Journal of Colloid and Interface Science 330 (2009) 77–83

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

Surface-grafting of phosphates onto a polymer for potential biomimetic functionalization of biomaterials

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ARTICLE INFO

Article history:
Received 4 August 2008
Accepted 9 October 2008
Available online 14 October 2008

Keywords:
Surface grafting
Phosphate
Poly(ethylene-*co*-acrylic acid)
Polymer
Biomaterials

ABSTRACT

In the human body, phosphate groups play important roles in signaling and the biological functions of proteins and peptides. Despite the importance of phosphate groups, polymer surfaces have not been directly grafted with phosphate groups by chemical reactions because the usual organic solvents used to graft phosphate groups can dissolve or swell polymers. We focused this study on grafting phosphate groups onto a poly(ethylene-*co*-acrylic acid) (PEAA) surface in an aqueous solution. *O*-phospho L-serine and *O*-phosphoethanolamine were grafted on PEAA surfaces to introduce phosphate groups by activating carboxylic acid groups of PEAA using *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in an aqueous environment. X-ray photoelectron spectroscopy (XPS) was used to elucidate the process by which surface grafting occurs and the process that the phosphate group is cleaved into a phosphate ion and a hydrolyzed molecule at high pH. It was found that under appropriate reaction conditions the phosphate groups could be successfully grafted on the polymer surfaces. The phosphate-grafted polymer surfaces showed lower water contact angles than the initial polymer surfaces likely due to their highly mobile and hydrophilic phosphate side groups. This work demonstrates a technique to successfully graft phosphate groups onto organic polymer surfaces in a biocompatible aqueous environment, which may open new avenues to functionalizing synthetic polymeric and natural macromolecule derived biomaterials.

1. Introduction

In the human body, phosphate groups play important roles in signaling and biological functions of proteins and peptides such as control of biomineral growth in bone [1], regulation of ion channel in membrane phospholipids [2], control of glycogen synthase in liver [3], regulation of the receptor in sphingosine 1-phosphate [4], and so on. Additionally, most of the coenzymes are esters of phosphoric acid or pyrophosphoric acid. The principal reservoirs of biochemical energy [adenosine triphosphate (ATP), creatine phosphate, and phosphoenolpyruvate] are phosphates [5]. As specific examples in the field of biotechnology, grafted phosphate groups have been used in enzyme-amplified immunosensors [6] and as bioactive sites for biomimetic mineral nucleation and growth [7].

Surfaces modified with hydrophilic chemicals have been studied for blood contacting applications because of their antifouling properties [8,9]. To improve the hydrophilic property of the surface of biomaterials, hydroxyl and carboxyl groups have been explored [10]. The luminal surface of the natural endothelial bilayer membrane is rich in phosphate groups [11,12]. Nevertheless, phosphate groups have not been well studied. In this study, to simulate the effect of this luminal surface, we focused our efforts on developing methods to graft phosphate groups on a polymer surface and to compare the hydrophilic properties between the phosphate group and the carboxyl group grafted polymer surfaces.

Phosphate groups have not been grafted on polymer substrates through pure chemical reactions previously because the usual solvents used to attach phosphate groups are incompatible with polymers. Surface grafting of phosphate groups is typically achieved in one of two ways; either direct coupling or by substitution of OH groups with phosphorus oxychloride ($POCl_3$) in organic solvents [13]. These solvents can either dissolve or swell polymer materials. Even when a solvent does not dissolve the substrate, the incomplete removal of the solvent and other residual chemicals that have penetrated the swollen materials during the surface reaction can be harmful in biomaterials.

Physico-chemical methods (such as ozone, plasma, gamma irradiation) have previously been used to graft phosphates on poly-

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0021-9797/$ – see front matter
doi:10.1016/j.jcis.2008.10.015

mer surfaces [14]. However, these methods are limited because they may cause polymers to degrade, result in non-homogeneous grafting, and are often only suitable for a material outer surface or a very thin layer of porous material; but not suitable for complex three-dimensionally porous structures such as scaffolds in tissue engineering. Therefore, surface-grafting of phosphate groups on polymers in an aqueous environment is attractive and has high potential for applications in biomaterials and biomedical devices. In spite of its importance, such reactions have not previously been reported. The aims of this research were to surface-graft phosphate groups onto a polymer surface in an aqueous environment and to analyze the chemical structures and their effects on wettability of the surface. Most natural macromolecules such as proteins and peptides have amine and carboxyl groups. For this reason, poly(ethylene-*co*-acrylic acid) (PEAA) was used as the substrate in this research because it contains carboxylic acid groups [15]. The coupling of phosphate groups on this surface was accomplished through the use of *O*-phospho L-serine and *O*-phosphoethanolamine. To prevent the chain reactions (polymerization) between amine and carboxylic acid groups of *O*-phospho L-serine or *O*-phosphoethanolamine, the carboxylic acid on the substrate polymer is first activated using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). Although phosphorus pentachloride [16] and thionyl chloride [17] have also been used to activate carboxylic acid for the amide formation between carboxylic acid and amine, these chemicals can only be used in organic solvents. Specifically, when phosphorus pentachloride in dichloromethane [18] and thionyl chloride in hexane [19] were used to modify PEAA, it was found that these chemicals penetrated into PEAA films and it was impossible to completely remove these chemicals after reaction. Therefore, NHS and EDC were chosen in this work to activate the carboxylic acid on the PEAA film for phosphate grafting in an aqueous solution.

To form the amide bond between the amine of the phosphate-containing molecule and NHS ester, the pH must exceed the critical pH, below which NHS ester is too stable to react [20]. This high pH causes an acylation reaction of NHS ester. However, the reagents that contain the phosphate and amine groups may also be cleaved to form a phosphate ion and a hydrolyzed molecule with the amine group at a high pH [21]. Therefore, the pH of the solution must be carefully chosen for the immobilization of phosphate onto a polymer surface in an aqueous environment. To determine an optimum pH for this reaction, the coupling reaction was carried out at various levels of pH, and the chemical structures of the resultant surfaces were analyzed by X-ray photoelectron spectroscopy (XPS). The surface wettability, which may be affected by both the amount and the distribution of the surface-grafted molecules, was analyzed using contact angle goniometry.

2. Experimental

2.1. Materials

Poly(ethylene-*co*-acrylic acid) (PEAA, acrylic acid 20 wt%) and *N*-hydroxysuccinimide (NHS) were purchased from Aldrich Chemical Co. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 4-morpholineethanesulfonic acid (MES), sodium chloride (NaCl), *O*-phospho L-serine, and *O*-phosphoethanolamine were obtained from Sigma Chemical Co. Sodium hydroxide (NaOH) and triethylamine (TEA) were purchased from Sigma-Aldrich Chemical Co. All chemicals were used without further purification.

PEAA thin films were prepared by melting PEAA beads on a glass mold. Prepared PEAA films were rinsed with distilled deionized water for one day. The rinsed PEAA films were dried under vacuum for 3 days at room temperature before use.

2.2. Activation of PEAA surface using EDC and NHS (PEAA-NHS)

PEAA films were activated in an MES buffer solution (pH 6.0) containing 50 mM NHS and 100 mM EDC for 4 h at room temperature with gentle stirring. MES buffer solution was prepared with 50 mM MES and 0.1 M NaCl. The pH of MES buffer solution was adjusted to 6.0 with NaOH [22]. The activated films were then rinsed with distilled deionized water to remove unreacted NHS and EDC. Thus activated film is denoted as PEAA-NHS.

2.3. Surface-grafting of *O*-phospho L-serine or *O*-phosphoethanolamine on PEAA-NHS (PEAA-PS or PEAA-PA)

The activated PEAA-NHS films were immersed in a 2 wt% *O*-phospho L serine or *O*-phosphoethanolamine dissolved in distilled deionized water for 24 h at room temperature with moderate stirring. The reaction was carried out at various pH levels. The pH of solution was adjusted with TEA. The reacted films were then rinsed with distilled deionized water three times to remove unreacted *O*-phospho L-serine or *O*-phosphoethanolamine. The films were dried under vacuum for 7 days at room temperature. These films are denoted as PEAA-PS or PEAA-PA.

2.4. X-ray photoelectron spectroscopy (XPS) analysis

To detect the phosphate groups on the surface, XPS surface characterization of the samples was carried out using a Kratos Axis Ultra photoelectron spectrometer. Spectra were obtained with a monochromatic Al $K\alpha$ X-ray source (1486.71 eV of photons). The analysis chamber was maintained at a pressure of less than 10^{-8} Torr during measurement. High-resolution spectra of the C 1s, O 1s, N 1s, and P 2p core levels were recorded using pass energy of 160 eV at a takeoff angle of 90°. All binding energies were referenced to the neutral C 1s peak at 285.0 eV to compensate for the surface-charging effects. Intensity ratios were converted into atomic ratios by using the sensitivity factors provided by the manufacturer. The peaks were deconvoluted using a curve-fitting method with a series of Gauss–Lorentzian curves allowing for adjustment of FWHM (full width at half maximum).

2.5. Contact angle measurement

The equilibrium water contact angle of the samples was measured at room temperature using the sessile drop method and image analysis of the drop profile with a CAM 100 Goniometer (KSV Instruments). Contact angles were calculated automatically by curve fitting with Young–Laplace equation. Four measurements for each specimen were carried out. Distilled deionized water (purified by MilliQ Plus system from Millipore) was chosen as probe liquid. The advancing (θ_a) and receding (θ_r) contact angles were measured by increasing or decreasing the water drop volume until three-phase boundary moves over the film surface.

3. Results and discussion

Fig. 1 is a schematic of the surface grafting process of *O*-phospho L-serine and *O*-phosphoethanolamine onto the surface of PEAA film. In the first step, PEAA film was activated with NHS and EDC. EDC intermediate can be used to form an amide between amine and carboxylic acid. However, NHS ester is more commonly used for such a reaction because EDC-activated carboxyl group is not stable and is easily deactivated [23]. NHS enhances the yield of EDC coupling reaction by the formation of the NHS ester. NHS esters are more stable in a solution with a pH of between 5 and 6 than at alkaline pH [20]. For this reason, activation

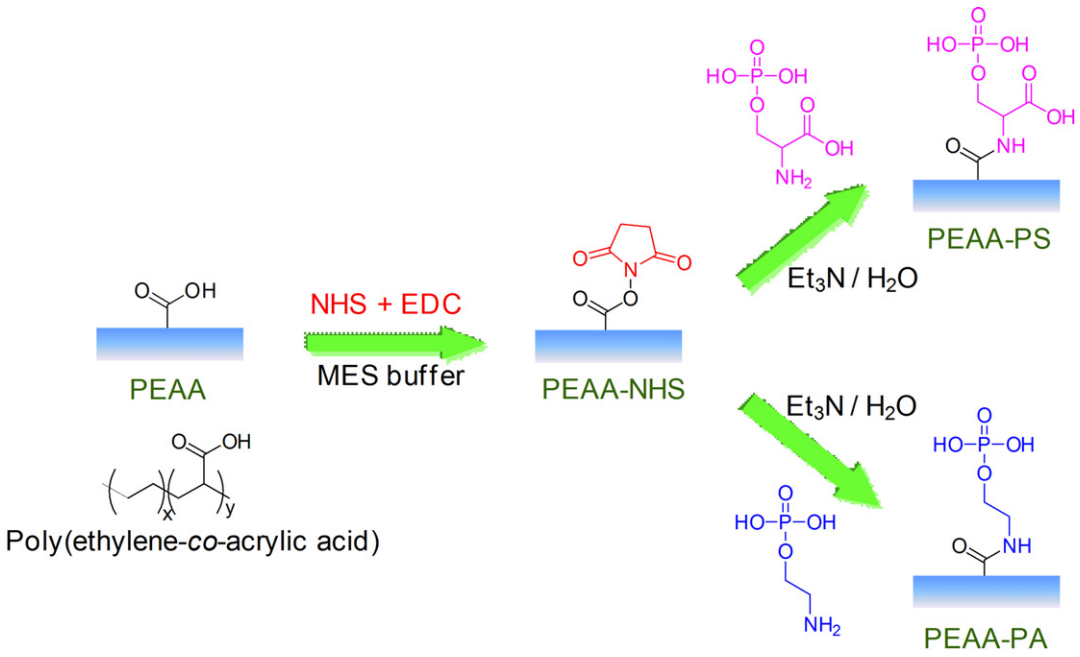

Fig. 1. Surface-grafting of *O*-phospho L-serine and *O*-phosphorylethanolamine onto the PEAA surface.

of PEAA films was carried out in pH 6.0 MES buffer solution in this study. After grafting NHS ester, TEA was used to increase pH in order to facilitate the grafting of *O*-phospho L-serine or *O*-phosphoethanolamine onto the surface of PEAA film. The grafting process requires the breaking and acylation of the NHS. Acylation was accomplished through a reaction of the NHS ester under a controlled pH range because the NHS ester only reacts with the deprotonated form of the amine [24]. Hydrolysis of NHS ester is a major competing reaction of the NHS-ester acylation. The rate of hydrolysis of an NHS ester increases with increasing pH. Low temperature is sometimes used to reduce the hydrolysis rate of the NHS ester and to obtain a high yield of surface grafting [25]. For convenience the grafting reaction was carried out at room temperature without significantly sacrificing the yield. The acylation reaction is favored in more concentrated solutions of grafting reagents. Therefore, a high concentration of *O*-phospho L-serine or *O*-phosphoethanolamine (2 wt%) was used in this study. The grafting of phosphate groups is also complicated by the fact that phosphate groups are unstable at high pH. High pH causes the phosphate group to degrade into phosphoric acid and a hydroxyl group [26]. One of the main efforts is to optimize the conditions including pH to maximize grafting efficiency of the phosphate onto the PEAA surface.

3.1. XPS study of the effect of pH on the surface grafting of phosphates

XPS, also called electron spectroscopy for chemical analysis (ESCA), is one of the most effective spectroscopic techniques available for surface analysis of polymers, and has many advantages for studying biomaterials. The advantages include the high sensitivity to surface, the high speed of analysis, the high information content, the low damage to the sample, and the ability to analyze samples without specimen preparation [27]. The latter advantage is particularly important because it allows biomedical devices (or parts of devices) to be inserted, as fabricated and sterilized, directly in the chamber for analysis. In this study, high-resolution XPS spectra were obtained to confirm the chemical structure of PEAA, PEAA-NHS, PEAA-PS, and PEAA-PA.

High-resolution C 1s scan of PEAA showed three peaks, attributable to COOH, C–COOH, and CH_2–CH_2 at 289.4 eV, 285.5 eV, and 285.0 eV, respectively (Fig. 2a). After the activation of PEAA with NHS and EDC, two new peaks appeared at 288.2 eV and 286.3 eV, which are characteristic of CON and C–CON as shown in high-resolution C 1s spectra (Fig. 2b). The peak of COO in NHS ester was observed at the same binding energy as that of COOH in PEAA. Those PEAA-PS and PEAA-PA films used for XPS analysis were synthesized at pH 7.10 and pH 7.59, respectively. In the PEAA-PS film, the peaks at 288.4 eV, 286.7 eV, and 286.0 eV represent CONH, CN, and COP, respectively (Figs. 2c and 2d). The peak of COOH in *O*-phospho L-serine appeared at the same binding energy as that of COOH in PEAA. After surface grafting of *O*-phosphoethanolamine on PEAA, peaks of CONH, CN, and COP were assigned at 288.4 eV, 286.7 eV, and 286.0 eV. A COOH peak was too weak to be observed. These peaks demonstrate that *O*-phospho L-serine and *O*-phosphoethanolamine were successfully grafted on the surface of the PEAA film.

To determine the optimum pH of the solution for surface grafting of *O*-phospho L-serine and *O*-phosphoethanolamine onto PEAA films, the surface grafting reaction was carried out at various pH levels. TEA was used to adjust the pH of the solution. XPS high-resolution P 2p spectra of all phosphate grafted PEAA films were obtained to analyze the phosphorus content (Figs. 3 and 4). In the case of PEAA-PS films, P 2p peak intensity of the film increased with pH up to pH 7.10, and decreased with pH after pH 8.16. The grafting efficiency of PEAA-PA changed with pH in a similar way to that of PEAA-PS. The highest phosphate intensity was observed for the grafted PEAA-PA synthesized at pH 7.59.

Elemental compositions of carbon and phosphorus for PEAA-PS and PEAA-PA synthesized at various pHs are shown in Fig. 5. Elemental composition of samples was obtained by converting XPS intensity ratios of the sample into atomic ratios using the sensitivity factors provided by the manufacturer. Theoretical atomic % of carbon in PEAA is ca. 92.18%. Atomic % of carbon in PEAA-PS and PEAA-PA decreased with increasing pH until pH 7.10 and pH 7.59, respectively. After that the atomic % of carbon on the sample films increased with pH. Conversely, phosphorus had the opposite tendency to that of carbon. The highest atomic % of phosphorus of PEAA-PS and PEAA-PA were 0.37% and 0.66%, respectively. These are lower than the theoretical atomic % of PEAA-PS (2.86%) and PEAA-PA (3.12%). However, this discrepancy is likely due to the

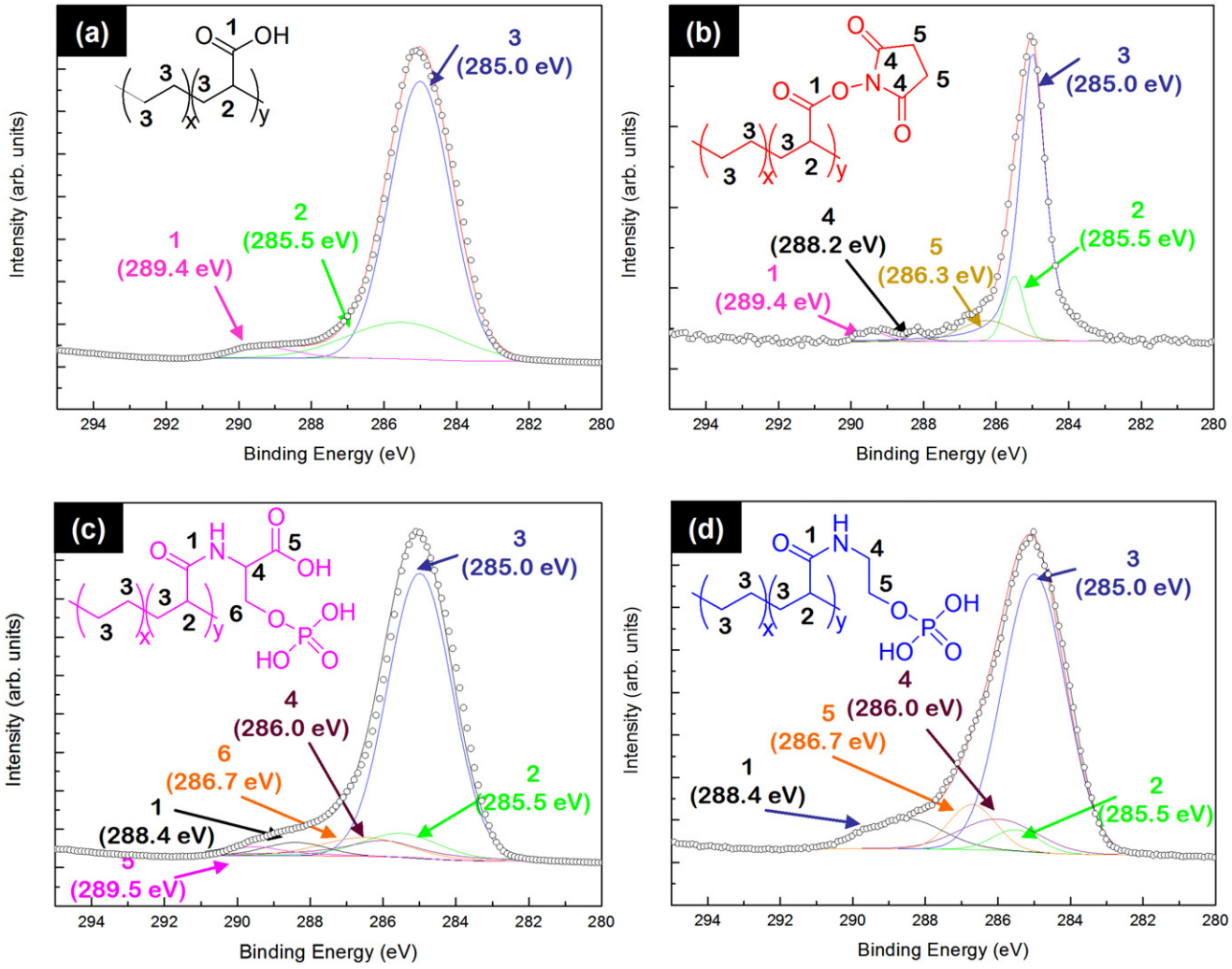

Fig. 2. XPS high-resolution C 1s spectra of (a) PEAA, (b) PEAA-NHS, (c) PEAA-PS, and (d) PEAA-PA.

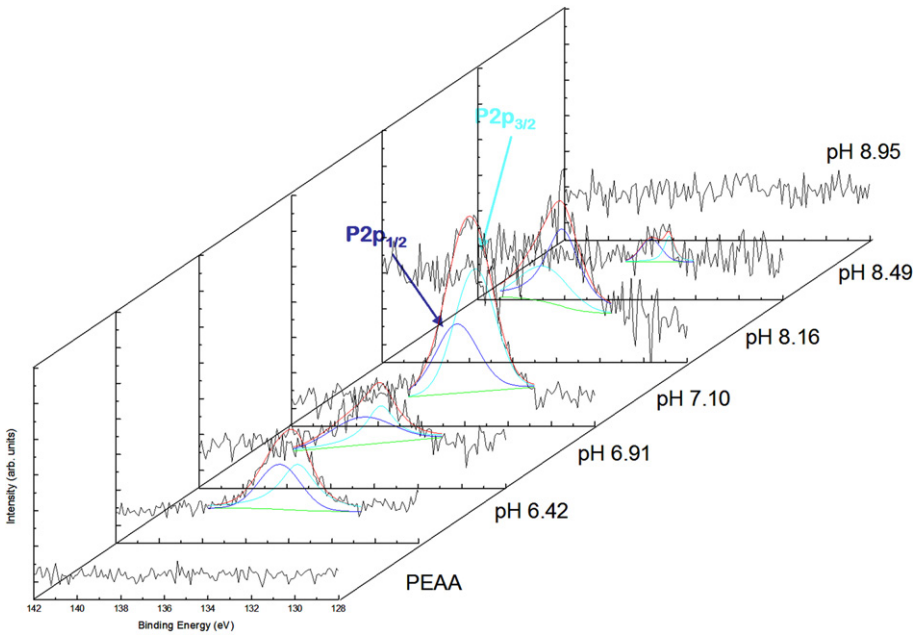

Fig. 3. High-resolution XPS P 2p spectra in relation to the pH of the reaction solution for surface grafting of *O*-phospho L-serine onto the PEAA surface; the pH values were 6.42, 6.91, 7.10, 8.16, 8.49, and 8.95.

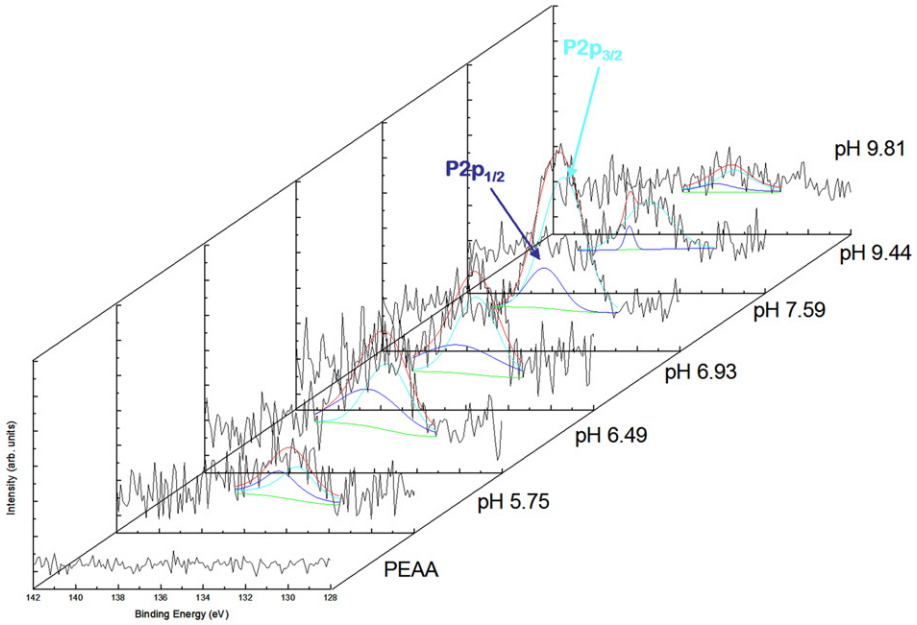

Fig. 4. High-resolution XPS P 2p spectra in relation to the pH of the reaction solution for surface grafting of *O*-phosphorylethanolamine onto the PEAA surface; the pH values were 5.75, 6.49, 6.93, 7.59, 9.44, and 9.81.

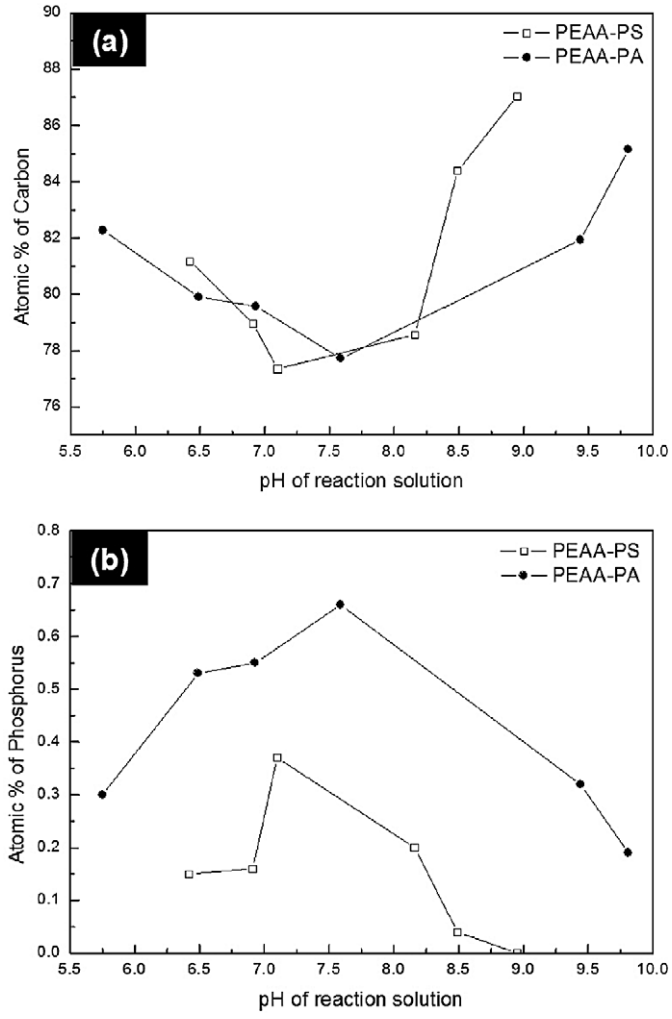

Fig. 5. Elemental composition of (a) C 1s and (b) P 2p for *O*-phospho L-serine and *O*-phosphorylethanolamine surface-grafted PEAA at various pHs of the reaction solution.

analysis depth of XPS. XPS analyzes a surface depth of greater than 10 Å [28], whereas the molecular length of surface-grafted *O*-phospho L-serine and *O*-phosphoethanolamine is ca. 5 Å if extended into a straight line [29].

Two possible explanations could account for the decrease in the amount of surface-grafted *O*-phospho L-serine and *O*-phosphoethanolamine at high pH. One is that PEAA-NHS was hydrolyzed during reaction at high pH and then converted back into PEAA. The other is that phosphate was broken into a hydroxyl group and a phosphate ion at high pH. In order to test this hypothesis, high-resolution C 1s spectra of PEAA-PS and PEAA-PA surface-grafted PEAA films at different pHs were analyzed (Fig. 6). For the comparison between samples, the peak intensity of C3 peak (carbon located in polyethylene part of PEAA) was fixed. C1 (amide), C6 (C–O–P), and C4 peaks of PEAA-PS increased with the reaction pH until pH 7.10. This means that the yield of surface-grafted *O*-phospho L-serine increased as the experimental pH increased. At pH 8.95, the peak intensity of C1 (amide) was equal to that of PEAA-PS surface-grafted at pH 7.10. However, a difference was noted at C6 (C–O–P). Therefore, there are equal amounts of amide bonds on PEAA-PS, but unequal amounts of phosphate at pH 8.95. This tendency was also observed in the case of PEAA-PA. The peak analysis results therefore support the concept that the decrease of phosphorus in PEAA-PS and PEAA-PA at high pH was caused by the degradation of phosphate.

3.2. Comparison of hydrophilic properties between PEAA-PS and PEAA-PA

Contact angle measurement is sensitive to the chemical composition of the top molecular layer (sampling depth: 3–20 Å [28]) and is a relatively simple, inexpensive, and effective technique for characterizing polymer surfaces. It is often desirable for biomaterials to be hydrophilic in nature in order to reduce the adhesion of bacteria, proteins, and platelets [30], to allow for improved cell seeding into a scaffold [31], and so forth. Equilibrium water contact angles for PEAA-PS and PEAA-PA that were synthesized at various pH are shown in Fig. 7. Both polymers showed the lowest contact angle at the reaction pH that resulted in the highest grafting yield. Notably, the contact angles of PEAA-PA were lower than

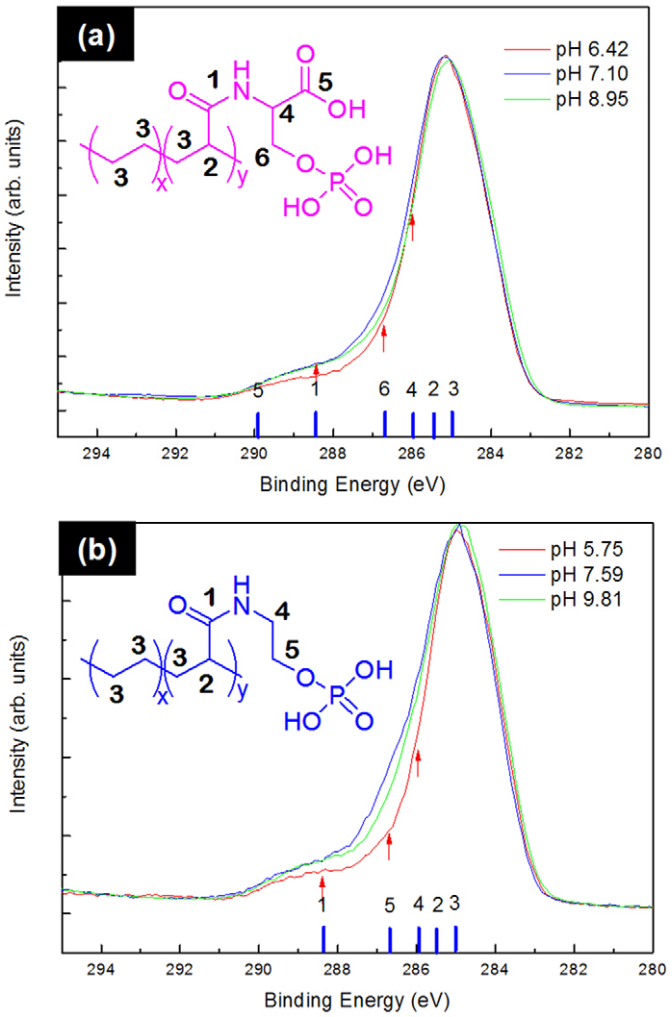

Fig. 6. High-resolution XPS C 1s spectra of (a) *O*-phospho L-serine and (b) *O*-phosphorylethanolamine surface-grafted PEAA at various pHs of the reaction solution.

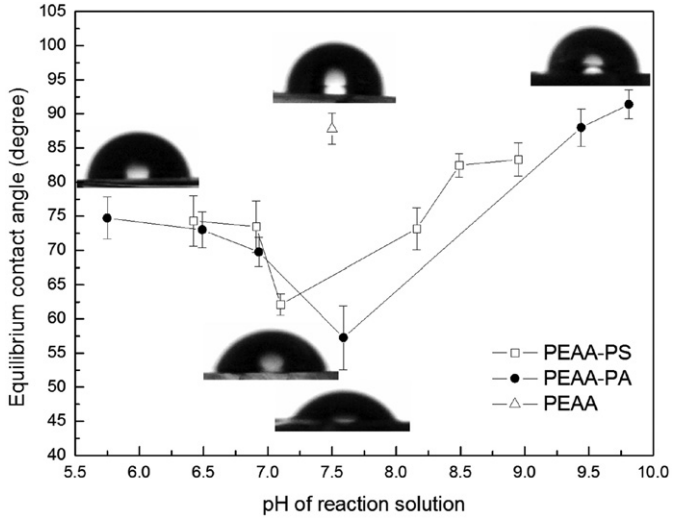

Fig. 7. Equilibrium contact angles for *O*-phospho L-serine and *O*-phosphorylethanolamine surface-grafted PEAA that were formed at various pHs of solution.

Table 1
Contact angles for *O*-phospho L-serine and *O*-phosphorylethanolamine surface-grafted PEAA that were formed at various pHs of the reaction solution.

Samples	Contact angles (degree)		
	θ_a	θ_r	H
PEAA	98	73	25
PEAA-PS			
pH 6.42	82	54	28
pH 6.91	77	45	32
pH 7.10	74	36	38
pH 8.16	78	49	29
pH 8.49	85	58	27
pH 8.95	84	60	24
PEAA-PA			
pH 5.75	79	48	31
pH 6.49	76	35	41
pH 6.93	75	28	47
pH 7.59	70	23	47
pH 9.44	95	70	25
pH 9.81	100	75	25

those of corresponding PEAA-PS in general. As characterized earlier (Fig. 5b), there were more phosphate groups grafted on PEAA-PA than on PEAA-PS. At the highest grafting percentage, the ratio of phosphate groups between PEAA-PA and PEAA-PS was about 2. The grafted molecule on PEAA-PA contains only a phosphate group, whereas the grafted molecule on PEAA-PS contains a phosphate group and a carboxylic acid group. Therefore the total number of grafted hydrophilic groups on PEAA-PA (phosphate) and PEAA-PS (carboxyl and phosphate) were similar at their highest grafting percentages. In several instances [32,33], it has been shown that phosphate groups are more hydrophilic than carboxylic acid group. One or two water molecules can form hydrogen bonds with a carboxyl group [34], whereas three or more water molecules can form hydrogen bonds with a phosphate group [35]. Increased hydrogen bonding between water and carboxyl or phosphate groups reduces interaction energy [34]. Contact angle measurement is the most common method of solid surface tension measurement. By the Young equation, low interfacial energy presents low contact angle [36]. Therefore, PEAA-PA is more hydrophilic than PEAA-PS.

Advancing contact angle (θ_a) and receding contact angle (θ_r) of each sample were measured by the sessile drop method (Table 1). PEAA-PS and PEAA-PA films showed the lowest advancing and receding contact angles at the reaction pH of the highest grafting condition. However, the change of θ_a value was smaller than that of θ_r value as the wettability of samples increase. Contact angle hysteresis, H, was obtained by the following equation; $H = \theta_a - \theta_r$. Thermodynamic hysteresis, which is obtained by this measurement, is independent of time. Surface roughness and heterogeneity are the primary causes of thermodynamic hysteresis. PEAA, PEAA-PS, and PEAA-PA are copolymers consisting of ethylene and acrylic acid or modified acrylic acid units. For this reason, the surfaces of these films are heterogeneous. In this study, flat PEAA films were used for modification. Therefore, the heterogeneous surface of unmodified and modified PEAAs is likely a reason to cause hysteresis. Johnson and Dettre proposed several qualitative conclusions about θ_a and θ_r on various heterogeneous surfaces [37,38]; (1) θ_a is more sensitive on predominantly hydrophilic surfaces, whereas θ_r is more sensitive on predominantly hydrophobic surfaces. (2) Either θ_a or θ_r alone is not a reliable measure of surface modification. We believe that the surface-grafted phosphate or carboxylic acid groups cover not only the domains of acrylic acid units but also the domains of ethylene units on the PEAA surface due to the high mobility and long chain-length of surface-grafted *O*-phospho L-serine and *O*-phosphoethanolamine. The highest H values of PEAA-PS and PEAA-PA were observed on films with the reaction pHs that resulted in the maximal surface-grafting, because θ_r decreased at a higher rate than θ_a at the initial grafting stage of *O*-phospho L-serine and *O*-phosphoethanolamine on PEAA films

although θ_a also decreased. Taken together, surface-grafted *O*-phospho L-serine and *O*-phosphoethanolamine molecules partly cover the hydrophobic domains (polyethylene) in PEAA. The initially low coverage of the hydrophobic regions of the surface by these molecules contributed to the slower initial decrease of θ_a, whereas phosphate and carboxyl groups contributed to the initial faster decrease of θ_r.

Hydrophilicity of PEAA has been effectively and significantly improved by successfully grafting phosphate groups as demonstrated by the water contact angle results in this study. For different biomedical applications, possibly different phosphate groups and likely different grafting percentages are required. Further grafting studies are warranted for various biomedical applications, including methods of increasing grafting yields as needed.

4. Conclusions

O-phospho L-serine and *O*-phosphoethanolamine were successfully grafted on PEAA surfaces in order to introduce phosphates in an aqueous solution. NHS and EDC were used to activate carboxylic acid in PEAA to form amides with *O*-phospho L-serine and *O*-phosphoethanolamine. Surface-grafting of *O*-phospho L-serine and *O*-phosphoethanolamine on PEAA were carried out at various pHs. From XPS high-resolution spectra, it was found that the phosphate group was broken into a hydroxyl group and a phosphoric ion in an aqueous solution at high pH values. However, a higher pH value increased the yield of amide formation. An optimal pH range of surface-grafting of *O*-phospho L-serine and *O*-phosphoethanolamine on PEAA was determined to be between 7 and 8. *O*-phosphoethanolamine grafted PEAA showed a lower contact angle than *O*-phospho L-serine grafted PEAA at a similar pH of grafting reaction. Surface-grafted phosphate and carboxylic acid likely covered not only the acrylic acid domains but also ethylene domains of the PEAA surface due to their high mobility and long chain-length after surface-grafting of *O*-phospho L-serine and *O*-phosphoethanolamine. This work on surface-grafting of phosphates in aqueous solution lays the foundation for future research into the introduction of phosphate groups onto the surfaces of synthetic polymeric and natural macromolecule derived biomaterials.

Acknowledgments

The authors would like to acknowledge the financial support from the National Institutes of Health (Research Grants DE015384, GM075840 and DE017689: P.X.M.). Y.G. Ko was partially supported by the Korea Research Foundation Grant from the Korean Government (MOEHRD, KRF-2006-214-D00207).

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