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Surface Modification and PEGylation of Branched Polyethyleneimine for Improved Biocompatibility

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ABSTRACT: In this article, we report the surface modification of branched polyethyleneimine (PEI) for improved biocompatibility. PEIs with different surface functionalities were synthesized via covalent modification of the PEI amines, including neutralized PEI modified with acetic anhydride, negatively charged PEI modified with succinic anhydride, hydroxylated PEI modified with glycidol, and PEI–poly(ethylene glycol) (PEG) conjugates modified with both PEG and acetic anhydride. The modified PEI derivatives were characterized with ^{1}H-NMR, Fourier transform infrared spectroscopy, and ζ-potential measurements. An *in vitro* cytotoxicity assay of mouse fibroblasts revealed that the biocompatibility of PEI was significantly improved after these modifications. The neutral and negatively charged PEIs were nontoxic at concentrations up to 200 μg/mL, whereas the pristine PEI was toxic to cells at concentrations as low as 10 μg/mL. The successfully modified PEIs with different surface charges and functionalities may provide a range of opportunities for various biomedical applications. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 128: 3807–3813, 2013

KEYWORDS: biocompatibility; dendrimers; functionalization of polymers; hyperbranched polymers and macrocycles

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INTRODUCTION

Polyethyleneimine (PEI) is a kind of cationic polymer synthesized by the acid-catalyzed polymerization of ethyleneimine. Because of its high density of amines,[1] PEI has been widely used in the fields of biology and medicine. For instance, PEI has been used as a very effective nonviral vector for gene delivery[2–6] and as an additive for effectively improving the specificity and efficiency of the polymerase chain reaction (PCR) for DNA amplification.[7] In addition, PEI has also been used as templates, stabilizers, and molecular glue for the synthesis, stabilization, modification, and assembly of metal nanoparticles (NPs),[8–11] metal oxide NPs,[12–14] semiconductor NPs,[15] and carbon nanotubes.[16,17]

On the other hand, the amine-rich functional groups make PEI distinctly cytotoxic, causing cell damage before cellular internalization by membrane destabilization.[3,18] Therefore, a variety of chemical modifications have been performed to neutralize PEI amines. For instance, acylation and PEGylation of PEI[19–22] and the modification of PEI with other molecules[23–28] have been used in an effort to reduce the cytotoxicity of the polymer. In addition to the benefits of improving the biocompatibility of PEI, the surface modification could also allow for a deep understanding of the interaction between PEI and the biomolecules that regulate its biological functionality. For instance, our previous work[7] showed that PEI can be used as an efficient PCR enhancer; however, the detailed mechanism was not clear. It is generally acknowledged that as a kind of cationic polyamine, PEI is able to interact or bind with the DNA template or DNA polymerase; this can significantly increase the local concentration of the polymerase or DNA template and improve the specificity and efficiency of PCR. Therefore, the surface modification of PEI is very important for not only possibly reducing its cytotoxicity but also providing PEI with different surface properties for the mechanistic study of PCR optimization or for other biomedical applications, especially gene delivery when PEI is partially modified.

Our previous studies related to the surface modification of polyamidoamine (PAMAM) dendrimers have shown that amine-terminated PAMAM dendrimers can be modified with acetic anhydride, succinic anhydride, and glycidol to generate acetamide, succinamic acid, and glycidol hydroxyl functionalized dendrimers.[29–32] In addition, the biocompatibility of amine-terminated dendrimer-entrapped gold NPs can be significantly improved by the modification of the dendrimer terminal amines with acetamide or hydroxyl groups.[33,34] These studies have led us to hypothesize that as a branched amine-rich polycationic polymer, PEI could also be

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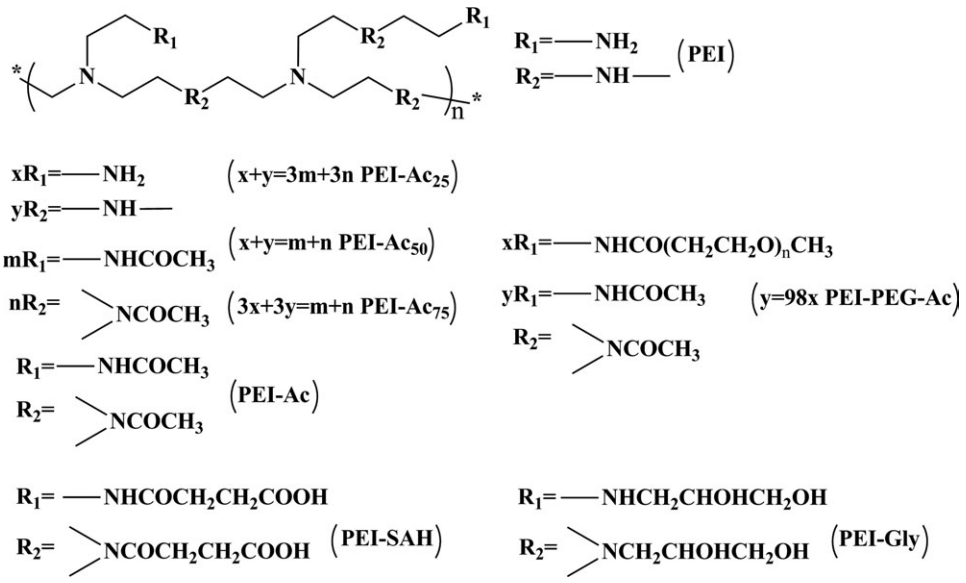

Scheme 1. Schematic illustration of the structures of PEI, PEI–SAH, PEI–Ac, PEI–Gly, PEI–PEG–Ac, and PEI–Ac_{25}, PEI–Ac_{50}, and PEI–Ac_{75}. *x*, *y*, *m*, and *n* represent the number of R_1 or R_2 groups in different PEI derivatives, respectively.

modified in a similar way to improve its biocompatibility for various biomedical applications. However, these systematic modifications to neutralize PEI amines have not been reported in the literature.

In this article, we report the systematic surface modifications of branched PEI amines to generate PEIs with defined acetylation degrees, with hydroxyl groups, with succinamic acid groups, and with both poly(ethylene glycol) (PEG) chains and acetamide groups (Scheme 1). For the acetylation, hydroxylation, and carboxylation of PEI amines, these approaches were similar to those used for PAMAM dendrimer modification.[29–32] For the modification of PEI with both PEG and acetamide, PEI was first reacted with poly(ethylene glycol) monomethyl ether with carboxyl end groups (*m*PEG-COOH) via amide bond formation, and then, the remaining PEI amines were reacted with acetic anhydride. The various PEI derivatives that were formed were thoroughly characterized by ^{1}H-NMR, Fourier transform infrared (FTIR) spectroscopy, and ζ-potential measurements. The *in vitro* cytotoxicity of the PEI derivatives was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay of mouse fibroblasts (L929 cells) treated with the PEI derivatives. To our knowledge, this is the first report dealing with the systematic surface modification of PEI with acetyl, carboxyl, hydroxyl, and PEG moieties in one single and complete study that allowed a direct comparison of the PEI structures with different surface modifications. In addition, there has been no report in the literature related to PEI hydroxylation with glycidol and the mixed PEGylation and acetylation of PEI. The generated PEI derivatives may be used for different biomedical applications.

EXPERIMENTAL

Materials

*m*PEG-COOH [weight-average molecular weight (M_w) = 2000] was from Shanghai Yanyi Biotechnology Corp. (Shanghai, China). Branched PEI ($M_w \approx$ 25,000 by light scattering method, number-average molecular weight ≈ 10,000 by gel permeation chromatography, catalog number 40,872-7, lot number 08910MH-088), acetic anhydride, succinic anhydride, glycidol, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, and all other chemicals and solvents were obtained from Aldrich (St. Louis, Missouri) and were used as received. Mouse fibroblasts (L929 cells) were obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Regenerated cellulose dialysis membranes (molecular weight cutoff = 3000) were acquired from Fisher (Pittsburgh, Pennsylvania).

Surface Modification of PEI with Acetic Anhydride

The amine groups of the branched PEI were acetylated to neutralize the positive charges. In brief, triethylamine (2.0 mL) was added to the solution of PEI (0.2 g, 8.0 μmol) dissolved in dimethyl sulfoxide (DMSO; 10 mL), and the solution was thoroughly mixed for 30 min. Excess acetic anhydride (1.41 mL, 12.8 mmol) was then added dropwise into the solution of the PEI/triethylamine mixture under vigorous magnetic stirring. The mixture was allowed to react at room temperature with stirring for 24 h. Then, the DMSO and the excess of reactants and byproduct were removed from the mixture by extensively dialysis against phosphate-buffered saline buffer (three times, 4 L) and water (three times, 4 L) for 3 days; this was followed by lyophilization to obtain the PEI–Ac (Ac denotes acetyl groups, yield = 90.7%). For partial acetylation of the PEI amines, acetic anhydride with different molar ratios to PEI amines (0.717, 1.434, and 2.161 mmol) was added to the same PEI/triethylamine mixture to achieve the desired degree (25, 50, or 75%) of acetylation. The formed PEI–Ac_{25}, PEI–Ac_{50}, and PEI–Ac_{75}, respectively, were purified and lyophilized according to the procedure used for the purification of PEI–Ac (yields = 92.0, 91.3, and 92.8%, respectively).

Surface Modification of PEI with *m*PEG-COOH and Acetic Anhydride

To modify the PEI amines with both PEG and acetyl groups, *m*PEG-COOH (14.19 mg, 7.1 μmol) was dissolved in 4.0 mL of DMSO. Then, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (13.60 mg, 71.2 μmol) dissolved in DMSO (2.0 mL) was added to the *m*PEG-COOH solution under vigorous magnetic stirring. After 3 h, the activated *m*PEG-COOH was added dropwise into the solution of PEI (0.1 g 4.0 μmol in 5.0 mL of DMSO) under vigorous magnetic stirring. The reaction was continued for 3 days to obtain the PEI–PEG conjugate. The remaining amines of the PEI–PEG conjugate were then completely acetylated according to the procedure described previously. The formed PEI–PEG–Ac (yield = 93.1%) was purified and lyophilized according to the procedure used for the purification of PEI–Ac.

Synthesis of PEI Succinamic Acid

The amine groups of the PEI were reacted with succinic anhydride to generate negatively charged PEI succinamic acid. PEI (0.20 g) dissolved in DMSO (10 mL) was added with excess succinic anhydride (1.523 g) dissolved in 5 mL of DMSO under vigorous magnetic stirring. The reaction was stopped after 2 days. Thereafter, the formed PEI–SAH (SAH denotes succinamic acid moieties, yield = 89.5%) was purified according to the procedure used to purify PEI–Ac.

Synthesis of Hydroxylated PEI

The amine groups of PEI were reacted with glycidol. PEI (0.20 g) dissolved in DMSO (10 mL) was added with excess glycidol (1.023 mL) dissolved in DMSO (2 mL) under vigorous magnetic stirring. The reaction was stopped after 24 h. Thereafter, the formed PEI–Gly (Gly denotes glycidol hydroxyl groups, yield = 88.0%) was purified according to the procedure used to purify PEI–Ac.

Characterization Techniques

The formed PEI derivatives were extensively characterized by ^{1}H-NMR, FTIR spectroscopy, and ζ-potential measurements. ^{1}H-NMR spectra were recorded on a Bruker DRX 400 nuclear magnetic resonance spectrometer (Rheinstetten, Germany). Samples were dissolved in D_2O before measurements. FTIR spectra were acquired with a Nicolet Nexus 670 FTIR spectrometer (Madison, Wisconsin) with the scanning wave-number range of 400–4000 cm^{-1} and a resolution of 1 cm^{-1}. Dry samples were mixed with grounded KBr crystals and pressed as pellets before measurements. The baseline correction and normalization of the obtained FTIR spectra were performed after measurement with a Nicolet Omnic 8.0. The ζ-potential values of the pristine and functionalized PEIs were measured with a Zetasizer Nano ZS system (Malvern, United Kingdom) equipped with a standard 633-nm laser.

MTT Viability Assay of Cells Treated with PEI Derivatives

L929 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin. An MTT assay was used to quantify the viability of the cells. Briefly, 1×10^4 L929 cells per well were seeded into a 96-well plate. After 24 h of incubation to bring the cells to confluence, the cell culture medium was replaced with fresh culture medium containing pristine and functionalized PEI derivatives at a concentration ranging from 0 to 200 μg/mL. After 24 h of incubation with different materials at 37°C, the MTT assay was performed according to the manufacturer's instruction to quantify the cell viability. The mean and standard deviation values for the triplicate wells were reported. Statistical analysis was performed by an analysis of variance method. In all evaluations, a value of $p < 0.05$ was considered to be statistically significant.

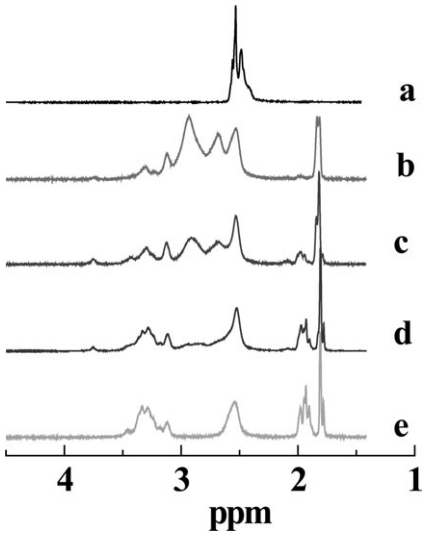

Figure 1. ^{1}H-NMR spectra of (a) pristine PEI, (b) PEI–Ac_{25}, (c) PEI–Ac_{50}, (d) PEI–Ac_{75}, and (e) PEI–Ac, respectively.

RESULTS AND DISCUSSION

^{1}H-NMR Spectra of the Modified PEI Derivatives

A previous study by Majoros et al.[35] showed that the acetylation of amine-terminated generation 5 PAMAM dendrimers was a controlled stoichiometric reaction, allowing for the defined acetylation of the dendrimer surface amines dependent on the initial molar ratio between the dendrimers and acetic anhydride. In our previous study,[33] we showed that dendrimer-entrapped Au and Ag NPs could also be modified with different degrees of acetylation. Therefore, in this study, apart from the complete acetylation of PEI (PEI–Ac), we also modified PEI with different degrees of acetylation (PEI–Ac_{25}, PEI–Ac_{50}, and PEI–Ac_{75}). ^{1}H-NMR was used to characterize these acetylated PEIs (Figure 1). We observed that PEI–Ac_{25} (Figure 1, spectrum b) showed a new peak at 1.82 ppm, and the peak above 2 ppm was separated into three main peaks at 2.93, 2.68, and 2.53 ppm, respectively, whereas the pristine unmodified PEI only displayed the —CH_2— proton signals at 2.25–2.60 ppm ($[CH_2CH_2N]_x[CH_2CH_2NH]_y[CH_2CH_2NH_2]_z$, ethylene backbone; Figure 1, spectrum a). With the increase of the acetylation degree, the intensity of the peaks at 2.93 and 2.68 ppm decreased sharply, whereas the one at 2.53 ppm did not show any significant changes. In contrast, the intensity of the peaks at 1.82, 1.97, and 3.33 ppm increased with the acetylation degree. This means that the peaks at 2.93, 2.68, and 2.53 ppm were associated with the —CH_2— proton signals linked with primary, secondary, and tertiary amines, respectively, whereas the new peaks below 2.0 ppm and above 3.0 ppm referred to the acetyl protons of primary and secondary amides, and the —CH_2—

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proton signals were linked with primary and secondary PEI amides, respectively. These results were consistent with those reported in the literature.[36,37] The peaks at 1.70–1.85 ppm were related to the acetyl protons linked with secondary amides ($R—NHCOCH_3$), whereas the ones at 1.85–2.00 ppm corresponded to acetyl protons linked with tertiary amides $—\overset{|}{N}—COCH_3$.[36,37] The peaks at 3.0–3.5 ppm were principally attributed to the $—CH_2—$ proton signals, which directly linked with the amide. From the spectra, we also observed that the peak of the acetyl protons linked with the secondary amides at 1.70–1.85 ppm did not change significantly with increasing acetylation degree from 50 to 100%, whereas the peak at 1.85–2.00 ppm, associated with the acetyl protons linked with the tertiary amides, increased significantly. This suggested that the primary amines reacted much more easily with acetic anhydride than the secondary amines, and the acetylation of PEI was a controlled, stoichiometric reaction. To further quantify the acetylation degree in each PEI derivative, the signals of the respective ^{1}H-NMR peaks were integrated. The acetylation degrees of the modified PEI were calculated through a comparison between the integration value of the peaks at 1.7–2.0 ppm (related to the acetyl protons linked with the PEI secondary and tertiary amides) with that at 2.4–3.5 ppm (related to the $—CH_2—$ proton signals of PEI backbone). The acetylation degrees of PEI–Ac_{25}, PEI–Ac_{50}, PEI–Ac_{75}, and PEI–Ac were estimated to be 23, 49, 72, and 100%, respectively.

The PEGylation of PEI has many advantages, including the introduction of biodegradable linkages, increased polymer solubility, and further improved biocompatibility.[19] In our study, we partially PEGylated PEI, and the remaining PEI amines were completely acetylated. To further modify PEI with negative charges or hydroxyl groups, the PEI amines were also modified with succinic anhydride and glycidol, respectively. The modified PEI derivatives were qualitatively confirmed by ^{1}H-NMR spectroscopy (Figure 2). For PEI modified with both *m*PEG-COOH and acetic anhydride (PEI–PEG–Ac), only two new proton signals at 3.54 and 3.21 ppm (Figure 2, spectrum a) were observed in the spectrum compared with the spectrum of PEI–Ac (Figure 1, spectrum e); these were assigned to the ethylene backbone and methoxyl protons of PEG, respectively. To quantify the PEGylation and acetylation degree in this PEI derivative, the signal of the respective ^{1}H-NMR peaks were integrated. The PEGylation degree of the PEI–PEG–Ac was calculated by comparison of the integration value of the peaks at 3.54 ppm (related to the $—CH_2—$ proton signals of PEG) with that at 2.4–3.5 ppm (related to the $—CH_2—$ proton signals of the PEI backbone). The acetylation degree was calculated according to the method described previously. The degrees of PEGylation and acetylation were estimated to be 0.48 and 99.1%, respectively.

The carboxylation and hydroxylation of PEI amines were also confirmed by ^{1}H-NMR. Compared with the spectrum of the PEI, the spectrum of the PEI–SAH showed additional peaks at 3.0–3.6 ppm (Figure 2, spectrum b), and the new peaks were associated with the $—CH_2—$ proton signals, which were directly linked with the succinamic acid moieties. The ^{1}H-NMR spectrum of the PEI–Gly clearly showed three new peaks (Figure 2, spectrum c), compared with that of the PEI. The peaks at 3.72–

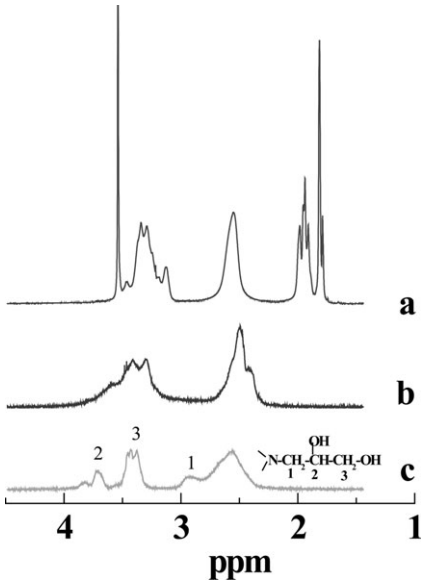

Figure 2. ^{1}H-NMR spectra of (a) PEI–PEG–Ac, (b) PEI–SAH, and (c) PEI–Gly, respectively.

3.82 ppm (peak 2) and 3.45 ppm (peak 3) were assigned to the protons of at position 2 and $—CH_2—$ at position 3 of the 2,3-dihydroxylpropyl group, respectively.[31,38] The peak at 2.93 ppm belonged to the protons of the $—CH_2—$ at position 1, which overlapped with peaks associated with the $—CH_2—$ proton signals of the PEI backbone. It should be noted that it was difficult to quantify the carboxylation and hydroxylation degrees of these PEI derivatives through the integration of the respective ^{1}H-NMR peaks because the main characteristic peaks of PEI–SAH and PEI–Gly overlapped with peaks associated with the $—CH_2—$ proton signals of PEI. In this case, ^{1}H-NMR could only be used to qualitatively confirm the structural transformation of the PEI amines. We also noted that some small molecular PEI may have existed in the PEI main product with an M_w of 25,000.[39,40] Because of the structural similarity, NMR techniques could not differentiate these small molecular derivatives.

FTIR Spectra of the Modified PEI Derivatives

FTIR spectroscopy was used to further confirm the chemical transformation of the PEI amine groups (Figure 3).[20] Because the structures of the PEI derivatives were characterized in detail with ^{1}H-NMR spectra, only changes in the FTIR spectra after the different modifications are discussed. In Figure 3, spectrum a, the typical absorption bands at 3357 and 3295 cm^{-1} were associated with the N—H stretch of the primary and secondary amines of PEI, respectively. The bands at 2935 and 2813 cm^{-1} were assigned to the symmetrical and asymmetrical C—H stretching vibrations, respectively. The broad bands at 1592 and 770 cm^{-1} corresponded to the N—H deformation vibration and wagging mode of the primary amines of PEI, respectively, which disappeared when PEI was modified with acetic anhydride, succinic anhydride, and glycidol, respectively (Figure 3, spectra b–e). The FTIR spectroscopic features of the amide bond formation for PEI derivatives with different surface modifications shown in Figure 3, spectra b–d, are listed in Table I. In the

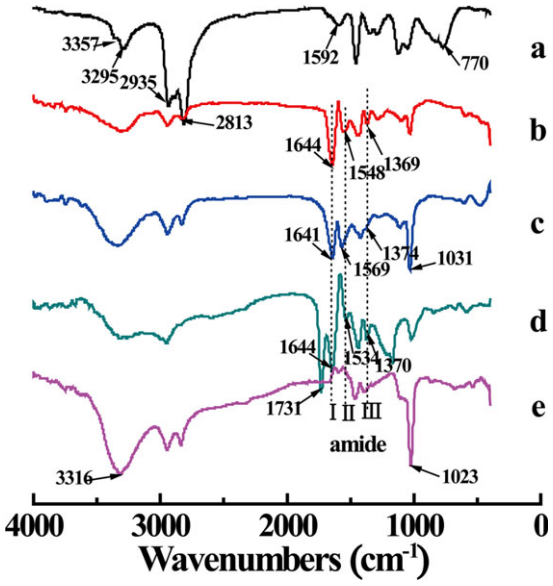

Figure 3. FTIR spectra of (a) pristine PEI, (b) PEI–Ac, (c) PEI–PEG–Ac, (d) PEI–SAH, and (e) PEI–Gly, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

spectrum of PEI–PEG–Ac (Figure 3, spectrum c), an intensive band around 1031 cm^{-1} could be assigned to the stretching vibration of the C—O—C groups of PEG ethers; this was different from reported data with a band at 1100 cm^{-1}.[20] This difference was likely due to the difference in the chemical environment because the reported data about the stretching modes of —CH_2—O—CH_2— were observed at 1067 cm^{-1} in another study.[41] A strong absorption band at 1731 cm^{-1}, which was attributed to C=O of the carboxyl group, was observed in PEI–SAH. The sharp band at 1031 cm^{-1} in Figure 3, spectrum c, indicated the presence of C—O—C groups in the PEI–PEG–Ac product. In Figure 3, spectrum e, the broad band at 3316 cm^{-1} was due to the O—H stretching of hydroxylated PEI, and the distinct sharp band of C—O groups at 1023 cm^{-1} also proved the successful hydroxylation of PEI. It should be noted that usually the primary and secondary alcohols give a band of C—O stretching vibrations at about 1035 and 1070 cm^{-1}, respectively. The band position difference was likely due to the structural differences between small molecular primary and secondary alcohols and macromolecular derivatives. These FTIR spectral data further confirmed that the designed surface-modified PEIs were successfully synthesized.

Table I. Characteristic Absorption of Amide Groups in Samples b, c, and d, as Shown in Figure 2

Bond	Sample	Wave number (cm^{-1})
Amide I	b	1644
	c	1641
	d	1644
Amide II	b	1548
	c	1569
	d	1534
Amide III	b	1369
	c	1374
	d	1370

Table II. ζ Potential Values of the Modified PEI Derivatives

Sample	ζ potential (mV)
Pristine PEI	23.1 ± 4.1
PEI–Ac	0.4 ± 1.1
PEI–PEG–Ac	−0.1 ± 1.8
PEI–SAH	−29.6 ± 5.6
PEI–Gly	1.2 ± 1.6

ζ-Potential Measurements

The surface modification of PEI was also confirmed by the surface potential changes (Table II). All PEI derivatives were dissolved in phosphate-buffered saline buffer (pH 7.4) before the measurements. As we expected, the surface potential of the positively charged pristine unmodified PEI (23.1 mV) became negatively charged (−29.6 mV) after modification with succinic anhydride (PEI–SAH). The acetylation (0.4 mV), PEGylation followed by complete acetylation (−0.1 mV), and hydroxylation (1.2 mV) of the PEI amines were able to neutralize the positive surface potential of PEI. These results suggest that the PEI amines were successfully transformed via different derivatization reactions.

Cytotoxicity Assay

The neutralization of PEI amines was expected to improve the biocompatibility. To prove our hypothesis, the cytotoxicity of all of the synthesized PEI derivatives with different surface properties was tested via MTT assay. After incubation of the pristine unmodified PEI, PEI–Ac, PEI–PEG–Ac, PEI–Gly, and PEI–SAH with L929 cells for 24 h, an MTT assay was performed to evaluate the viability of the L929 cells (Figure 4). It was clear that pristine PEI started to exhibit cytotoxicity at 10 μg/mL ($p <$

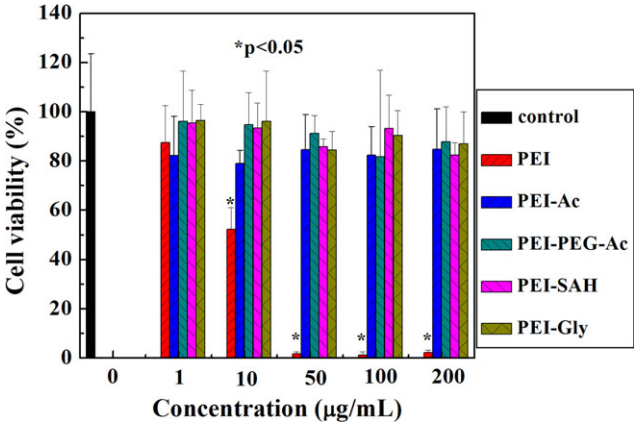

Figure 4. MTT assay of L929 cell viability after treatment with differently functionalized PEI derivatives for 24 h. Mean and standard deviation values for the triplicate wells are reported. The data are expressed as mean plus or minus standard deviation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

0.05). In contrast, the modified PEI derivatives, including PEI–Ac, PEI–PEG–Ac, PEI–Gly, and PEI–SAH, did not display cytotoxicity even at concentrations of up to 200 μg/mL. In general, the cytotoxicity of PEI stems from the strong electrostatic interaction between the positively charged PEI and negatively charged cell membranes.[16] After modification to neutralize the PEI amines, the positive charges of PEI were shielded; this significantly improved the biocompatibility of PEI. This study underlines the fact that through the neutralization of PEI amines by acylation, PEGylation, or hydroxylation, the biocompatibility of the formed neutral or negatively charged PEI derivatives was significantly improved.

CONCLUSIONS

In summary, the amine groups of PEI were modified with acetyl, carboxyl, hydroxyl groups, and PEG chains in a systematic and complete study. Our results show that the acetylation of PEI is a controlled, stoichiometric reaction and that partial acetylation of PEI may enable multiple modifications of PEI with different functionalities. Importantly, the formed PEI derivatives (PEI–Ac, PEI–PEG–Ac, PEI–SAH, and PEI–Gly) did not display apparent cytotoxicity at concentrations of up to 200 μg/mL, whereas the pristine PEI was cytotoxic at concentrations as low as 10 μg/mL. Our study indicated that through relatively simple modification strategies to neutralize the surface amines, PEI can be functionalized to have improved biocompatibility. With the ability to modulate the PEI surface charge to be positive, negative, or neutral and to afford PEI with desired surface functional groups, the formed PEI derivatives were found to be very useful for the mechanistic study of PCR optimization.[42] In addition, the PEI derivatives with partial neutralization of the surface amines may be used for low-toxicity gene delivery. Furthermore, these surface modification strategies may also provide the means for the modification of PEI-based hybrid composite materials.

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