Phosphonic acid-functionalized poly(amido amine) macromers for biomedical applications

Seckin Altuncu ¹, Ece Akyol ¹, Melek Naz Guven ¹, Gozde Demirci ², Havva Yagci Acar ²,
Duygu Avci ^{1,*}

¹*Department of Chemistry, Bogazici University, 34342 Bebek, Istanbul, Turkey*

²*Department of Chemistry, Koc University, 34450 Sariyer, Istanbul, Turkey*

E-mail: avcid@boun.edu.tr

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jbm.a.36969

Abstract

Novel phosphonic acid-functionalized poly(amido amine) (PAA) macromers are synthesized through aza-Michael addition of 2-aminoethyl phosphonic acid or its mixture with 5-amino-1-pentanol at different ratios onto N,N' -methylene bis(acrylamide) to control the amount of phosphonic acid functionality. The macromers were homo- and copolymerized with 2-hydroxyethyl methacrylate at different ratios to obtain hydrogels with various hydrophilicities. The hydrogels' swelling, biodegradation and mineralization properties were evaluated. The swelling and degradation rates of the gels can be tuned by the chemical structure of PAA macromer precursors as well as pH and CaCl_2 pre-treatment. The hydrogels show composition-dependent mineralization in SBF and 5xSBF, as evidenced from Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDX) analyses. The degradation products of the hydrogels have no effect on U-2 OS, Saos-2 and NIH 3T3 cells, suggesting their cytocompatibility. Overall, these materials have potential to be used as nontoxic degradable biomaterials.

Keywords: macromers; phosphonic acid; poly(amido amine)s; biomaterials; mineralization

INTRODUCTION

Hydrogels, which are 3D networks of hydrophilic polymers capable of holding large amounts of water or biological fluids, hence resembling in some respects biological tissue, have great potential for biomedical applications in fields such as drug delivery systems, biosensors, self-healing materials and tissue engineering.^{1,2} Synthetic and biodegradable hydrogels are preferred to naturally formed hydrogels since their properties can be tuned by choice of chemical composition as well as methods of preparation.³ The controllable nanopore size may enable selective migration of cells and nutrients, and tailorable mechanical properties and degradation times make hydrogels suitable for various biomedical applications.⁴

Poly(amido amine)s (PAAs) are a promising class of synthetic biodegradable polymers. They are easily synthesized via Michael-type polyaddition of primary amines or bissecondary amines to bisacrylamides.⁵⁻⁷ PAAs show minimum cytotoxicity and are suitable for use in biomedical applications such as gene and drug delivery, imaging, and tissue engineering. They can be crosslinked using different methods including photopolymerization and they can even serve as crosslinkers to synthesize hydrogels.^{8,9}

The use of various types of amines and bisacrylamides with different substituent groups during PAA synthesis enables incorporation of additional functionalities; the biodegradability of the PAAs can be arranged by changing the nature of starting materials, their hydrophobic/hydrophilic balance and the amount of charged groups on them.¹⁰ These properties can carry over to their hydrogels, too. For example, bioactive and biomimetic compounds such as arginine (RGD mimicking structure) overcome the poor cell adhesion and proliferation problem of PAA hydrogels.^{11,12} Martello et al. synthesized HEMA hydrogels crosslinked with RGD-mimicking PAA by changing monomer ratio and reaction time to investigate the effect

of these parameters on final properties for tissue engineering; they observed improved cell adhesion and control on mechanical properties and degradation rate.¹³

It is well known that negatively charged groups such as the phosphate, (bis)phosphonate, carboxylate, sulfonic group on surfaces of hydrogels act as nucleating centers for the deposition of hydroxyapatite-like calcium phosphate and enhance mineralization which is a fundamental step for bone regeneration.¹⁴⁻²⁰ Additionally, phosphorous based groups promote cell adhesion and osteogenic differentiation.²¹⁻²⁴ Liu et al. synthesized alendronate functionalized methacryloyl gelatin and observed upregulation of the osteogenesis-related genes as well as improvement in mineralization.²⁵

In addition to the effect of anionic functional groups on mineralization, pH, temperature, polymer concentration and polymer chemistry control type of mineralized inorganic crystals.²⁶ For example, Phadke et al. showed that hydrophilicity also affects calcium binding and morphology of the mineral phase.²⁷

In this study, we designed new PAA macromers functionalized with phosphonic acid groups and their hydrogels for biomedical applications. We expect to combine advantageous properties of the phosphonic acid group, such as mineralization, mechanical and cell adhesion with biodegradability and biocompatibility of PAAs to obtain degradable hydrogels. The effect of the macromer structure on hydrogel properties such as swelling, degradation and mineralization of the hydrogels were investigated.

MATERIALS AND METHODS

Materials

N,N'-methylene bis(acrylamide) (MBA), 5-amino-1-pentanol (AP), 2-aminoethyl phosphonic acid (AEPA), 2-hydroxyethyl methacrylate (HEMA), 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) and all solvents were purchased from Aldrich Chemical Co. and used as received. Dulbecco's Modified Eagle Medium (DMEM) (with l-glutamine and high glucose (4.5g/l)), penicillin/streptomycin (pen-strep), fetal bovine serum (FBS) and trypsin-EDTA were purchased from Diagnosticon, Ebsdorfergrund (Germany). Thiazolyl blue tetrazolium bromide (MTT) was provided by Gold Biotechnology (USA). Phosphate buffered saline (PBS) tablets were provided by BBI Life Sciences (China). 96-well plates were purchased from Nest Biotechnology (China). NIH 3T3 mouse embryonic fibroblast cells and Saos-2 human osteosarcoma cells were a kind gift of Dr. Halil Kavakli (Department of Molecular Biology and Genetics, Koc University, Istanbul, Turkey). U-2 OS human bone osteosarcoma epithelial cells were a kind gift of Prof. Devrim Gozuacik (Department of Molecular Biology, Genetics and Bioengineering, Sabanci University, Istanbul, Turkey).

Characterization

^1H NMR spectra were recorded with a Varian Gemini 400 MHz spectrometer at ambient temperature in deuterated solvents. FTIR spectra were obtained with a Nicolet 380 FTIR spectrometer (Thermo Scientific) equipped with an attenuated total reflection (ATR) element. Potentiometric titrations were carried out using a WTW Inolab 720 pH meter and WTW SenTix 41 epoxy pH electrode at room temperature. The thermal transitions were verified by differential scanning calorimetry (DSC) with the DSC Q100 apparatus (TA Instruments) using heating rate of $10\text{ }^{\circ}\text{C min}^{-1}$ under nitrogen. Degradation studies of the hydrogel samples were

performed using an incubator shaker (VWR) operating at 37 °C and 200 rpm. The morphologies of the hydrogel samples were examined using scanning electron microscopy (SEM) (FEI-Philips XL30) under an accelerating voltage of 7.0 kV after sputter coating of the lyophilized samples with a gold layer. The EDX analysis was used to identify the elemental composition of materials. Thermogravimetric analysis (TGA) studies were performed under nitrogen with a TGA STA 6000 (Perkin Elmer) using a heating rate of 10 °C min⁻¹.

Synthesis of PAA macromers

Three PAA macromers were prepared by combining MBA with AP and/or AEPA at mole ratios MBA:AP:AEPA of 1.1:1.0:0, 1.1:0.7:0.3 and 1.1:0:1.0; and were denoted as PAA0, PAA30 and PAA100, respectively. The synthesis was carried out using a total concentration of 1.5 M in water/methanol mixture (v/v= 1/3) for the two/three starting compounds; at 50 °C for 3 days. For example, for the synthesis of PAA30, first AEPA was neutralized with potassium hydroxide by stirring a mixture of AEPA (265.1 mg, 2.12 mmol) and potassium hydroxide solution (0.13 g, 2.33 mmol in 2.5 mL water) at room temperature for one hour. Then, AP (0.51 g, 4.95 mmol) and MBA (1.2 g, 7.78 mmol) dissolved in 7.5 mL methanol were added to the solution under nitrogen atmosphere. The solution was stirred at 50 °C for 3 days and then methanol was removed under reduced pressure. The solution was then diluted with water and dialyzed using a membrane with a molecular weight cut-off of 1000 in distilled water to remove the unreacted components. The macromer was obtained as a white solid in 47 % yield after lyophilization. The synthesis of PAA0 and PAA100 follow the same procedure, without AEPA or AP. The yields of the products were 52 and 44 %, for PAA0 and PAA100, respectively.

pH-sensitivity of PAA macromers

PAA macromers (50 mg) were dissolved in deionized water at a concentration of 1.0 mg/mL and the pH of the solution was adjusted to around 2.0 using 2.0 mol/L of HCl solution. Then the solution was titrated with 0.1 mol/L NaOH solution at increments of 0.5 mL until pH 11.0. The pH values of the solution were monitored by a pH meter (WTW Inolab pH 720) at room temperature.

Synthesis of PAA and PAA-crosslinked HEMA hydrogels

HEMA and PAA macromer mixtures with weight ratios between 72:28 to 0:100 (HEMA:PAA) were dissolved in water at total monomer concentration of 25 w %. Irgacure 2959 (1 w % of total monomer weight) was added as a photoinitiator and the mixture was polymerized in a syringe by exposure to UV light (365 nm) for 30 min in a cylindrical photoreactor (inner radius 15 cm, height 30 cm) containing 12 Philips TL 8W BLB lamps on the perimeter. The hydrogel samples (diameter: 4mm, length: 7mm) were dried in lyophilizer and weighed (W_1). The samples were then immersed in ethanol (12 h) and in ultrapure water (24 h) to remove unreacted components. The samples were lyophilized and weighed again (W_2). The gelation percentage (GP) was calculated as

$$GP = \frac{W_2}{W_1} \times 100 \quad (1)$$

Degradation of the hydrogels

Each lyophilized hydrogel was weighed as W_i and then immersed in PBS (pH 7.4) or 0.5 M CaCl₂ solution at 37 °C. After the incubation at 37 °C for 1, 2 and 4 weeks, the samples were

removed, rinsed with water, freeze-dried and re-weighed as W_f . The degradation % was calculated according to

$$Degr adat i o n(\%) = \frac{W_i - W_f}{W_i} \times 100 \quad (2)$$

Swelling of the hydrogels

The dry hydrogel samples were weighed (W_i) and immersed in PBS (pH 5) or 0.5 M CaCl_2 solution at 37 °C. The samples were removed from the swelling medium after 24 h, blotted on filter paper and weighed (W_s). The swelling % was calculated using the following equation

$$Swell i n g(\%) = \frac{W_s - W_i}{W_i} \times 100 \quad (3)$$

Mineralization of the hydrogels

The hydrogels were mineralized using two different methods. In the first method, the samples were immersed in simulated body fluid (SBF)²⁸ for a period of four weeks, replacing the solution with a fresh one in every two days, rinsed with ultrapure water and lyophilized. In the second method, the samples were immersed in a 5xSBF solution prepared as described in literature²⁹ for 2 days, replacing the solution with a fresh one after one day. The samples were then immersed in ultrapure water for 24 h by refreshing the water in 6 h and lyophilized.

In vitro Cytotoxicity Assay

NIH 3T3, U-2 OS and Saos-2 cells were used to evaluate the cytotoxicity of the degradation products of the prepared gels. The cells were cultured in DMEM complete medium supplemented with 10% (v/v) FBS and 1% (v/v) pen-strep in 5% CO₂-humidified incubator at 37 °C and passaged every 2-3 days. For the cytotoxicity assay, the degradation products of the hydrogels in different concentrations ranging from 12.5-200 µg/mL by serial dilution in 100 µl were introduced into 96-well plates. Then, the cells were seeded at a density of 15x10³ cells/well in 100 µl DMEM complete medium and incubated at 37 °C in 5% CO₂ atmosphere. After 48 h further incubation, the cell viability was assessed using MTT colorimetric assay by the addition of 50 µl of MTT solution (5 mg/mL in PBS) into each well with 150 µL of culture medium and incubated for 4 h. The purple formazan crystals formed as a result of mitochondrial activity in viable cells were dissolved with ethanol:DMSO (1:1 v/v) mixture. Each sample's absorbance at 600 nm with a reference reading at 630 nm was recorded using a Synergy – H1 microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Cells which were not exposed to degradation products of gels were used as controls. 100% viability is assumed for the control cells. The relative cell viability was calculated from equation (4):

$$Cell\ viability(\%) = \frac{Absorbance_{sample}}{Absorbance_{control}} \times 100 \quad (n = 4) \quad (4)$$

Statistical Analysis

Statistical analyses of the degradation products were conducted by using ordinary one-way ANOVA analysis of variance followed by multiple Dunnett's comparison test of GraphPad

Prism 8.3.0 software package (GraphPad Software, Inc., USA). All measurements were expressed as mean values $\pm$ standard deviation (SD) based on 4 replica. $p < 0.05$ was accepted as statistically significant difference.

RESULTS

Synthesis and characterization of the PAA macromers

Three biodegradable PAA macromers were synthesized by aza-Michael addition of primary amines (AEPA and/or AP) and MBA as shown in Fig. 1. Herein we selected AEPA and AP alone and both together to react with MBA to investigate effect of phosphonic acid functionality on material properties. The molar ratio of the MBA to amines was fixed at 1.1:1 to obtain acrylamide terminated products which can be used as crosslinkers. AEPA was first activated towards aza-Michael addition by neutralization with potassium hydroxide. The solvent was selected as methanol:water (3:1 v/v) because potassium salt of AEPA being soluble only in water and methyl bisacrylamide highly soluble in methanol, all three macromers are soluble in the mixture. Also, it is known that the presence of water facilitates efficiency of the aza-Michael reaction by activating both the amine and the acrylate through hydrogen bonding.³⁰

The composition and properties of the macromers are given in Table 1. As discussed in the Materials and Methods section, they were designated as PAA_w, where *w* is the mole fraction of AEPA in the feed. The macromers were obtained as white solids in 44-52 % yields. They are highly soluble in polar (water, ethanol) solvents. Their structures were confirmed by ¹H-NMR and FTIR spectroscopy. For example, as seen in Fig. 2, ¹H NMR spectra of all macromers show the characteristic peaks of acrylamide double bond protons at 5.65 (b) and 6.10 (a) ppm. The peak at 1.88 (g) ppm (in the spectrum of PAA100) belongs to methylene protons adjacent

to phosphorous. This peak shifted from 1.88 to 1.56 (g) ppm in the spectrum of PAA30. The peaks at 1.14 (j), 1.31 (k) and 1.37 (i) ppm (in the spectrum of PAA0) are ascribed to the methylene protons of AP. The peaks at 4.44 and 4.51 (c and n) ppm are the characteristic methylene (at the end and middle of the macromer) peaks of the MBA. The molecular weights and average number of repeating units of PAA macromers were calculated using $^1\text{H-NMR}$ by integrating acrylamide double bond protons at 5.65 and 6.10 ppm with respect to AP peaks at 1.14-1.37 ppm and/or AEPA peak at 1.56 ppm (Table 1). The FTIR spectra of the macromers (Fig. 3) show strong peaks at around 1631 and 1528 cm^{-1} due to C=O and NH stretchings. Also, the peaks at 1043 and 963 cm^{-1} corresponding to the symmetric and asymmetric vibrations of P-O are present in the spectra of PAA30 and PAA100.

The thermal properties of the macromers have been investigated using DSC, and their T_g 's have been determined as 1, 15 and 64 $^{\circ}\text{C}$ (taken as averages) for PAA0, PAA30 and PAA100, respectively (Table 1, Figure S1). The pH sensitivity of PAA macromers was evaluated by the acid-base titration, as shown in Fig. 4. The pH value increased gradually with the addition of NaOH solution, reaching a plateau in the pH range of about 5.8-7.8 for PAA0 and PAA30, which is characteristic for poly(amido amine)s as seen in the literature.^{31,32} The plateau is wider for PAA0, whereas PAA100 does not show a distinct plateau region.

Synthesis of PAA and PAA-crosslinked HEMA hydrogels

HEMA containing different amounts of PAA0, PAA30 and PAA100 was photopolymerized in water using Irgacure 2959 as photoinitiator. Table 2 summarizes initial compositions and gelation percentages of the synthesized hydrogels. The samples are named as H-PAAx-y%

where “H” stands for hydrogel, PAAx indicates crosslinker, x is the mol% of AEPA in PAA (in the feed) and y stands for weight% of PAAx. Gelation % (GP) values of the hydrogels were found to be between 72 and 87.

Swelling and degradation studies of the hydrogels

Mass swelling percentages of the synthesized hydrogels were studied in 0.5 M CaCl_2 and two different buffers with pH 5 and pH 7.4. Swelling percentages of hydrogels after 24 h are shown in Figure 5; they range from about 400% to about 950%. The hydrogels reached equilibrium very fast, in a period of the order of 15 minutes.

The degradation of the hydrogels with 50 w% of PAA0 and PAA30 crosslinkers were investigated in PBS (pH 7.4) for 4 weeks, including the case with pre-treatment by immersion in 0.5 M CaCl_2 solution; with results shown in Fig. 6, together with SEM micrographs of the PAA30 hydrogel before and after degradation. It is seen that the degradations for both hydrogels are similar and around 20 % after 4 weeks if not pre-treated with the CaCl_2 solution. The SEM micrograph of degraded H-PAA30-50% shows that the hydrogel seems to have degraded choosing an orientation somehow.

Mineralization of the hydrogels networks

The mineralization abilities of the synthesized hydrogels were evaluated in two ways: (a) immersion in SBF for 4 weeks, (b) immersion in 5xSBF for 2 days. SBF was chosen because it closely mimics the ionic concentrations and pH typically observed in plasma.²⁷ Hence, formation of apatite like structure on a scaffold in SBF indicates its capacity for bone tissue engineering applications. However, the mineralization was slow under SBF conditions.

Therefore 5xSBF with higher concentrations of Ca^{2+} and HPO_4^{2-} was used to increase rate of mineralization. Before soaking in the mineralization solutions, some of the hydrogels were immersed in 0.2 M CaCl_2 solution to exchange potassium ions bound to phosphonic acid groups with calcium, expecting acceleration of mineralization because of increase in Ca^{2+} release from the polymer.³³ Mineralization of the hydrogels was studied by using SEM, FTIR and TGA.

SEM micrographs of H-PAA0-50% (control) and H-PAA30-50% after soaking in SBF for 4 weeks are presented in Fig.7, together with the associated FTIR spectra. Surprisingly, the control hydrogel containing only AP groups showed mineral layers (Fig. 7b) and its FTIR spectrum showed strong peaks at 1002 and 548 cm^{-1} corresponding to P-O bond, similar to HAP.

For H-PAA30-50 % hydrogels, we could not observe any CaP formation unless we immerse the hydrogels in CaCl_2 solution before mineralization (Fig. 7c and 7d). EDX was used to determine the elemental composition of the inorganic complex shown in Fig. 7e. The stoichiometric ratio of Ca/P was calculated as 1.67 which is same with theoretical value of HAP. Additionally SEM mapping analysis demonstrates the deposition of Ca, P and O atoms on this part.

For studies in 5xSBF, hydrogels with three different compositions of PAA30 (50%, 75% and 100%) were used. This way we also evaluated the effect of matrix structure (concentration of phosphonic acid and AP) on formation of apatite-like phases on a hydrogel. These hydrogels have increasing concentration of both AEPA and AP (5.8, 8.7 and 11.6 w% AEPA and 13, 20 and 26.5 w% AP). The mineralized samples were characterized by FTIR analysis (Fig 8a). The FTIR spectra of the PAA30-100% hydrogels contained two broad peaks at around 1015 and

556 (P-O) cm^{-1} which is related with HAP. The TGA thermograms of HPAA30-100% before and after mineralization show the initial weight loss around 100 °C due to removal of water, the main weight loss between 200 and 400 °C because of main chain degradation and char yield at 600 °C (Fig. 8b). The char yield of the hydrogels after mineralization were higher (27%) than those (19%) of before mineralization, confirming mineral deposition. SEM images of these hydrogels showed mineral layers (Fig. 8d and e). FTIR spectrum of the hydrogel samples synthesized with PAA30-75% also showed an apatite-like mineral (Fig. 8a). However, the hydrogel samples prepared from PAA30-50% showed no noticeable mineralization, with no change in FTIR spectrum after mineralization (Fig 8a). Surprisingly, the hydrogels (H-PAA100-50%) containing only AEPA showed no mineralization, either.

Cytotoxicity of PAA hydrogels

To evaluate cytotoxicity of the degradation products a dose dependent study was performed on NIH 3T3, U-2 OS and Saos-2 cells using the standard MTT assay (Fig. 9). Based on ISO 10993-5, the cell viability above 80% is defined as noncytotoxic and between 80 and 60 as weakly cytotoxic.³⁴ Viability of all type of cells treated with degradation products were above 75%, indicating no significant toxicity.

DISCUSSION

Synthesis and characterization of the novel PAA macromers

We have illustrated that PAA macromers with desired amount of phosphonic acid functionalization can be synthesized; the NMR and FTIR spectroscopies have confirmed that

the syntheses have been successful. The AEPA:AP mol ratio in the macromer (2.6:7.4) was found to be similar to the AEPA:AP mol ratio (3.0:7.0) in the feed (PAA30).

The thermal properties of the macromers investigated using DSC showed the effect of chemical structure of the macromers on their T_g (Table 1). We observed that incorporation of AEPA on the macromer structure increases T_g , from 1 °C for PAA0 to 64 °C for PAA100 (Figure S1). This increase can be explained by a decrease in the mobility of the macromer chains significantly due to electrostatic interactions of AEPA side groups.

The plateaus in the titration curves (Fig. 4) indicate the buffering capacities of PAA0 and PAA30 in the physiologically relevant range of pH 6-7.4. When the pH is below the pK_b of the macromer the protonation of the tertiary amine groups occurs, whereas the same deprotonates when the pH is above the pK_b . As shown in Fig. 4, the pH-responsiveness plateau is influenced by the composition or hydrophilicity of the macromers. The wider plateau of PAA0 indicates its better pH-responsive performance, whereas PAA100 with higher hydrophilicity does not show a distinct plateau region.

Synthesis of PAA and PAA-crosslinked HEMA hydrogels

PAA macromers were used as crosslinkers in different amounts for the preparation of HEMA hydrogels as potential biomaterials and to investigate the effect of phosphonic acid functionalization on the final properties of the materials (Table 2). The syntheses were carried out successfully and the gelation % (GP) values of the hydrogels steadily decreased from 87% to about 72% when the phosphonic acid functionalized component was increased to 50%, but did not decrease further when the ratio was increased towards 100%.

Swelling and degradation studies of the hydrogels

In general, both molecular structure and molecular weight of the macromers are expected to affect the swelling properties of gels obtained from them. For example, the hydrogel with 50 w% of PAA30 ($M_n = 1943$) is observed to show slightly higher water uptake than the corresponding PAA0 ($M_n = 2650$)-crosslinked hydrogel at pH 7.4, indicating that the anionic character of the crosslinker dominates over the crosslink density. The incorporation of PAA30 into the hydrogel was increased to investigate the effect of the concentration of the phosphonic acid functionality. As seen from Figure 5, the equilibrium swelling percentages are almost same for all compositions probably due to relatively insignificant amount of phosphonic acid functionality (5.8, 8.7 and 11.6 w%). The hydrogel prepared from PAA100 showed highest swelling at pH 7.4 because of the highest charge or hydrophilic property. In this figure, the clearest trend is the higher swelling values in the more acidic medium (pH 5) at lower AEPA concentrations and its steady decrease with increase of the same. The high swelling comes from the increase in total charge due to the protonation of the amine groups, whose effect is increasingly ameliorated by the zwitterionic nature of the AEPA functionality attracting the chains together electrostatically. For example, the hydrogel with 50 w% of PAA100 is observed to show the lowest water uptake among all the hydrogels. When the hydrogels were immersed in 0.5 M CaCl_2 solution, exchange of potassium ions with calcium is expected. The interaction between phosphonic acid groups and Ca^{2+} ions may form physical crosslinking.^{18,19} However, it was observed that phosphonic acid content in the hydrogel needs to reach a certain level to

observe a noticable shrinkage. The hydrogel (H-PAA100-50%) containing the highest AEPA (21.2 w%) swelled 45.8 % less in CaCl_2 solution compared to PBS.

PAA degradation occurs by hydrolytic cleavage of amide bonds.³⁵ Therefore, the control hydrogels synthesized from the higher molecular weight macromer (PAA0) are expected to degrade faster because of the higher amount of amide linkages between the crosslinks. However, the hydrophilic character of the anionic group in the hydrogels synthesized from PAA30 macromer compensates this effect, explaining the similar degradations for the two hydrogels as seen in Figure 6a. For the pre-treated samples, one might expect similar degradation since their swelling was the same, and the water content would determine the effect of hydrolysis. Less degradation of the acid containing hydrogels than the control one shows that H-PAA30-50% forms chelates with Ca^{2+} ions whereas the control hydrogel does not. The Ca^{2+} -phosphonic acid complex acts as a second crosslinked network and slows down the mass loss.¹⁶ The oriented pattern of degradation in the SEM micrograph of degraded H-PAA30-50% may prove its homogeneity.

Mineralization of the hydrogels networks

The control hydrogel's (H-PAA0-50%) better mineralization performance might be explained with the observation in the literature that not only polyanions, but also polycations such as linear PEI have a strong influence on CaP mineralization.³⁶ LPEI accelerates the transformation brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) (DCPD) to HAP by acting as a proton acceptor, which takes up the protons released from either the calcium phosphate precipitate during the phase transformation

or from ions present in solution during a presumed, but so far not directly observed, dissolution–reprecipitation process.³⁷

The premineralization treatment making CaP formation possible for H-PAA30-50 % hydrogels probably formed limited nucleation points on phosphonic acid groups which support mineralization.^{16,38} The EDX and SEM analyses strongly suggest that HAP has actually formed. Studies in 5xSBF did give results in 2 days, confirming the importance of the ion concentration in SBF. From the results given above, it can be concluded that macromer concentration and its composition is very important for mineralization. The AEPA:AP ratio in H-PAA30-100% homogel is the most proper one due to hydrophilicity and architecture for mineralization.

The *in vitro* cytotoxicity studies of the degradation products on NIH 3T3, U-2 OS and Saos-2 cells showed no significant toxicity.

Conclusion

It was shown that phosphonic acid functionalized primary amines can be used for the synthesis of novel phosphonic acid-functional PAA macromers and these macromers can be incorporated into hydrogels by homo- or copolymerization with HEMA at different ratios to control the phosphonic acid concentration, thus hydrophilicity. The T_g of the PAA macromers increased with incorporation of phosphonic acid on the macromer structure. Swelling of the hydrogels is mostly governed by the amount of phosphonic acid in their structures, pH of the medium and CaCl_2 -pre-treatment. The degradability of hydrogels decreases when they are pre-treated with CaCl_2 , due to the presence of Ca^{2+} -phosphonic acid complex, parallel to their swelling behavior. It was observed that small changes in composition of the hydrogels can influence the generation of hydroxyapatite-like mineral phases. While hydrogels prepared from only AP functionalized

macromers promote mineralization, the hydrogels obtained from only AEPA functionalized ones have no apatite nucleation ability. The hydrogels which have both AP and AEPA in their structures showed different mineralization abilities depending on their ratios, probably due to hydrophilicity. The pre-treatment of hydrogels with CaCl_2 increases the capacity of the hydrogels for mineralization. The degradation products of these new materials showed no toxicity on cells and therefore they can be considered as potential candidates in various biomedical applications.

Acknowledgments: This work was financed by TUBITAK grant number 117Z330.

Conflict of Interest: All authors have no conflict of interest.

REFERENCES

1. Ferreira NN, Ferreira LMB, Cardoso VMO, Boni FI, Souza ALR, Gremião MPD. Recent advances in smart hydrogels for biomedical applications: From self-assembly to functional approaches. *Eur Polym J* 2018;99:117-133.
2. Kumar A, Rao MK, Han SS. Synthesis of mechanically stiff and bioactive hybrid hydrogels for bone tissue engineering applications. *Chem Eng J* 2017;317:119-131.
3. Gyles DA, Castro LD, Silva JOC Jr., Ribeiro-Costa RM. A review of the designs and prominent biomedical advances of natural and synthetic hydrogel formulations. *Eur Polym J* 2017;88:373-392.

4. Chen S, Shi J, Xu X, Ding J, Zhong W, Zhang L, Xing M, Zhang L. Study of stiffness effects of poly(amidoamine)–poly(n -isopropyl acrylamide) hydrogel on wound healing. *Colloids Surf B Biointerfaces* 2016;140:574-582.
5. Ferruti P, Bianchi S, Ranucci E, Chiellini F, Caruso V. Novel poly(amido-amine)-based hydrogels as scaffolds for tissue engineering. *Macromol Biosci* 2005;5(7):613-622.
6. Jacchetti E, Emilriti E, Rodighiero S, Indrieri M, Gianfelice A, Lenardi C, Podesta A, Ranucci E, Ferruti P, Milani P. Biomimetic poly(amidoamine) hydrogels as synthetic materials for cell culture. *J Nanobiotechnology* 2008;6(1):14.
7. Emilriti E, Guizzardi F, Lenardi C, Suardi M, Ranucci E, Ferruti P. Novel Poly (amidoamine)-based hydrogels as scaffolds for tissue engineering. *Macromol Symp* 2008; 266(1):41-47.
8. Gevorgyan S, Rossi E, Cappelluti MA, Tocchio A, Martello F, Gerges I, Lenardi C, Milani P, Argenti S. Photocrosslinked poly(amidoamine) nanoparticles for central nervous system targeting. *Colloids Surf B Biointerfaces* 2017;151:197-205.
9. Tocchio A, Martello F, Tamplenizza M, Rossi E, Gerges I, Milani P, Lenardi C. RGD-mimetic poly(amidoamine) hydrogel for the fabrication of complex cell-laden micro constructs. *Acta Biomater* 2015;18:144-154.
10. Peng B, Lai X, Chen L, Lin X, Sun C, Liu L, Qi S, Chen Y, Leong KW. Scarless Wound Closure by a Mussel-Inspired Poly(amidoamine) Tissue Adhesive with Tunable Degradability. *ACS Omega* 2017;2(9):6053-6062.

- Accepted Article
11. Shi J, Ouyang J, Li Q, Wang L, Wu J, Zhong W, Xing MMQ. Cell-compatible hydrogels based on a multifunctional crosslinker with tunable stiffness for tissue engineering. *J Mater Chem* 2012;22(45):23952.
 12. Ferruti P, Bianchi S, Ranucci E, Chiellini F, Piras AM. Novel agmatine-containing poly(amidoamine) hydrogels as scaffolds for tissue engineering. *Biomacromolecules* 2005; 6:2229-2235.
 13. Martello F, Tocchio A, Tamplenizza M, Gerges I, Pistis V, Recenti R, Bortolin, M, Fabbro MD, Argenti S, Lenardi C. Poly(amido-amine)-based hydrogels with tailored mechanical properties and degradation rates for tissue engineering. *Acta Biomater* 2014;10(3):1206-1215.
 14. Chen L, Yang K, Zhao H, Liu A, Tu W, Wu C, Chen S, Guo Z, Luo H, Sun J, Fan H. Biomaterialized hydrogel with enhanced toughness by chemical bonding of alkaline phosphatase and vinylphosphonic acid in collagen framework. *ACS Biomater Sci Eng* 2019; 5(3):1405-1415.
 15. Salama A. Chitosan based hydrogel assisted spongelike calcium phosphate mineralization for in-vitro BSA release. *Int J Biol Macromol* 2018;108:471-476.
 16. Yang X, Akhtar S, Rubino S, Leifer K, Hilborn J, Osipov D. Direct "Click" synthesis of hybrid bisphosphonate-hyaluronic acid hydrogel in aqueous solution for biomineralization. *Chem Mater* 2012;24(9):1690-1697.
 17. Lin Z, Cao S, Chen X, Wu W, Li J. Thermoresponsive hydrogels from phosphorylated ABA triblock copolymers: A potential scaffold for bone tissue engineering. *Biomacromolecules* 2013;14(7):2206-2214.

18. Guven MN, Altuncu MS, Bal T, Oran DC, Gulyuz U, Kizilel S, Okay O, Avcı D. Bisphosphonic acid-functionalized cross-linkers to tailor hydrogel properties for biomedical applications. *ACS Omega* 2018;3(8):8638-8647.
19. Dey RE, Zhong X, Youle PJ, Wang QG, Wimpenny I, Downes S, Hoyland JA, Watts DC, Gough JE, Budd PM. Synthesis and characterization of poly(vinylphosphonic acid-co-acrylic acid) copolymers for application in bone tissue scaffolds. *Macromolecules* 2016;49(7):2656-2662.
20. Mai T, Wolski K, Puciul-Malinowska A, Kopyshv A, Gräf R, Bruns M, Zapotoczny S, Taubert A. Anionic polymer brushes for biomimetic calcium phosphate mineralization-A surface with application potential in biomaterials. *Polymers* 2018;10(10):1165.
21. Dey RE, Wimpenny I, Gough JE, Watts DC, Budd, PM. Poly(vinylphosphonic acid-co-acrylic acid) hydrogels: The effect of copolymer composition on osteoblast adhesion and proliferation. *J Biomed Mater Res Part A* 2017;106(1):255-264.
22. Wang QG, Wimpenny I, Dey RE, Zhong X, Youle PJ, Downes S, Watts DC, Budd PM, Hoyland JA, Gough JE. The unique calcium chelation property of poly(vinyl phosphonic acid-co-acrylic acid) and effects on osteogenesis in vitro. *J Biomed Mater Res Part A* 2017;106(1):168-179.
23. Kim S, Cui Z-K, Fan J, Fartash A, Aghaloo TL, Lee M. Photocrosslinkable chitosan hydrogels functionalized with the RGD peptide and phosphoserine to enhance osteogenesis. *J Mater Chem B* 2016;4(31):5289-5298.

24. Nuttelman CR, Benoit DSW, Tripodi MC, Anseth KS. The effect of ethylene glycol methacrylate phosphate in PEG hydrogels on mineralization and viability of encapsulated hMSCs. *Biomaterials* 2006;27(8):1377-1386.
25. Liu L, Li X, Shi X, Wang Y. Injectable alendronate-functionalized GelMA hydrogels for mineralization and osteogenesis. *RSC Adv* 2018;8(40):22764-22776.
26. Bleek K, Taubert A. New developments in polymer-controlled, bioinspired calcium phosphate mineralization from aqueous solution. *Acta Biomater* 2013;9(5):6283-6321.
27. Phadke A, Zhang C, Hwang Y, Vecchio K, Varghese S. Templated mineralization of synthetic hydrogels for bone-like composite materials: Role of matrix hydrophobicity. *Biomacromolecules* 2010;11(8):2060-2068.
28. Kokubo T, Takadama H. How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* 2006;27(15):2907-2915.
29. Chou YF, Chiou WA, Xu Y, Dunn JCY, Wu BM, The effect of pH on the structural evolution of accelerated biomimetic apatite. *Biomaterials* 2004;25:5323-5331.
30. Manfredi A, Ranucci E, Suardi M, Ferruti P. Polymerization kinetics of poly(amidoamine)s in different solvents. *J Bioact Compat Pol* 2007;22(2):219-231.
31. Mateos-Timoneda MA., Lok MC, Hennink, WE, Feijen J, Engbersen JFJ, Poly(amido amine)s as gene delivery vectors: Effects of quaternary nicotinamide moieties in the side chains. *ChemMedChem* 2008;3:478-486.

32. Lin C, Engbersen JFJ, PEGylated bio-reducible poly(amido amine)s for non-viral gene delivery. *Mater Sci Eng C* 2011;31:1330-1337.
33. Elliott JC. *Structure and Chemistry of the Apatite and Other Calcium Orthophosphates*; Elsevier: Amsterdam, The Netherlands, 1994.
34. Wallin RF, Arscott E. A practical guide to ISO 10993-5: Cytotoxicity. *Med Device Diagn Ind* 1998;20:96-98.
35. Ferruti P, Marchisio MA, Duncan R. Poly(amido-amine)s: Biomedical applications. *Macromol Rapid Commun* 2002;23:332-355.
36. Shkilnyy A, Friedrich A, Tiersch B, Schöne S, Fechner M, Koetz J, Schlapfer CW, Taubert A, Poly(ethylene imine)-controlled calcium phosphate mineralization. *Langmuir* 2008;24:2102-2109.
37. Shkilnyy A, Schöne S, Rumplach C, Uhlmann A, Hedderich A, Günter C, Taubert A, Calcium phosphate mineralization with linear poly(ethylene imine): a time-resolved study. *Colloid Polym Sci* 2011;289:881-888.
38. Vieira S, da Silva Morais A, Garet E, Silva-Correia J, Reis RL, González-Fernández Á, Miguel Oliveira J. Self-mineralizing Ca-enriched methacrylated gellan gum beads for bone tissue engineering. *Acta Biomater* 2019;93:74-85.

List of Tables

Table 1. Compositions, number average molecular weights (M_n), yield and T_g of the macromers

Table 2. Composition of PAA and PAA-crosslinked HEMA hydrogels

List of Figures

Figure 1. Syntheses of the PAA macromers.

Figure 2. ^1H -NMR spectra of PAA0, PAA30 and PAA100.

Figure 3. FTIR spectra of PAA0, PAA30 and PAA100.

Figure 4. Acid-base titration curves of the 1 mg/mL aqueous solutions of PAA0, PAA30 and PAA100.

Figure 5. Swelling percentages of PAA hydrogels after 24 h. All the experiments were carried out in triplicate ($n=3$) and mean average values with standard error ($\pm\text{SD}$) were represented in the error bars.

Figure 6. (a) The degradation of H-PAA0-50% and H-PAA30-50% hydrogels in pH 7.4 (one pre-treated with 0.5 M CaCl_2); (b) SEM micrographs of H-PAA30-50%, (b) before and (c) after 4 weeks degradation.

Figure 7. (a) FTIR spectra of H-PAA30-50% before (top); and H-PAA30-50% and H-PAA0-50%, treated as labeled, with HAP inset, (b) (c) (d) SEM micrographs of the top three cases listed in (a), in order, (e) 8000x and mapped image of (d).

Figure 8. (a) FTIR spectra of samples as labeled, after 2 days treatment in 5xSBF, (b) TGA thermograms of H-PAA30-100% before and after the same treatment, (c) (d) SEM micrographs of the same, before and after treatment, (e) 5000x image of (d).

Figure 9. The effect of degradation products on cell viability of (a) U-2 OS, (b) Saos-2 human osteosarcoma cells, (c) NIH 3T3 mouse embryonic fibroblast cells. Cells were treated with different concentrations of the products for 24 h. The cell viability test was performed by MTT assay ($\pm$ SD; $n = 5$; $p < 0.05$ compared with all concentrations).

Table 1. Compositions, number average molecular weights (M_n), yield and T_g of the macromers

Macromer	AEPA:AP in feed (mol)	AEPA:AP in macromer (mol)	M_n^a (g/mol)	Yield (%)	AEPA (w %)	T_g (°C)
PAA0	0:1.0	0:1.0	2650	52	0	4
PAA30	3.0:7.0	2.6:7.4	1943	47	11	24
PAA100	1.0:0	1.0:0	2946	44	42	68

^aCalculated from ^1H NMR spectra

Table 2. Composition of PAA and PAA-crosslinked HEMA hydrogels

Hydrogels	HEMA:PAA (w %)	AP & AEPA (w %)	Gelation (%)
H-PAA0-50%	50 : 50	18.8 & 0	87
H-PAA30-50%	50 : 50	13 & 5.8	72
H-PAA30-75%	25 : 75	20 & 8.7	75
H-PAA30-100%	0 : 100	26.5 & 11.6	72
H-PAA100-28%	72 : 28	0 & 11.6	80
H-PAA100-50%	50 : 50	0 & 21.2	74

PAA0	<i>n</i> =9.7	<i>m</i> =0
PAA30	<i>n</i> =5	<i>m</i> =1.8
PAA100	<i>n</i> =0	<i>m</i> =10