

# Improved cell adhesion and proliferation on synthetic phosphonic acid-containing hydrogels

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## Abstract

Hydrogels with tissue-like mechanical properties are highly attractive scaffolds for tissue engineering. In this study, copolymers containing vinyl phosphonic acid (VPA) and acrylamide (AM) were tested for their swelling, protein uptake in serum supplemented medium, and cell adhesion and proliferation. The swelling of the gels in serum containing culture medium increased with increasing VPA content. The presence of VPA also increased protein uptake of gels in medium; gels polymerized with more than 50% of VPA absorbed as much as 100  $\mu\text{g}/\text{cm}^2$  of protein, twice the amount absorbed by gels made with only acrylamide. The adhesion and growth of the three types of cells, NIH 3T3 fibroblast, osteoblast-like MG-63 and Saos-2, were significantly improved on the gels made with 50% or more VPA; the number of adherent Mg-63 cells increased three-fold while the growth rate increased four-fold. Similar results were obtained for Saos-2 and 3T3 cells. The adhesion and growth of the three cell types on gels with sufficient phosphonate content were at least comparable to, or even better than, that on commercially available tissue culture plates. These results suggest great potential of anionic gels in bone tissue engineering.

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## 1. Introduction

Hydrogels are highly hydrated polymer networks that can be made from either natural or synthetic components. Hydrogels have a wide range of biomedical applications, ranging from biomacromolecule separation to contact lenses, coatings of blood contacting materials, controlled drug delivery devices, as well as tissue engineering scaffolds [1–3]. In general, hydrogels have excellent mechanical properties that match soft tissues. The physical shape of a hydrogel can be easily manipulated to fit any particular application site and the pore size of the gel network can be controlled to allow

optimal diffusion and transport of biological molecules [1,3–5]. All these features make hydrogels an excellent choice for use in tissue engineering scaffolds.

The ability to support cell adhesion and proliferation is a prerequisite for any material to serve as a tissue engineering scaffold. The interactions between cell membrane receptors and specific ligands on the supporting substrate play a primary role in cellular behavior. In a natural environment, such interactions could be acquired through the adhesion peptide sequences of extracellular matrix proteins, including RGD, YIGSR, REDV, etc. [6]. During *in vitro* culture, adhesive molecules on the surface of a synthetic material, which are either chemically or physically attached, are mainly responsible for cell adhesion. Unfortunately, most synthetic hydrogels do not have built-in cell adhesive ligands, nor do they readily attract proteins or peptides to physically adsorb on the surfaces due to the low

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interfacial energy between the highly hydrated gel phase and the aqueous biological media. As a result, cells do not usually adhere to hydrogels, especially those with a neutral or anionic nature [7], although some anionic hydrogels have been identified with increased protein uptake [8].

Extensive efforts have been made to improve hydrogel biocompatibility and usefulness for cell culture in the last 30 years [1]. Many researchers have focused on attaching whole ECM proteins [4] or adhesion peptides to hydrogels through chemical modifications. The adhesion peptide RGD has been incorporated into various gels, such as poly(ethylene glycol) diacrylate [9], poly(ethylene glycol) [10], poly(propylene fumarate-co-ethylene glycol) [11], poly(vinyl alcohol) [12], and poly(2-hydroxypropyl methacrylamide) [13] to promote cell adhesion, proliferation, migration, and tissue regeneration. While these studies have been important, optimal hydrogel surfaces have not yet been identified. For example, covalent bonding to the gel can change the conformation of ECM proteins and adhesion peptides, thus leading to substantial loss of biological activity [6].

We explored alternative ways of improving hydrogel biocompatibility and control of cell interactions. Anionic hydrogels have been extensively used in the development of controlled drug delivery devices because their swelling behavior depends strongly on the local environment, such as pH, ionic strength and solvent conditions [2,14]. But anionic hydrogels are often claimed to be “non-fouling” materials, with little protein absorption and thus little cell attachment [15–17]. For this reason, they have not been explored as tissue engineering scaffolds. At physiological conditions, pH=7.4, anionic gels are negatively charged and the electrostatic repulsion among charges on the polymer chain result in significant swelling [2,14]. Anionic hydrogels in highly swollen state should be able to trap a large amount of proteins and other bioactive molecules in biological fluids, either by protein entanglement in the surface or electrostatic interactions [18–20]. We speculated that this phenomenon, if it occurs, might provide essential molecular interaction sites for gels to support cellular activities. Moreover, the physically entrapped molecules might adopt a conformation similar to their natural form so that the bioactivity could be retained. Conformational stability should be high for protein embedded in the hydrogel,

because the molecular interactions are similar to ECM–protein interactions [21].

Based on the above hypothesis, we synthesized a series of hydrogels, namely, poly(vinyl phosphonic acid-co-acrylamide) gels, p(VPA-co-AM), and tested their ability to support cell adhesion and growth. First, anionic hydrogels were produced by copolymerization of neutral monomer acrylamide (AM) and acidic monomer vinyl phosphonic acid (VPA) at various ratios (Fig. 1). Polymers of vinyl phosphonic acid are non-toxic [22]. Vinyl phosphonic acid has been included in dental cement to improve adhesion to enamel or dentin [23–25], because phosphates are expected to interact favorably with the mineral component of hard tissues. Phosphate-containing hydrogels are of considerable interest to applications in cartilage and bone tissue engineering for the similar reason [26]. Second, we tested if the incorporation of VPA could increase the swelling and the protein uptake of gels in cell culture medium. Third, we studied the adhesion and proliferation of cells on gels with various compositions. To validate the potential of the gels in orthopedic applications, we chose several cell lines that are relevant to bone tissue engineering in our study.

## 2. Materials and methods

### 2.1. Preparation of poly(acrylamide)-poly(vinyl phosphonic acid) hydrogels

Monomers of acrylamide (AM) and vinyl phosphonic acid (VP), crosslinker tetraethylene glycol dimethacrylate (TEGMA) and initiator ammonium persulfate were all purchased from Sigma Chemical (St. Louis, MO). They were used as received without any further purification. Poly(AM-co-VPA) was synthesized similarly to the synthesis of poly(AM-co-AA) [27]. First, all the chemicals were dissolved or diluted with de-ionized (DI) water to serve as stock solutions (Table 1a). Next, various amounts of stock solutions were mixed at room temperature to achieve a range of VPA/AM molar ratio in the monomer feed, while the amount of total monomers (VPA + AM in mole), crosslinkers and initiators were kept constant (Table 1b). The mixed monomer solutions were carefully poured into the gap between two glass plates that was maintained by a

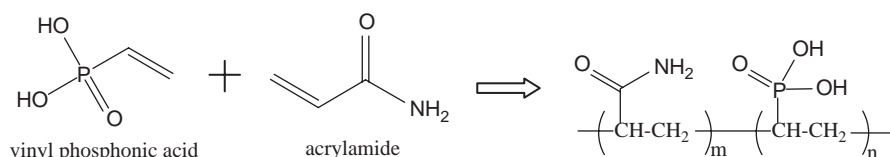


Fig. 1. Chemical structures of vinyl phosphonic acid, acrylamide and poly(vinyl phosphonic acid-co-acrylamide).

Table 1a

Concentration of chemicals as stock solutions for making poly (vinyl phosphonic acid-co-acrylamide) hydrogels

Chemical	Abbreviation	FW (g/mol)	Concentration (mol/L)
Vinyl phosphonic acid	VPA	108.03	9.0
Acrylamide	AM	71.08	7.0
Tetraethylene glycol dimethacrylate	TEGMA	330.3	3.0
Ammonium persulfate	APS	228.2	1.0

Table 1b

Chemical compositions for hydrogel preparation

Name	Composition (mol %)		Amount of chemicals (ml of stock solutions)				
	VPA	AM	VPA	AM	TEGMA	APS	H <sub>2</sub> O
VPA0% <sup>a</sup>	0	100	0	9.0	0.398	0.144	0
VPA10%	10	90	0.70	8.10	0.398	0.144	0.20
VPA30%	30	70	2.11	6.30	0.398	0.144	0.59
VPA40%	40	60	2.81	5.40	0.398	0.144	0.79
VPA50%	50	50	3.52	4.50	0.398	0.144	0.98
VPA60%	60	40	4.22	3.60	0.398	0.144	1.18
VPA70%	70	30	4.92	2.70	0.398	0.144	1.38

The concentration of each gradient of the monomer solution: Monomers (VPA + AM): 6.6 mol/L, crosslinker (TEGMA): 0.125 mol/L and initiator (APS): 0.015 mol/L.

<sup>a</sup>This abbreviation indicates a poly(vinyl phosphonic acid-co-acrylamide) gels polymerized with a certain percentage of VPA.

1-mm-thick silicone rubber gasket. The reactants were allowed to polymerize for 4 h at 60 °C in a water bath. At the end of the reaction, thin sheets of gel were removed from between the plates and washed extensively with DI water during a 2 h period to remove any residual reactants. The chemical structure of the copolymer is shown in Fig. 1. Gels were stored in DI water at 4 °C with 1% of penicillin and streptomycin (Pen/Strep, Life Technologies, Grand Island, NY) before use. From this point on, gels were handled under sterile conditions for biocompatibility studies.

### 2.2. Swelling of gels in cell culture media

Small disks of each gel, while still immersed in DI water, were cut with a metal bore (1.3 cm in diameter). Each disk was rinsed three times with complete cell culture medium (DEME and 10% fetal bovine serum) and transferred to a 12-well plate that contained 2 ml of medium in each well. The plate was placed in a humidified incubator at 37 °C with 5% CO<sub>2</sub> for 24 h to allow complete swelling of the gels. Experiments in triplicate were repeated three times for each gel composition and averaged.

### 2.3. Protein uptake by hydrogels

Protein uptake into gels containing various VPA was examined by measuring the total amount of proteins associated with the small gel disks. The disks were cut

from gels in DI water with 1.3 cm metal bore. Before protein uptake, the disks were extensively washed with phosphate buffer saline (PBS) solutions and serum-free DEME medium containing 1% of Pen/Strep for 2 h. They were allowed to swell in DEME media without serum in a humidified incubator at 37 °C with 5% CO<sub>2</sub> for 24 h. Expansion of gel disks was taken into account when calculating the protein concentration in the gel or on the surface. Next, gels were rinsed three times with serum-free medium before being placed in a 12-well plate with 2 ml DEME supplemented with 10% serum. After 24 h in a humidified incubator at 37 °C with 5% CO<sub>2</sub>, the gels were transferred to a clean 12-well plate and gently rinsed with PBS for 10 s to remove loosely attached proteins. An aliquot, 0.5 ml, of 1% sodium dodecyl sulfate solution was pipetted into each gel and the gels were placed on a shaker for protein desorption for 30 min; preliminary study showed that desorption was complete within 20 min. The concentration of proteins in SDS solution was measured using Micro BCA<sup>TM</sup> Protein Assay (Reagent Kit 23235, Pierce, Rockford, IL). In addition, serum protein adsorption on TC cultures plates was also measured for comparison. Each measurement in triplicate was repeated twice.

### 2.4. Cell culture conditions

Mouse fibroblast NIH/3T3, human osteoblast-like Saos-2 and MG-63 cell lines were obtained from ATCC. They were maintained in tissue culture flasks at 37 °C in a

humidified incubator with 5% CO<sub>2</sub>. 3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, ATCC), Saos-2 cells were cultured in McCoy's 5A Medium (ATCC) supplemented with 15% FBS and MG-63 cells were cultured in Eagle's Minimal Essential Medium (EMEM, ATCC) supplemented with 10% FBS. Penicillin/streptomycin (1%) were added to all three types of media to prevent bacterial growth. All experiments were performed using cells between 6 and 20 passages.

### 2.5. Biocompatibility studies

Before cell seeding, gels were extensively washed with sterile PBS solutions and cell culture medium to minimize contamination from residual chemicals and bacteria, as described earlier. Gels were then preconditioned with 2 ml of complete growth medium in a clean 12-well plate for 2 h before seeding cells. Each gel was seeded with  $1 \times 10^4$  cells/cm<sup>2</sup> cells and kept at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. The medium was carefully refreshed every 48 h.

Cells were monitored hourly for their behavior in the first 8 h and then every 24 h for 7 days. Digitized images of cells were then collected after 24 h using an inverted phase contrast light microscopy (Nikon-Diaphot, Garden City, NY). At least four randomly selected fields of view were collected for each gel disk. The extent of cell adhesion to gels with different VPA content was compared using the percentage of attached cells after 24 h. Briefly, the number of attached cells (characterized by elongated polygon shape and large projected area) and total cells (attached and un-attached at ~50 cell/field) were counted and the percentage was calculated for each four view field. Then the average was determined for each gel. The experiment was performed three times in triplicate gels.

Cell proliferation on various gels was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay, Promega). After 7 days of culture, gels were transferred to a clean 12-well plate and rinsed gently with PBS three times. Adherent cells were dislodged from the gels using 0.5 ml trypsin-EDTA before adding 1 ml of serum-containing media to stop enzymatic reaction. Cell suspensions were then collected and centrifuged at 500 rpm for 5 min. Cell pellets were resuspended with 100 µl of medium and the number of viable cells was determined using the standard MTS assay protocol. Each experiment was repeated in triplicate. Statistical analyses were performed using the two-tailed *t*-test assuming equal variance for two samples.

### 3. Results

In this study, we investigated a hydrogel system that swelled in biological fluid. Therefore, we examined the

change in volume of each gel after they were transferred from de-ionized water to cell culture medium. Not surprisingly, we noted that gels with more than 50% of VPA were acidic enough to change the pH indicator in the culture medium from pink (pH > 7) to orange (pH < 7), while the color of gels polymerized with 40% or less VPA was still pink when transferred to complete growth media. Changes in the medium had a significant influence on the swelling of p(VPA-co-AM) gels (Table 2). The diameter of gels without any VPA content remained the same regardless of conditions ( $P > 0.03$ ); no further swelling was observed due to medium change. In contrast, the diameter of gels with VPA increased after immersion in culture medium. The expansion of gels increased with increasing VPA content until it reached a maximum for gels polymerized with 50% of VPA. Further increase in VPA content did not lead to any further increase in diameter ( $P > 0.03$ ). The maximum diameter, 1.88 cm, obtained for gels polymerized with 50–70% VPA represents a 45% increase over the materials with 100% p(AM).

The presence of VPA had a substantial influence on the ability of gels to attract proteins from the culture medium. The total amount of protein uptake, normalized by the surface area of the corresponding gel, was determined for each gel composition (Table 2). Protein uptake on materials with less than 40% VPA did not change with composition ( $P > 0.03$ ), but protein uptake was dramatically higher for materials with 50% VPA. However, further increase of VPA content in p(VPA-co-AM) gels (to 60% and 70%) did not result in greater amounts of uptake.

Images of MG-63 cells cultured on gels polymerized with 0%, 40%, 50% and 70% VPA at 24 h, 3 and 7 days were compared to images of cells on tissue culture plates (Fig. 2). During the first 8 h, no significant attachment of MG-63 cells was observed on most of the gel surfaces. Most cells were rounded with minimal spreading on gels without any VPA after 24 h (Figure 2ai). Cells appeared to be contacting each other and forming aggregates. At

Table 2  
Swelling and protein uptake properties of hydrogels

Name	Diameter <sup>a</sup> (cm)	Protein surface concentration (µg/cm <sup>2</sup> )	Protein bulk concentration (µg/cm <sup>3</sup> )
VPA0%	1.32 ± 0.04	47.5 ± 1.4	0.47 ± 0.01
VPA10%	1.38 ± 0.04	43.0 ± 1.3	0.41 ± 0.01
VPA30%	1.65 ± 0.07	52.7 ± 1.0	0.42 ± 0.01
VPA40%	1.81 ± 0.06	52.2 ± 0.1	0.38 ± 0.001
VPA50%	1.88 ± 0.04	111.3 ± 4.5	0.79 ± 0.03
VPA60%	1.89 ± 0.06	95.9 ± 1.5	0.67 ± 0.01
VPA70%	1.87 ± 0.08	95.9 ± 1.9	0.68 ± 0.01
TC plate		3.2 ± 0.2	

<sup>a</sup>The initial diameter for each disc was 1.30 cm.

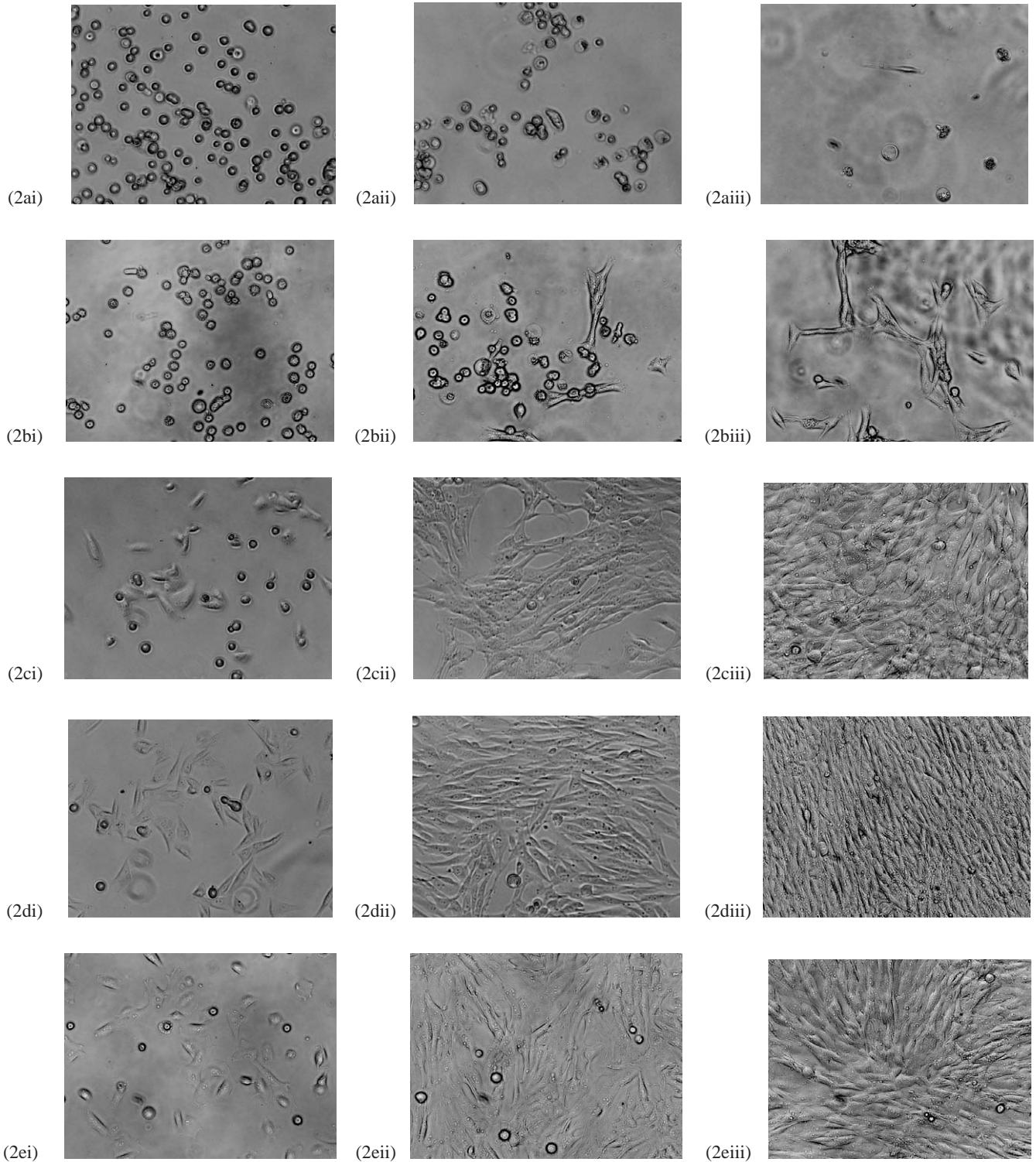


Fig. 2. Morphology of MG63 cells seeded on hydrogel surfaces polymerized with various amounts of VPA: (a) 0%; (b) 40%; (c) 50%; (d) 70%; (e) Tissue culture plate; and (i) Day1; (ii) Day3; (iii) Day7

day 3, most cells were clumped, with few single cells adherent to the surface. Overall, there were visibly fewer cells on the gels after 3 days of culture. By day 7, the majority of the cell clumps disappeared and only few attached cells were present on the surface. Cell behavior

on gels polymerized with 10–30% VPA was similar to that without any VPA (images not shown). There was a slight improvement in adhesion when 40% VPA was copolymerized into the gel (Fig. 2b). At 3 days, cells appeared to be divided into two groups: clumped or

adherent. At 7 days, clumps disappeared, as those on 100% p(AM) gels, but apparently more cells attached and spread on the surface with 40% VPA than on the 100% p(AM). The biggest difference in cell behavior occurred when the VPA content was increase to 50% (Fig. 2c). After 24 h of culture, most cells were already adhered to the surface with normal spreading. In contrast to the gels with lower amounts of VPA, cells that remained round were not forming clumps but stayed as single cells. After 3 days, all the cells were well attached and spread on the gel surface, and by 7 days, cells became completely confluent. Further increases in VPA content did not change cell behavior to any significant degree (Fig. 2d). Cell behavior on gels polymerized with sufficient VPA (50–60%) were comparable to that on tissue culture plates, in which the surface conditions are optimized for cell culture during manufacture (Fig. 2e). Parallel experiments were performed with Saos-2 and 3T3 cells and similar results were obtained (data not shown).

We determined cell adhesion at 24 h as a function of VPA content quantitatively (Fig. 3). On gels polymerized with 0–30% of VPA content, less than 10% of the cells were adhered and the adhesion was independent of cell type ( $P > 0.03$ ). A transition in adhesion phenomenon appeared as VPA content approached 40% VPA where 20–30% of cells were adherent. The transitional VPA content was independent of cell type. When more than 50% VPA was added in polymerization of gels, a sharp increase in adhesion was obtained. The percent of adherent MG63 and 3T3 cells was tripled. In contrast, the changes were milder for Saos-2 cells with about 50% adherent cells on gels polymerized with 50% VPA. As the VPA content increased to 60%, the number of adherent cells of all three types continued to increase ( $P < 0.03$ ). Gels with 60% and 70% of VPA incorporated were similar with respect to cell adhesion ( $P > 0.03$ ). In comparison, the adhesion of all three

types of cells on gels with sufficient VPA was comparable to tissue culture plastic ( $P > 0.03$ ).

The ability of each gel to support cell growth was evaluated. The density of each cell type on gel surfaces at 7 days, e.g. the number of cells normalized by the surface area of the gel was determined as a function of VPA content (Fig. 4). Similar to adhesion, cell proliferation was strongly dependent on the amount of VPA used during polymerization. On gels polymerized with little or no VPA (0–40%), MG-63 cell density was significantly less than the initial seeding density ( $1 \times 10^4$  cells/cm<sup>2</sup>), which is consistent with the microscopic observations (Fig. 2). Significant growth was obtained only on gels polymerized with sufficient amount of VPA, e.g. more than 50%. On gels polymerized with 50% VPA, cell density was more than twice of initial seeding amount. Further increase in VPA content continuously enhanced cell proliferation. It should be noted that the growth rate of Mg-63 on gels polymerized with 60% or 70% VPA content was comparable to that on TC plastic ( $P > 0.03$ ), with an overall four-fold increase in cell number over the course of the experiment. Similar trends were observed for 3T3 and Sao-2 cells although the growth rate was faster for 3T3 (six fold) and slower for Saos-2 (three-fold).

In a comparative study, cells were cultured on gels in completely serum-free medium (Table 3). After 24 h, none of the three cell types were spread on any gel surfaces. Cells appeared round and aggregated. After 3 days of culture in a serum-free environment, more than 95% of 3T3 and Saos-2 cells were already dead regardless of gel composition. On the other hand, MG-63 cells were alive but not spread on any gel surface after 3 days. At this point, serum-supplemented medium was added to each culture. After 2 days of serum-supplemented culture, cells adhered, spread and proliferated only on gels polymerized with 50–70% of

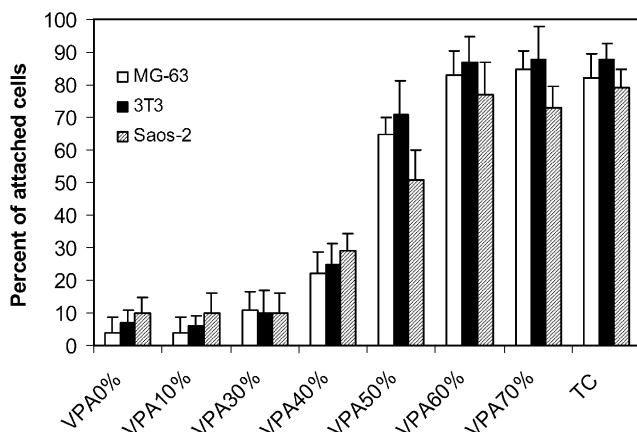


Fig. 3. Cell adhesion as a function of VPA content after 24 h culture.

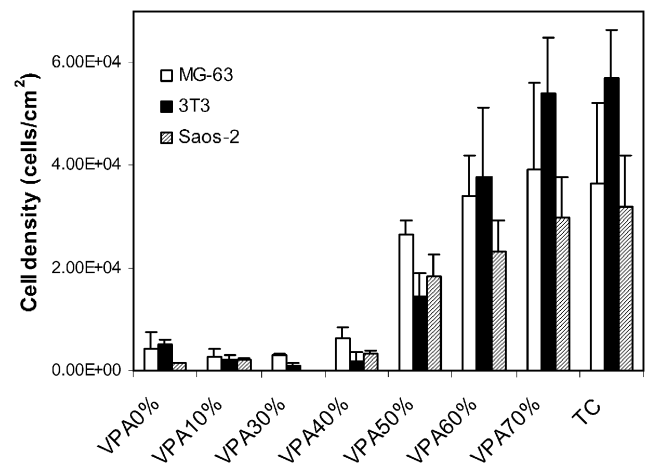


Fig. 4. Cell proliferation as a function of VPA content after 7 days of culture.

Table 3  
Cell adhesion on p(VPA-co-AM) gels in the absence of serum proteins

	Serum-free		Serum supplemented later			
	MG-63	3T3	Saos-2	MG-63	3T3	Saos-2
VPA0%	0	0	0	0	0	0
VPA10%	0	0	0	0	0	0
VPA30%	0	0	0	0	0	0
VPA40%	0	0	5%	0	0	0
VPA50%	0	0	49%	0	2%	2%
VPA60%	0	0	53%	0	2%	2%
VPA70%	0	0	45%	0	2%	2%
TC	77%	0	1%	82%	0	3%

Cells were cultured on hydrogels in serum free medium for the first 24 h. The percentage of adherent cell after 3 days of culture is shown under column “serum-free”. After 3 days, the serum-free medium was removed and complete growth medium (with serum) was introduced to cells. The percentage of cells attached to gels (% of attached over original cell number) 48 h later, this change is shown under column “serum supplemented later”.

VPA. Cells on gels polymerized with less than 40% VPA did not survive. It should be noted that the absence of serum did not change the swelling behavior of gels and thus the surface area or volume of the gels. Therefore, the pore size of the polymer network was assumed unchanged. This experiment provided strong evidence to support our hypothesis that serum protein uptake by the gels was crucial to cell adhesion and proliferation.

#### 4. Discussion

Biomaterials are usually exposed to various biological molecules during applications, either as implants in the body or as supporting materials for cells *in vitro*. The adsorption of proteins and other molecules is assumed among the first few events that occur at the cell or tissue/material interface [28]. The adsorbed molecules can then play an important role in the regulation of cellular response depending upon the extent of adsorption, orientation following adsorption, and conformation following adsorption. Therefore, the interaction of biological molecules, especially proteins, with biomaterials has been studied extensively. Much work has been done to investigate the influence of surface properties, such as charge and hydrophobicity, on the adsorption of proteins and subsequently, the responses of cells [8,19,20,28–33]. However, the results have been confusing (for a recent review, see Chapter 12 of [34]). Most investigators report that positive charge on surface promotes cell adhesion while negative charged and neutral surfaces repel cell adhesion [19,31–33]. Such results were typically explained by the fact that cell membrane is negatively charged and therefore attracted to the positively charged surfaces. Some studies,

however, showed that negatively charged surfaces could also enhance cell adhesion [8,35]. This was explained by the specific protein adsorption onto the negatively charged surfaces, such as fibronectin onto hydroxylated polystyrene [35] and BSA onto styrene sulphonic acid modified polyethylene [8]. It is therefore possible that specific proteins from serum may adsorb onto materials containing a net negative charge, and that these proteins can then mediate cell interactions.

In this study, we examined the behavior of our anionic gels in both the presence and the absence of serum proteins. Our study demonstrated that, in serum-supplemented culture, the incorporation of anionic constituents into non-adhesive poly(acrylamide) hydrogels could significantly increase protein uptake, cell adhesion, and cell proliferation on the gels. These results were confirmed with three different types of cultured cells. The strong correlation between protein uptake by the gels and cellular activities on gels supported our hypothesis that modification of the interaction between protein molecules and gels by anionic groups could be an effective means of improving material biocompatibility.

The increase in swelling of p(VPA-co-AM) gels in culture medium with increasing VPA content is probably due to charge repulsion, which is the major driving force for swelling of ionic gels [14,18]. The influence of VPA content on protein uptake was more complicated. We noted that the apparent surface concentration of protein on hydrogels, 40–110  $\mu\text{g}/\text{cm}^2$ , was much greater than the typical value of monolayer protein adsorption, which is in the range of 1  $\mu\text{g}/\text{cm}^2$  [28]. Because of this, we suspect that proteins penetrate into the hydrogel matrix, which has a mesh size of 1–10 nm [36]. Therefore, surface concentration might not an appropriate parameter to describe the protein uptake observed in these gels. We therefore calculated the bulk concentration of proteins in gels, assuming that they were evenly distributed throughout the entire gel volume (although such assumption is unlikely to be valid, e.g. there might be a gradient of protein distribution in the gel). The relationship between protein uptake and VPA content was not affected by the calculation, shown in Table 3. The bulk protein concentration within gels ranged from 0.4 to 0.7 mg/ml, about ten times less than the total protein concentration of the serum supplemented medium, ~6 mg/ml, indicating that VPA hydrogels can act as sponges to absorb proteins from biological fluid [16]. This high absorption of protein should promote cell adhesion and proliferation on the surface of the material.

The protein absorption decreased slightly when a low amount of VPA was added, increased dramatically at intermediate amounts of VPA, and then decreased slightly at the highest VPA content. This biphasic pattern could be explained by a competitive effect of

VPA on protein uptake. It has been suggested that the anionic constituent of hydrogels could alter protein uptake in two ways: (1) electrostatic attraction/repulsion and (2) increasing the infiltration of protein molecules due to increased porosity [18]. Since albumin ( $pI=4.9$ ), the dominant protein in serum, is negatively charged under physiological conditions, a decrease in protein uptake due to the increasing repulsion between albumin and VPA would be expected as the VPA content was increased. On the other hand, the greater pore size associated with swelling upon the addition of VPA should facilitate protein penetration [15]. The combination of the two factors probably led to the observed biphasic curve.

The transition for enhanced protein uptake and enhanced cellular activities occurred on the gels polymerized with the same amount of VPA, i.e. when it was changed from 40% to 50%. This correlation, along with the lack of cell viability cultured on gels in the absence of serum, suggests that protein uptake by the gels is important in supporting cellular activities. However, this can not be the only effect, because cell adhesion and growth were not greatest on gels polymerized with 50% VPA even though the protein uptake was maximum for these gels. The disparity between cellular response and total protein absorption probably reflects the complexity of serum protein composition and competitive absorption into gels. The measured total amount of the absorbed proteins in this study reflects the summation of many proteins present in serum. However, only certain proteins, including fibronectin, vitronectin and others, influence cell adhesion and growth. The differential absorption of these molecules as a function of VPA content, which is determined by physical and chemical properties of each individual molecule, is likely to be different from that of the total serum protein measurement. For example, the uptake of lysozyme ( $pI=11$ ) is known to increase with the anionic content of gels [18]. On the other hand, anionic moieties on other proteins could influence the absorption and conformation of molecules, and hence their biological activity. Detailed studies of the uptake of each individual protein molecule are needed to gain a better understanding of the mechanism. Such studies are extremely difficult, due to both the complexity of serum proteins and the likelihood that only a small fraction of the relevant proteins are known, and therefore beyond the scope of the present study.

## 5. Conclusion

We synthesized a series of anionic hydrogels, p(VPA-co-AM), that supported cell adhesion and proliferation. The effect of the anionic moieties on cellular activities was mediated through the interaction of serum protein

molecules with the gel network. With appropriate compositions of the materials, the adhesion and growth rate of cells was comparable to, or even better than, commonly used tissue culture materials, which are optimized for cell cultures. Our study indicated that it was possible to tune the adhesion and proliferation behavior of bone-related cells by simply changing the anionic constituent of the gels. The results of this study suggest potential uses of anionic gels as orthopedic tissue engineering scaffolds.

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