–ASPSP

A 1 mmole portion of polyethylene glycol methyl ether (MW = 2000) was dissolved in 5 ml of ionic liquid and reacted with 1 mmole of 1,1-carbonyl diimidazole at 20°C for 3 hours. This reaction modifies the terminal hydroxyl group of polyethylene glycol methyl ether. The reaction system was then supplemented with 1mmole of ASSP and the mixture incubated at 20°C for 16 hours. The product was then precipitated in DCM and the ionic liquid is recovered by evaporating the DCM.

2.6. Characterization of ASSP-PEG

NMR spectra of the compound were recorded using 300-MHz Brucker NMR spectrophotometer in D_2O. Infrared spectra of the polymers were recorded using an IR prestige-21 FT-IR Shimadzu spectrophotometer in the range of 4000–400 cm^{-1}. The thermal stability of the polymers was determined using DTG-60 Shimadzu thermo- gravimetric analyzer at a heating rate of 10°C/min in nitrogen. The thermal transitions of the polymers were determined using a Perkin-Elmer DSC-7 differential scanning calorimeter under nitrogen purge. The samples were heated at a rate of 10°C/min. The MALDI TOF MS were run using a Micro mass TofSpec 2E instrument using a nitrogen 337 nm laser (4 ns pulse). At least 40–50 shots are summed up. The matrix used is 2,5-dihydroxy benzoic acid dissolved in water. The sample ASSP is dissolved in water and the matrix spotted in MALDI target and allowed to dry before introducing into the mass spectrometer. The solution of the polymer ASSP and ASSP-PEG in water was prepared in a concentration of 1 mg/1 ml and the primary amine content was determined according to the procedure described elsewhere [45].

2.7. DNA-ASPSP-PEG complex formation

A solution of the polymer ASPSP-PEG in Tris-HCl buffer (pH 7.4) was prepared at a concentration of 1 μg/μl. Calf thymus and Plasmid DNA solutions were also prepared in the same buffer in at a concentration of ~1 μg/μl. A series of block ionomer complexes (BIC) at various charge ratios were prepared by mixing ASPSP-PEG solution with a DNA solution. The mixture was mixed by vortexing for 2–3 min and incubated for 30 min. at room temperature for completing the complex formation. It was noted that during complex preparation, the volume of DNA was kept constant and volume of polymer solution was adjusted for the theoretical charge ratio. The dispersive stability of the complexes was evaluated by turbidity measurement at 500 nm using UV-Vis spectrophotometer (PerkinElmer-Lamda-35).

2.8. Particle size measurement

The polyplexes were prepared at increasing weight ratios from 1:0.25 to 1:1.5 (DNA/Polymer) in Tris-HCl buffer. Ten microgram plasmid DNA was used and the final volume was set to 2 ml with H_2O. A helium–neon lamp was used as the light source (10 mW) on a Malvern Zetasizer 3000HAs system (Malvern Instruments Ltd, Worcestershire, U.K.). Values were obtained 3 cycles per 10 time measurements.

2.9. Circular Dichroism (CD)

For CD measurement, the chitoplex solutions with weight ratio from 1:0.25 to 1:1.5 (DNA/Polymer) were prepared using ASSP-PEG in terms of the method described above. CD spectra were collected with a 1 cm path length cuvette using J-810 spectropolarimeter in a range of wavelengths of 350–

200 nm at 25°C. The spectra were corrected by subtracting the background of sodium acetate/ acetic acid buffer

2.10. Transmission electron microscopy (TEM)

Transmission electron microscope (TEM) images were recorded using a Hitachi H-600 instrument at 75 kV. For TEM measurements a drop of the polyplex solution in the weight ratio of 1:1 (10 μg in 1 ml of trisbuffer) were deposited directly of Formvar coated copper grid.

3. Results and discussion

The present study was aimed to design and evaluate a novel, potentially safe non-viral gene delivery vector based on the cationic copolymer of spermine and aspartic acid using butylmethylimidazolium hexafluorophosphate ionic liquid as the medium [45]. Vygodskii *et al*. [46] have shown that 1,3-dialkylimidazolium based ionic liquids (IL) are suitable reaction and activating media for the synthesis of high molecular weight polymers, including polyamides. Low temperature solution polycondensation of diamines and diacid chlorides was used for preparing polyamides. The polyamide synthesis was carried out using Boc-aspartic acid and spermine under mild conditions. N-Boc protection was removed by washing the polymer with 50% [v/v] TFA/DCM. As the copolymer (ASSP) was found to be highly hydrophobic and insoluble in many of the organic solvents, it was coupled with PEG, using the same ionic liquid as solvent and 1,1-carbonyl diimidazole as condensing agent (see Figure 1), to make it hydrophilic and water soluble. A commercial PEG polymer with one end capped by a methoxy group was used for this purpose. The polymers thus obtained (BASSP, ASSP and ASSP-PEG) were structurally characterized by means of FTIR spectroscopy (Figure 2). The vanishing of the peak at 645 cm^{-1} and enhanced visibility of the peaks of primary amino group at 3440 cm^{-1} indicate the effective removal of Boc group from BASSP. The strong peaks at 843 cm^{-1} may be due to the N–H deformation vibrations of aliphatic primary amines. The peak at 1596 cm^{-1} is due to the asymmetric NH_3^+ deformation vibration. In addition, the weakening of the carbonyl peaks at 1726 and 1661 cm^{-1} might be due to the coupling of PEG at the acid groups the polymer other than amino group. Therefore, the amino groups are free for undergoing electrostatic interaction between the negatively charged phosphate groups of DNA [47]. We next determined the thermal characteristics of the copolymers using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). It can be seen from Table 1 that the polymer ASSP has the lowest thermal stability due possibly to the presence of the chain terminal groups (–COOH and $–NH_2$) in comparison to that of BASS having the thermally stable Boc protecting group. In contrast, ASSP-PEG has stable chain terminal groups ($–OCH_3$) leading to a higher thermal stability. Therefore, the observed differences in the

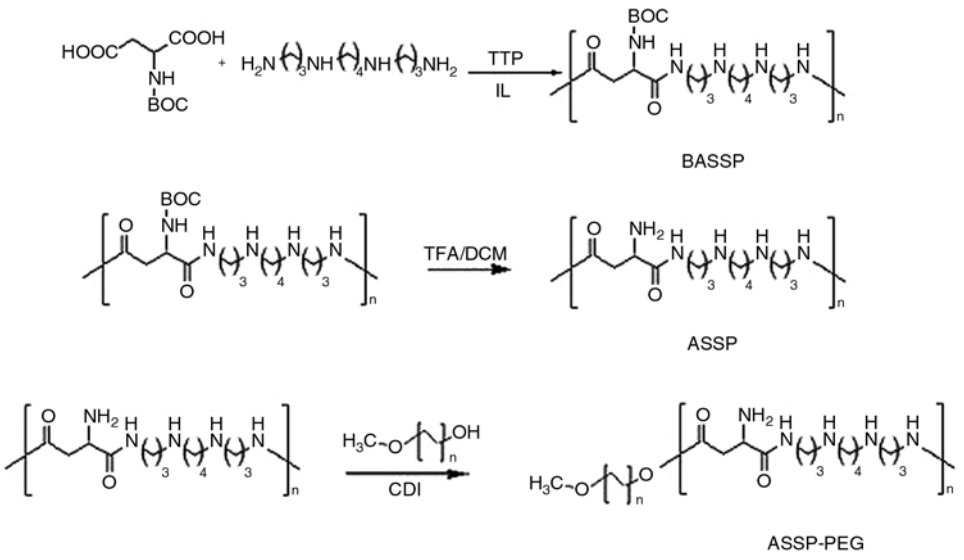

Figure 1. The chemical structure of polymers BASSP, ASSP, ASSP-PEG

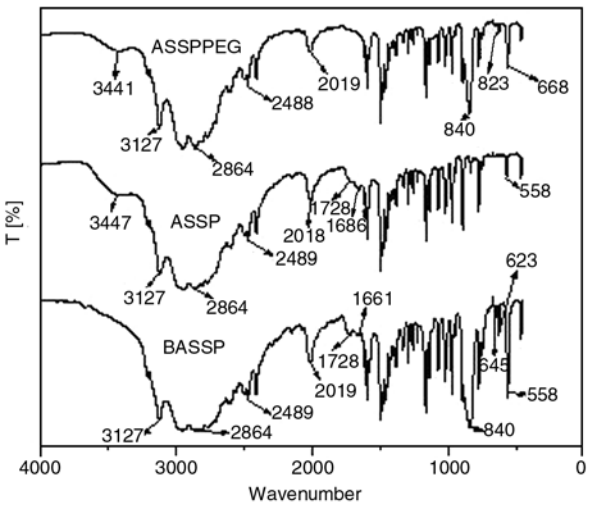

Figure 2. FTIR of polymers

Table-1. TGA and DSC results of the polymers

Polymer	10% wt loss T [°C]	T_g [°C]	ΔH [J/g]
BASSP	276	157	21
ASSP	234	179	21
ASSP-PEG	276	175	21

thermal stabilities of the synthesized polymer are consistent with their structures. The glass transition temperature and heat of transition of these polymers were determined from DSC data. Table 1 shows that ASSP-PEG exhibited a T_g value between those of ASSP and BASSP.

ASSP-PEG was soluble in water at a concentration of 1 µg/1 µl and the primary amine content was determined using TNBS (Trinitro benzene sulphonic acid) method (Table 2, Figure 3). It was observed that the number of primary amino group of ASSP-PEG was higher than that of ASSP. It may be due to the higher solubility of the polymer in aqueous medium after PEGylation [48]. This result further confirms that the PEG is coupled to the acid end group of the polymer, and not to the amino group. The molecular weight of the polymer was above 10 kDa as obtained from measurements by MALDI TOF MS.

A successful gene carrier is one which can effectively condense negatively charged DNA into nanosized particles [1, 2, 5–7, 47]. The condensation behaviors were studied by using IR spectroscopy, CD spectra, turbidity measurements, particle size analyzer and TEM. The IR spectrum the complex of the copolymer with calf thymus DNA (Figure 4) reveals that as the concentration of polymer increases the intensity of peaks at 1024 cm^{-1} due to P–O–H stretching vibration goes on decreasing and at the charge ratio of P/N = 1.5, the peaks are almost vanished. Similar observation was also found in the case of N–H stretching vibrations of purines and pyrimidines also. It indicates that as the concentration of polymer increases, there are no more negative centers on DNA.

The effect of the condensation of the copolymer with DNA on the secondary structures of DNA was evaluated by CD measurement. It can be seen from Figure 5 that the pure DNA appears in a typical

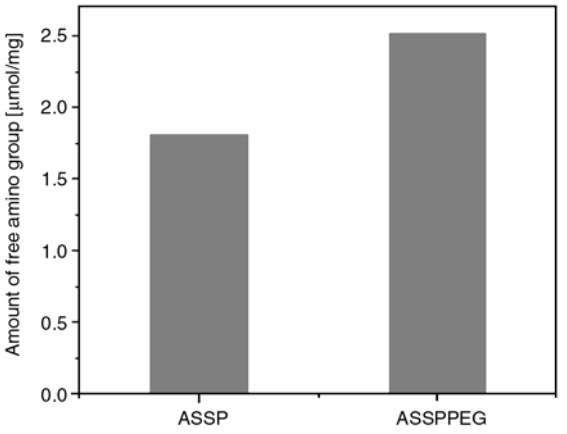

Figure 3. Number of primary amino group in µmol/mg

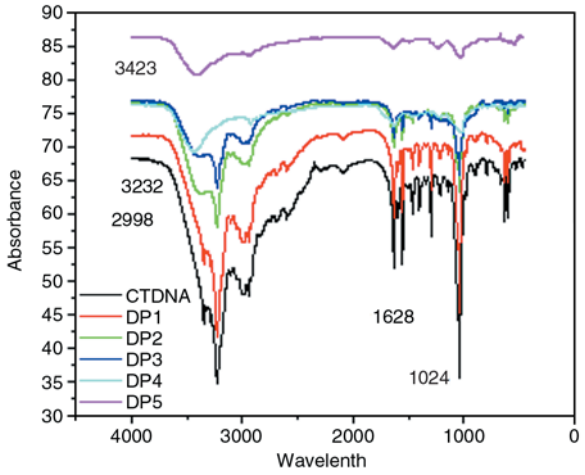

Figure 4. FTIR spectra of ASSP-PEG/CTDNA complexes

Table 2. Amount of primary amino group of the polymers

Polymer	Absorbance at 410 nm	Corresponding conc. of spermine	Corresponding no. of moles of spermine	No. of 1° NH_3 group/µl
ASSP	0.1098	91.27	$2.62 \cdot 10^{-7}$	$1.805 \cdot 10^{15}$
ASSP-PEG	0.1271	111.59	$3.20 \cdot 10^{-7}$	$2.510 \cdot 10^{15}$

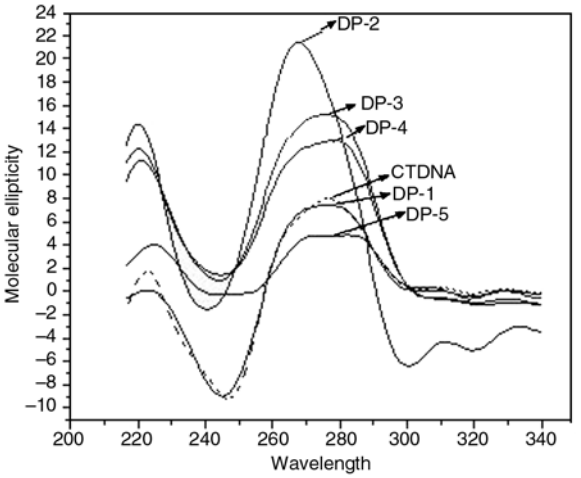

Figure 5. CD spectra of ASSP-PEG/CTDNA complexes

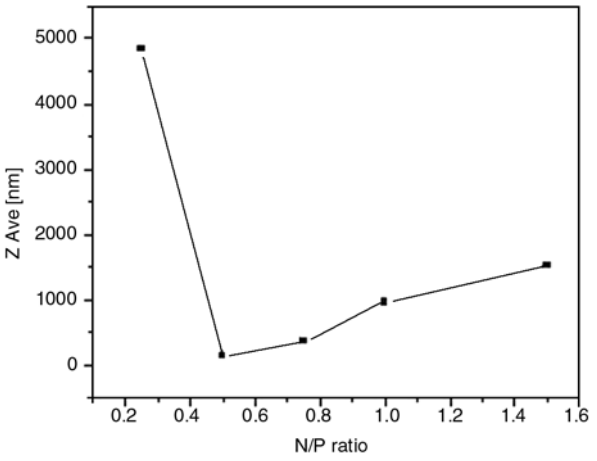

Figure 7. Polyplex sizes as CTDNA/ASSP-PEG complexes

B conformation with approximately equal positive (275 nm) and negative (245 nm) components centered on 258 nm. While plain spermine copolymer does not show any characteristic pattern, its complexes with DNA show changes in the intensity of peaks indicating electrostatic interaction with the negatively charged phosphate groups of DNA [49], but the 'Cotton effect' remains nearly unchanged, demonstrating that DNA remains in B conformation upon complexing with ASSP-PEG polymer.

The dispersive stability of ASSP-PEG polymer –DNA complex was measured using turbidity measurements in which the increase in turbidity indicates self-aggregation. Figure 6 shows that at P/N = 0.5 the turbidity was found to be the least and on increasing the charge ratio of the polymer, turbidity goes on increasing. And at the ratio 1:2 turbidity is found to be decreased without precipitation indicating enhanced stability.

The formation of the nanoparticles was confirmed by measurement of particle size and TEM. The polyplexes were prepared at increasing weight ratios from 1:0.25 to 1:1.5 (DNA/Polymer) in Tris-HCl buffer. Figure 7 shows that the formation of the complex is inversely related to the charge ratio. As the charge ratio increases, the size decrease sharply and becomes a minimum value of 142.5 nm at charge ratio of 0.75 (P/N = 0.75). The charge density and the copolymer structure have remarkable effects on the DNA condensation and size of the nanoparticles that are formed [5].

Figure 8 exhibits TEM the images of the particles of plasmid DNA in the charge ratio of P/N = 1. Toroid like structure with a core-shell arrangement indicates the formation of the DNA-chitosan nanoparticle. The particle size value observed in the TEM micrographs was in the same magnitude as observed by the particle size analyzer. Thus, our

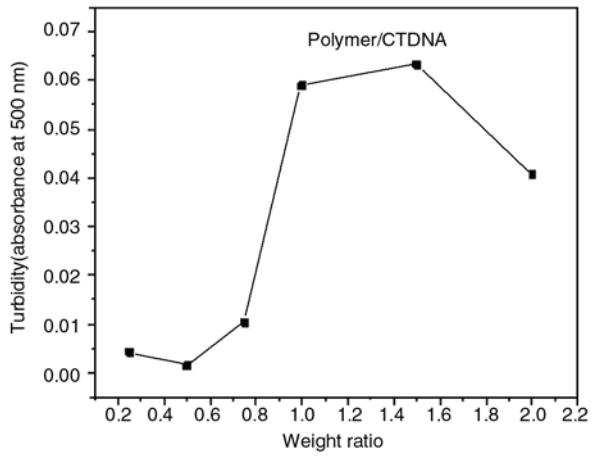

Figure 6. Turbidity curve of CTDNA/ASSP-PEG complexes

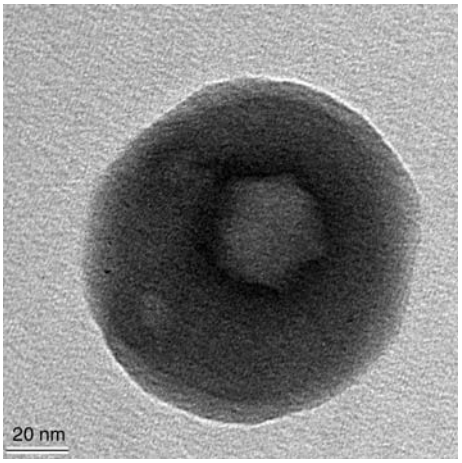

Figure 8. Electron microscope image of PLDNA/ASSP-PEG

data indicate that the copolymer ASSP-PEG condenses with DNA to form nanoparticles in the size range reported for safe transfection. The feasibility in gene transfection by spermine conjugates and derivatives has been established by a number of researchers [40–44, 50, 51]. The copolymerization of spermine with aspartic acid followed by coupling with PEG might be expected to substantially improve the low transfection efficiency of spermine [6]. This strategy also reduces cytotoxicity associated high cationic charge of spermine moieties [5]. The incorporation of PEG in gene vectors in various creative capacities is a widely used strategy for improving the polyplex solubility and reduce polyplex aggregation and prolong the circulation time [2, 5, 7, 12, 28, 48]. Both spermine and aspartic acid, being natural materials are biodegradable and its copolymer with an aliphatic amide bonding is expected to be also biodegradable [50].

4. Conclusions

A novel and convenient method for the synthesis of a potentially safe non-viral gene delivery vehicle based on the cationic block copolymer of spermine and aspartic acid (ASSP) coupled with polyethylene glycol (PEG) was developed. The polycationic ASSP-PEG was found to condense with calf thymus and plasmid deoxyribonuclceic acids (DNAs) in Tris-HCl buffer (pH 7.4) to give a series of block ionomer complexes with various charge ratios. Since DNA condensation to nano/micrometer sized particles are essential for gene delivery, our results indicate a potential use of the copolymer in gene delivery applications.

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