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Random branched poly(hydroxyetheramine): a novel polycation with proton sponge effect and high density of discrete charge

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Summary

A random branched poly(hydroxyetheramine) was synthesized by ring-opening reaction of tetraethylenepentamine (TEPA) to ethylenediglycidol (EG), which was denoted as PTEG. PTEG has not only high charge density but also discrete charge distribution due to the introduction of hydrophilic hydroxyoligoether. In addition, PTEG contains all grades of protonable amines. Acid base titration indicated that PTEG was protonated in wide pH range from 5.0 to 11.0, which showed that PTEG had proton sponge effect as polyethylenimine and protonation ability at human physiological pH condition. In dilute aqueous solution, PTEG was complexed with weak anionic polymer, poly(acrylic acid) (PAA), into nanoparticles.

Introduction

Interpolymer complex is such a kind of hybrid as achieved through specific interactions such as electrostatic interactions, hydrogen bonding and hydrophobic interactions etc [1,2]. The interpolymer complex is of significant interest due to its unique characteristics that are different from those of individual components and has found wide applications in industries and medicine [3-5]. As one kind of interpolymer complex, polyelectrolyte complex is formed under the electrostatic interaction of oppositely charged polyelectrolytes in solution [6]. Polyelectrolyte complexes were developed for the wide use in industrial and biological fields [7-12]. For example, negatively charged biologic component, DNA, is complexed with polycation into nanoparticle, which provides a promising way of transferring exogenous therapeutic DNA into cell to realize gene therapy [13]. As gene vector, polycation prevents DNA from degrading and facilitates the transfer of DNA in body and cell. Due to the easy preparation and versatile modification, large loading capacity and negligible immunogenicity, polycation receives great study interests as gene vector [14-17].
Polycation's characteristics such as charge density, protonation range and nitrogen profile exert significant effect on the formation and characteristics of complex. Charge density is related to whether compact or loose complex particle is obtained. Broad protonation range and nitrogen grade profile contribute to transfer in cell because they endow polycation with proton sponge effect. Polyethylenimine is one of the most efficient and popular polymeric gene vectors due to the highest charge density and

unique proton sponge effect [18-21]. However, in the case of polyethylenimine, excessively high charge density results in serious cytotoxicity, and further decreases transfection efficiency. In order to solve this problem, long hydrophilic chains were ever conjugated to polyethylenimine to shield charge and increase biocompatibility. This way decreased cytotoxicity but decreased simultaneously transfection efficiency due to the shielding for charge [22-24]. Compared to above method, chemically linking low molecular weight polyethylenimine by neutral unit was a desirable approach [25, 26]. Most outstandingly, Li et al reported a family of artistic supramolecular polycations in which oligomeric polyethylenimine derived cyclodextrins were threaded on poly(ethylene glycol). The supramolecular polycation with optimal structure showed low cytotoxicity and high transfection efficiency [27].
In modified polyethylenimines, acetylated polyethylenimine is very notable to mention. Gene delivery efficiency of 25 kDa branched polyethylenimine increased upon acetylation of up to 43% of the primary amines with acetic anhydride due to easy release and decreased cytotoxicity resulting from decreased charge density [28]. This shows that high charge density facilitates complexation and protection of DNA, but overly high charge density may compromise transfection efficiency.
Absence of secure and effective gene vector is a bottleneck for gene therapy into clinical application [29]. So it is necessary for novel polycation with optimized structure to be synthesized and introduced into gene therapy. In terms of polycation synthesis, an idea of polycation with high density of discrete charge was put forward ever [30, 31]. Under the drive of this idea, we synthesized a novel random branched poly(hydroxyetheramine), PTEG. In terms of molecular structure, PTEG has four outstanding characteristics, non-symmetric branched structure similar to fractured dendritic poly(amidoamine), broad nitrogen grade profile comprising primary, secondary and tertiary amines, a lot of short hydrophilic hydroxy groups and slightly lower charge density than polyethylenimine due to the introduction of not charged structure unit. The polyelectrolyte complex of PTEG was prepared by choosing PAA as a model polyanion.

Experimental

Materials

Ethylene glycol and epichlorohydrin were dried over 4A molecular sieve and distilled under vacuum prior to use. Tetrabutylammonium bromide was washed with ethyl ether and dried at 50°C under vacuum for 10 h. EG was synthesized from ethylene glycol and epichlorohydrin under catalysis of NaOH and tetrabutylammonium bromide. Acrylic acid was distilled under vacuum over cuprous chloride. Potassium peroxydisulfate was recrystallized from water. Dichloromethane was washed with NaOH solution, dried with anhydrous magnesium sulfate and distilled. TEPA was dried with NaOH followed by vacuum distillation. Other reagents were used without further purification. PAA was synthesized by aqueous free radical polymerization under catalysis of potassium peroxydisulfate. The Mw and PDI of PAA are 5.4×10^4 and 1.94 separatively by aqueous GPC.

Synthesis of PTEG

TEPA (1.87 g) and equimolar EG (1.72 g) were dissolved in 20 mL dichloromethane and reacted in 50°C oil bath. After 24 h, 20 mL ethanol was added to make reaction

mixture homogenous. The reaction proceeded at 50°C for 24 h followed by distillation of dichloromethane and reaction at 80°C for another 24 h. Ethanol was partly removed by distillation and concentrated solution was added with ethyl ether to precipitate PTEG under drastic stirring. The product was washed with ethyl ether three times and dried under vacuum at 80°C.

Complexation of PTEG with PAA

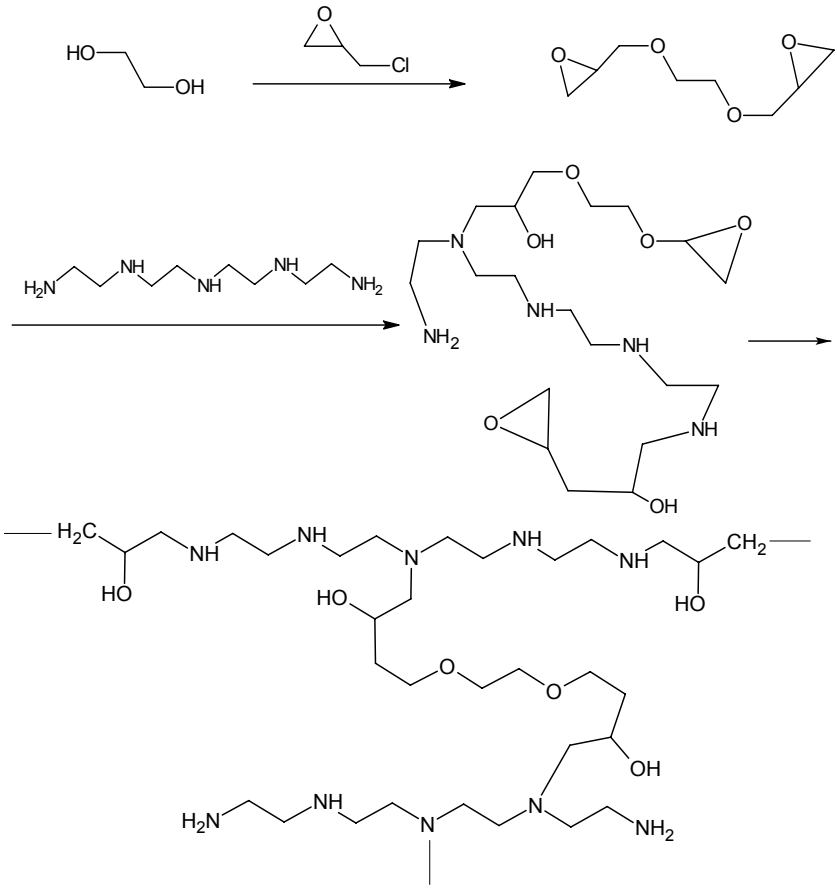
0.68 g/L PTEG and 0.73 g/L PAA aqueous solutions were prepared respectively by dissolving polymer in appropriate amount of deionized water. Varied amounts of PTEG solution were added slowly to 30 mL PAA solution under gentle votexing. After addition, the mixture was votexed for 5 min. The mass ratio of PTEG to PAA was represented as θ. The θ values studied here were 0.3, 0.4, 0.5, 0.8 and 1.0 . Complex solutions were left at room temperature and at certain intervals aliquots of supernatant liquid were withdrawn to determine complex stability by UV-Vis spectroscopy.

Measurements

^{1}H NMR spectra were recorded on Brucker Avance DPX 300 NMR spectrometer (Bruker, Germany) using D_2O as solvent for PTEG, $CDCl_3$ for EG. Protonation characteristic of PTEG was determined by acid-base titration. 0.5 g PTEG was dissolved in 10 mL 150 mmol/L NaCl solution and then added dropwise with 0.58 mL 37% HCl aqueous solution to protonating all amino groups of PTEG under ice cooling and continuous stirring. The obtained PTEG chloride solution was titrated with 0.3 mol/L NaOH aqueous solution. The titrations of 10mL 150 mmol/L NaCl solution and 10 mL 0.69 mol/L HCl solution containing 150 mmol/L NaCl were used as references. The pH changes were measured with Leici PHS-25 numerical pH meter (Leici Scientific Instrument Com., Shanghai, China) at room temperature. The sizes of complex particles were measured by dynamic light scattering at room temperature on Malvern MASTERSIZER 2000 (Malvern Instruments Ltd., Malvern, U.K.). Samples were inhaled with Hydro 2000MU and deionized water was selected as dispersion medium. Between two measures, the instrument was washed three times with deionized water. Transparence of complex solutions formed at various θ values was measured on UV–2401PC photospectrometer (Shimadzu, Japan). Aqueous GPC was performed using a Waters 515 HPLC pump and a Waters 2410 refractive index detector. Data were collected and integrated using Waters Empower software. Morphology of complex nanoparticle was observed by transmission electron microscopy on JEM-100CX (JEOL, Japan). Before measurement, complex solution was added with phosphotungstic acid.

Results and discussion

PTEG was prepared through ring-opening reactions of epoxy groups of EG by amino groups of TEPA. Since both primary and secondary amines could take part in ring opening reaction, part of secondary amines were converted to tertiary ones and some primary amines into secondary ones. So PTEG had a broad nitrogen-grade profile and was a random branched polymer (Scheme 1). EG was synthesized from epichlorohydrin and ethylene glycol under the catalysis of NaOH. Its ^{1}H NMR spectrum and assignments were shown in Fig. 1.



Scheme 1. Synthetic procedure of PTEG

In order to avoid side reaction of EG, well dried dichloromethane was used as medium of first reaction stage. When reaction proceeded to certain extent, precipitate came into being and stirring became difficult. Ethanol was selected as second stage solvent because of its solubility for monomers and PTEG. After ethanol was added, reaction proceeded at lot temperature to insure amino groups participate in reaction as completely as possible. Then reaction at high temperature made epoxy groups be exhausted. The ^{1}H NMR spectrum of PTEG was shown in Fig.2.

Molecular weight of PTEG was measured with aqueous GPC and polyethyleneglycol was used as reference. It can be seen from Fig. 3 that the peak value of molecular weight is 1.3×10^4.

Since PEC was obtained through electrostatic interaction following protonation, the protonation characteristics of PTEG had important effect on ionic complex formation. Polycation with a wide protonation range is needed by gene transfer because there will be some free amine groups left intact after complexation. The left amine groups continue to protonize in an environment with lower pH value, endosome, and make the complex swell. This effect is known as “proton sponge effect”, dominates the high transfection efficiency without additional endosome-disrupting agent in case of

polyethylenimine. In contrast, polylysine as gene carrier often resulted in low transfection efficiency when no adjuvant additive was used together because of absence of this effect [32]. Protonation characteristics of PTEG was determined by acid base titration. From Fig. 4, it can be seen that when NaCl aqueous solution and HCl solution are titrated with NaOH solution, in the pH range of 5 to 7.4, pH curves rise abruptly. That is, both solutions had no buffering capability in this range. However the titration curve of PTEG rises flatly over the entire measured range. This indicates that PTEG can be protonated within the pH range where polyethylenimine shows "proton sponge effect". Broad nitrogen grade profile possibly contributes to the buffering capability.

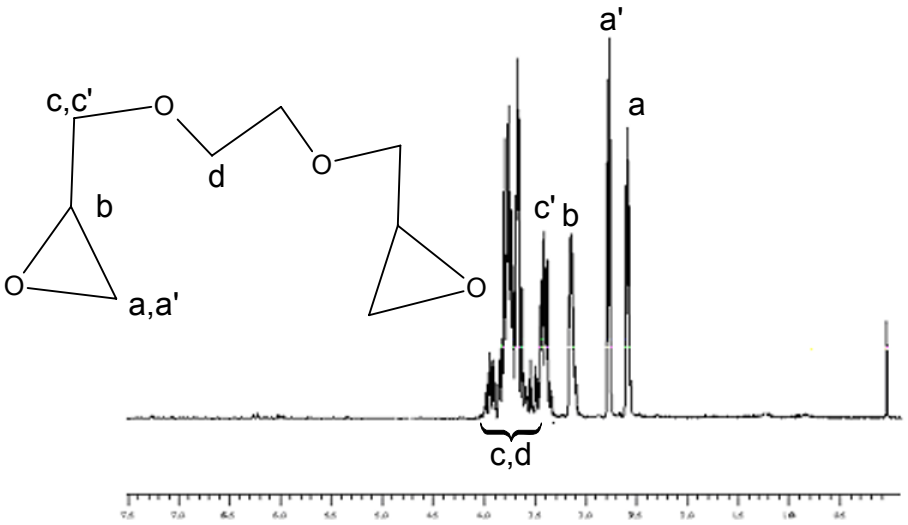


Fig. 1. ^{1}H NMR spectrum of EG in $CDCl_3$.

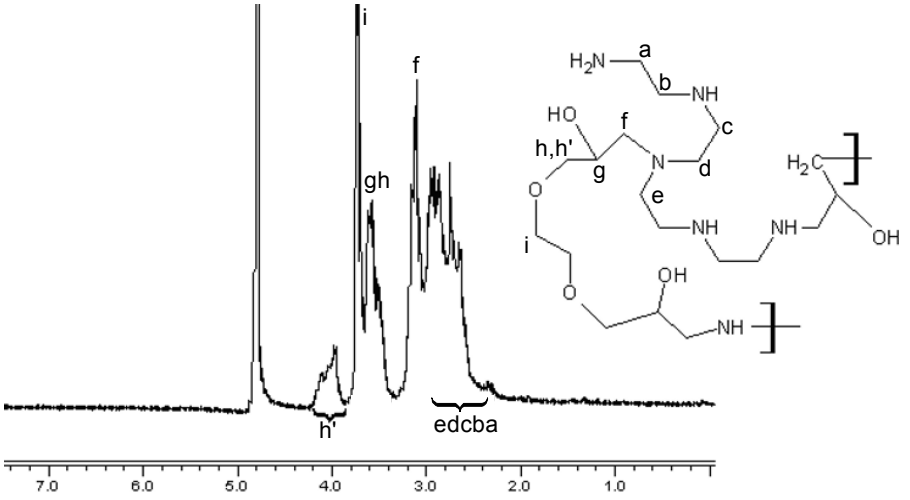


Fig. 2. ^{1}H NMR spectrum of PTEG in D_2O.

PTEG derived from TEPA had high nitrogen density, discrete charge distribution, many hydrophilic hydroxy groups and broad nitrogen grade profile, which suggests strong complexation ability and biocompatibility of PTEG complex with polyanion. When PTEG solution was mixed with PAA solution, complex particles were obtained right now. Complexing process was depicted in Scheme 2. When one component A is added into the other charged contrarily component B, extensive A molecular chain becomes collapsed into an initial charged nuclear due to electrostatic compress. The nuclear grows by adsorbing charged contrarily component A and changes its charge type. Then B and A components are adsorbed onto nuclear in turn until one of them is exhausted. At different mass ratios, three types of complex particle of PTEG and PAA can be obtained (Scheme 3).

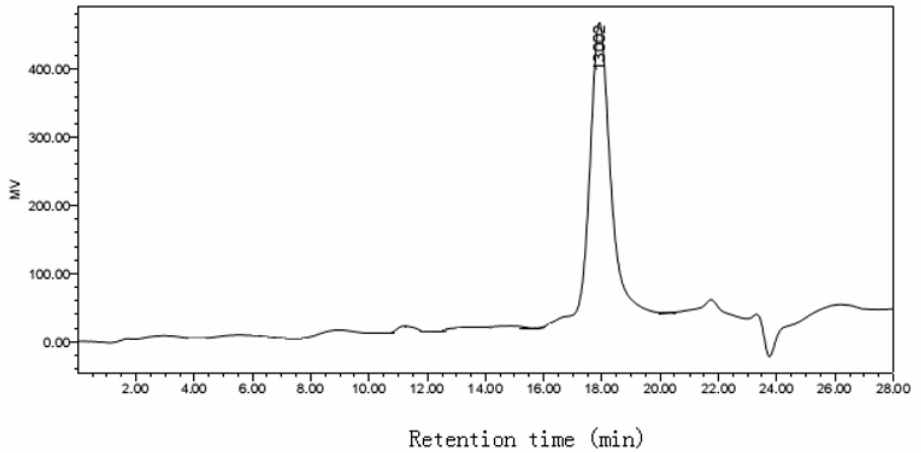


Fig. 3. GPC spectrum of PTEG in aqueous mobile phase.

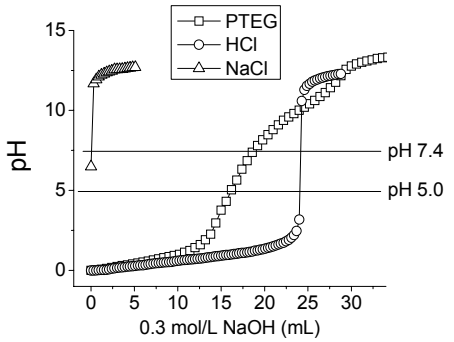
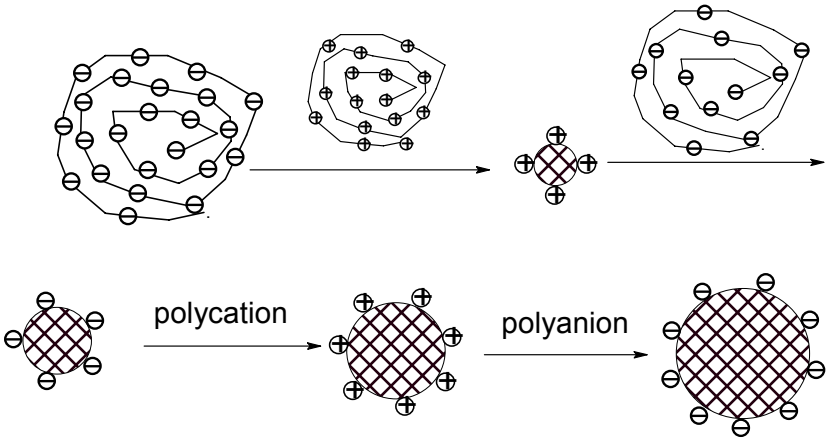


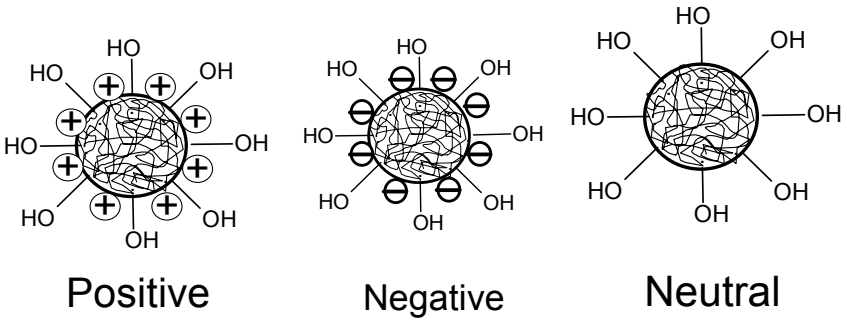
Fig. 4. Protonation property of PTEG.

Particle diameter and its distribution are different at different θ values as shown in Fig. 5. Mean particle diameter first increases and then decreases with increasing θ values. This was mainly because complex particles obtained at different θ values had different charge types. At middle θ values, approximately neutral complex particles formed due to nearly equal amount of ionizing amino and carboxylic groups. Newly produced

small particles were prone to aggregate due to hydrophobic nature and surface tension. So, in complex solution, small and big particle coexisted, which resulted in broad diameter distribution. While at higher or lower θ values, either excess component endowed complex particles with corresponding charge. Charged complex nanoparticles did not aggregate due to electrostatic repulsion. So there were only small particles in complex solution. Complex particle presents approximate sphericity. Unexpectedly, at θ value of 0.5 complex particle diameter exceeds 1000 nm indicating that short chained hydrophilic hydroxy groups is difficult to effectively prevent aggregation at electrically neutral conditions. But this doesn't possibly damage transfection characteristics because gene vector is largely excess in transfection. Hydrophilic hydroxy groups mainly are intended to contribute to biocompatibility.



Scheme 2. Formation process of polyelectrolyte complex particle.



Scheme 3. Three types of complexing particle of PTEG and PAA.

Complex solutions formed at different θ values had various apparent transparences. (Fig. 6). This difference reflected the discrepancy of diameter. Complex solution formed at θ of 0.3 and 0.4 had higher apparent transparence than that at 0.5 of θ in the wavelength range from 300 nm to 800 nm. This possibly resulted from light scattering of particle with different mean diameter. Big particles scatter light more strongly than small ones.

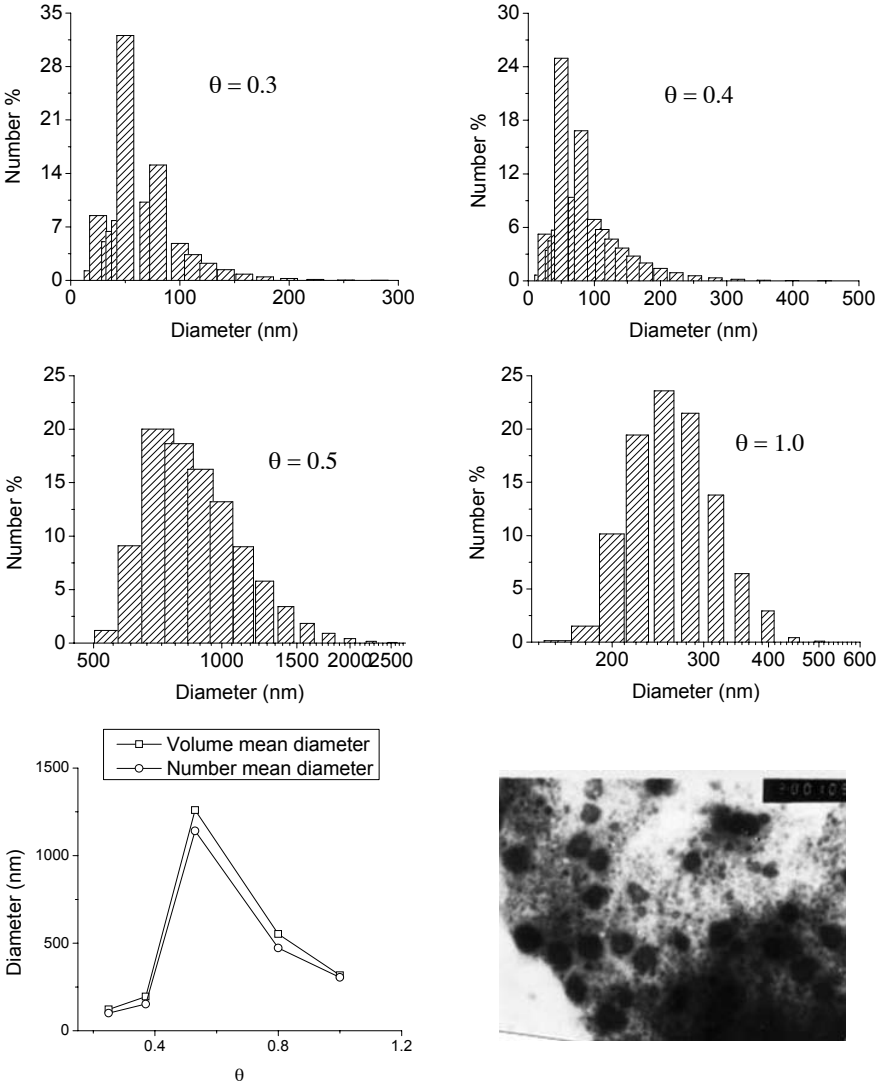


Fig. 5. The diameter distributions, sizes of complex particles at different θ values and microscopic morphology (×20000) at θ of 0.4.

The stability of complex solutions was reflected by the change of absorbance (Fig. 7). If complex solution was not stable, small particles will aggregate into big ones and precipitate during static incubation. Thus supernatant liquid apparent absorbance will decrease, which can be reflected by UV-Vis spectroscopy. Formed at θ of 0.3, the absorbance curve of complex solution at 7th day entirely overlaps with that at 1st day. With increasing θ, difference of apparent absorbances at 1st and 7th day becomes more and more obvious. It is somewhat unexpected that the absorbance changes of solutions formed at θ of 0.8 and 1 are more obvious than that at θ of 0.5. The cause is possibly that the positive charge is not excessive enough to stabilize particles.

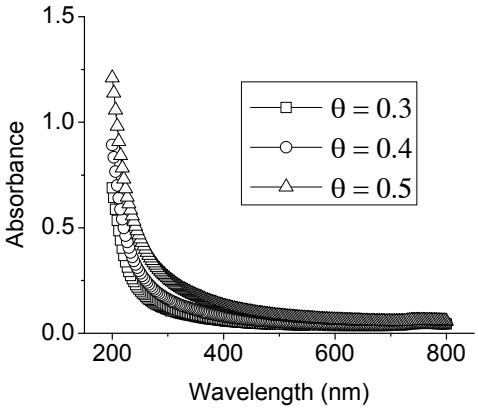


Fig. 6. Absorbance change with mass ratio.

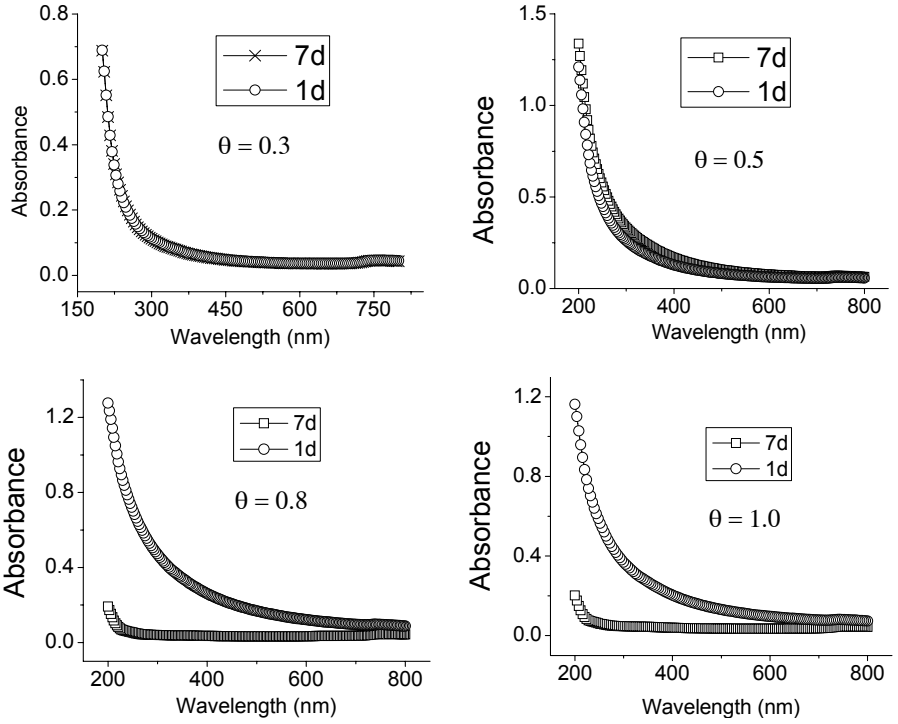


Fig. 7. UV-Vis absorbance changes of complexation solutions formed at various θ values.

Conclusions

A novel charge-dispersed cationic poly(hydroxyetheramine) with short chain of hydrophilic hydroxy groups was synthesized by ring opening copolymerization of TEPA and EG. PTEG contains broad nitrogen grade profile composed of primary, secondary and tertiary amino groups. Because all amino groups of TEPA are active in

ring opening, PTEG has random branched molecular structure. PTEG can protonize both in pH 7.4 ~11 and pH 5~7.4 indicating that PTEG has similar "proton sponge effect" as polyethylenimine. PTEG complexed with PAA into nanoparticles with mean diameter smaller than 400 nm at tested optimal conditions. Complex particle size first increased and then decreased with increasing mass ratio due to electrostatic interaction, surface tension and hydroxy stability contribution. It is worth noting that hydrophilic hydroxy groups only partially contribute to stability of particles at most in spite of the expected increase of biocompatibility.

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