Synthesis and characterisation of poly(2-hydroxyethyl methacrylate) polyelectrolyte complexes

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Copolymerisation of charged and neutral monomers is a well-known methodology to introduce charged moieties in a polymeric chain to obtain polyelectrolytes. New polyelectrolyte complexes have been synthesised by radical copolymerisation of neutral methacrylic monomer 2-hydroxyethyl methacrylate (HEMA) with cationic 2-methacryloyloxyethyltrimethyl ammonium chloride and anionic 2-acrylamido-2-methylpropane-sulphonic acid monomers in 10 : 1 : 1 and 10 : 1 : 2 stechiometric ratio. Chemical structure of the synthesised terpolymers was confirmed by FT-IR spectroscopy, moreover, X-ray photoelectron spectroscopy showed the presence of a cationic charge excess on the 10 : 1 : 2 terpolymer surface with respect to 10 : 1 : 1 terpolymer.

Swelling studies for 10 : 1 : 2 terpolymers showed a high water content in the swollen state and a ''smart behaviour'' upon changes in external stimuli such as pH, while, 10 : 1 : 1 terpolymer presented the behaviour of a neutral polymer.

Mechanical and differential scanning calorimetry analysis confirmed that terpolymer networks were stabilised by ionic co-operative interactions. Infact, the inclusion of oppositely ionic charges in the polymeric network of p(HEMA) represent a way to achieve higher elastic modulus as they stabilise the terpolymer networks.

Cytotoxicity and cytocompatibility studies demonstrated that all materials were not toxic, moreover, the presence of a cationic charge excess on 10 : 1 : 2 terpolymer surface was able to promote fibroblast adhesion.

1. Introduction

Interpolymer complexes or polymer–polymer complexes are formed by the association of various macromolecules [1]. These complexes are stabilised by several types of interactions (hydrogen bonding, van der Waals forces, ionic bonds and hydrophobic interactions) that can act together or alone depending on the chemical structure of macromolecules [2].

In the past, polyelectrolytes were used to generate interpolymer complexes which form between macromolecular acids and bases or their salts and are stabilised by ionic bonds that occur between oppositely charged macromolecules [3].

Polyelectrolytes are a class of polymers in which the charge nature gives rise to properties distinct from those of non-ionic polymers. Polyelectrolytes may be anionic, cationic or amphophilic; they occur naturally and can be synthesised. Amphophilic polyelectrolytes are polymers which contain both anionic and cationic species. In general, the positive and negative charges may be located on the same pendent group, or on different pendent groups that are dispersed regularly and randomly on the backbone chain [4].

In polyelectrolyte complexes, complexation process is co-operative enhancing the stability of the polymeric network [2]. The formation of polyelectrolyte complexes can have a strong effect on polymer solubility, rheology, turbidity and conductivity. The stability of the polyelectrolyte complex is dependent on a variety of factors, such as charge density, ionic strength, the nature and type of solvent, pH and temperature [2].

Generally, the polyelectrolyte complex is insoluble in

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water because the charged groups responsible for solubility are involved in the complex [5].

Many biomedical applications of polyelectrolytes and their complexes came from their ability to bind with oppositely charged surfaces and to associate to form complexes with oppositely charged polymers. Polymer-bound ionic groups may interact electrostatically with one another, with mobile charges in the solution and with biological polymers, such as collagen, trypsin and insulin [4].

The interactions between polymers and cell membranes is one aspect of the role of matrices, both biological and artificial, in tissue organisation. Most naturally occurring macromolecules present in extracellular environments, whether proteins, polynucleotides or complex carbohydrates, are polyelectrolytes. Electrostatic interactions with the charged lipid bilayers of cellular or subcellular membranes are crucial for the biological functioning of many natural polyelectrolytes.

It has long been known that the growth of many cell lines, both *in vivo* and *in vitro*, is an anchorage-dependent phenomenon [6]. The first step of the cell-surface interaction is attachment, in which the cells retain the round shape they had in suspension. Attachment of a cell to a surface is usually followed by a conformational change known as ''spreading'', in which the area of the cells in contact with the surface increases. Spreading is essential for correct attachment and early proliferation [7, 8].

The attachment phase depends on the physical and chemical properties of the polymer surface, i.e. interfacial free energy, hydrophylicity and hydrophobicity, mobility of the polymer chains, and ionic nature [9, 10]. If these properties can be manipulated by altering the molecular design of the polymer, we can obtain both cell-adhesive and non-adhesive polymers, and biocompatible polymers [11–13]. One strategy by which to modulate the first phase of cell adhesion to a substrate is to introduce electric charges into the polymer network [14].

This idea work is focused on the synthesis, chemical–physical, mechanical and biological characterisation of a new polyelectrolyte complexes obtained by radical copolymerisation of neutral methacrylic monomer 2-hydroxyethyl methacrylate (HEMA) with cationic 2-methacryloyloxyethyltrimethyl ammonium chloride (METAC) and anionic 2-acrylamido-2-methylpropane-sulphonic acid (AMPS) monomers, aimed at keeping such properties as high mechanical strength and good cell adhesion.

Finally, the behaviour of these polyelectrolyte complexes at the change of external stimuli is also investigated with a view to using these ''smart materials'' as biomedical devices.

2. Materials and methods

2.1. Polymers: synthesis and characterisation

HEMA, AMPS and METAC monomers were purified according to previously described procedures [4] and freshly used in the polymerisation steps.

HEMA/AMPS/METAC terpolymers at 10 : 1 : 1 and 10 : 1 : 2 molar ratio and p(HEMA) were prepared in bulk via radical chain polymerisation. 2,2′-Azoisobutyronitrile (AIBN) 0.1% w/w respect to monomers, was used as initiator.

Monomers, in the appropriate molar ratio, were mixed together under magnetic stirring; a small amount of distilled water was added for the complete solubilisation of the monomers. The compositions of the monomer solutions are shown in Table I. The monomer solutions were loaded on sealed glass chambers separated by a silicon frame, and cured as follows: 2 h at 60 °C, 4 h at 70 °C and 1 h at 85 °C. After polymerisation, the gels were extensively washed with sterile distilled water.

FT-IR spectroscopy (FT–IR Mod. Paragon 500, Perkin Elmer) was employed to determine the chemical structure of the synthesised materials.

X-ray photoelectron spectroscopy (XPS, Physical Electronics Mod. 5500-PHI) was used to determine the atom compositions of the surfaces of the synthesised polymers. The photoelectron releasing angle for each atom was fixed at 68° (about 10 nm of depth).

Swelling studies were carried out at 25 °C using distilled water, NaCl (0.1, 0.5 and 1 M) solutions and buffer solutions at different pH values (from 3.0 to 5.0 citrate/phosphate buffer 50 mM; from 5.0 to 9.0 phosphate buffer 50 mM).

The quantity of absorbed water at equilibrium was expressed by

$$\%\ \text{water swelling} = \frac{w_s - w_0}{w_0} \times 100$$

where w_0 is the dried sample weight and w_s is the swollen sample weight.

Differential scanning calorimetry (DSC) was employed to evaluate the transition temperature (T_g) of dried polymers. The tests were performed using a calorimeter of a DuPont under nitrogen flow, from − 20 to 200 °C, with a flow rate of 10 °C/min.

The mechanical analysis was performed in a tensile mode by INSTRON dynanometer, model 4204 according to ASTM D638 on swollen materials both in distilled water or in 1 M NaCl water solution, to analyse the mechanical behaviour of materials in different conditions of ionic strength. Tensile tests were conducted at the speed of 5 mm/min to evaluate the elastic modulus (E), the stress at break (σ) and the strain at break (ε).

2.2. Cell cultures

Primary human fibroblast cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), penicillin/streptomycin (10 000 units penicillin/ml, 10 000 mg streptomycin sulphate/ml), and 2 mM glutamine, in a humidified atmosphere at 37 °C (5% CO_2). Raji cells

TABLE I Swelling percentage at the equilibrium of p(HEMA), 10 : 1 : 1 and 10 : 1 : 2 terpolymers in water and in NaCl solutions

Samples	Water	0.1 M NaCl	0.5 M NaCl	1 M NaCl
p(HEMA)	56.7	55.6	54.1	53.2
10 : 1 : 1 terpolymer	121.3	116.1	112.5	106.2
10 : 1 : 2 terpolymer	287.2	266.3	189.5	134.9

(American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin/streptomycin (10 000 units penicillin/ml, 10 000 mg streptomycin sulphate/ml) and 2 mM glutamine, in a humidified atmosphere at 37 °C (5% CO_2).

2.3. Cytotoxicity assay

Cytotoxic effects was evaluated indirectly by quantification of mitochondrial dehydrogenase activity via the enzymatic conversion of MTT tetrazolium (Sigma, Milan, Italy) to a coloured formazan product. Since reduction of MTT occurs only in metabolically active cells, the level of activity is a measure of cell viability. The MTT colorimetric assay is a routine cytotoxicity test that is useful for the preliminary screening of biomaterial toxicity. The MTT assay was performed according to the manufacturer's instructions. We used Raji cells inoculated at 2×10^5 cells/ml on the test materials placed at the bottom of 24-well tissue culture plates. After 24 h, mitochondrial dehydrogenase activity was detected spectrophotometrically by absorbance at 570 nm of coloured formazan product [15].

2.4. Cell adhesion

Primary human fibroblasts were used for the assessment of the kinetics of cell adhesion on the test materials. Confluent cells were trypsinised using 0.05% trypsin–0.2% EDTA, centrifuged at 2000 rpm for 3 min and resuspended in the same culture medium. Single cell suspensions were seeded on the testing materials at a density of 1×10^5 cell/well (24-well plate) and left at 37 °C to attach to the substrates. As positive control, cells were plated at the same density onto tissue-culture polystyrene plates (TCPS). Three independent adhesion experiments were run for each test material. The interaction between the cell and the diverse polymers was studied with a phase optical microscope 3, 6, 24 and 48 h after plating. Phase contrast micrographs were taken of fibroblastic cells attached to the polymers tested after 48 h of seeding.

2.5. Scanning electron microscopy (SEM) preparation

Cells seeded on the various polymers were fixed by 2.5% gluteraldehyde solution in 0.1 M cacodylate, pH 7.4 buffer for 1 h at 4 °C. The samples were then rinsed three times with the same buffer, slowly dehydrated using increasing concentrations of ethanol, critical point-dried using CO_2, mounted on aluminium specimen stubs and coated with gold-palladium. Finally, the surfaces of the samples were examined with a Hitachi scanning electron microscope.

3. Results and discussion

3.1. Polymers: synthesis and characterisation

The synthesis of p(HEMA) via radical chain polymerisation is a well-established procedure [16]. Statistical terpolymers p(HEMA-co-AMPS-co-METAC) 10 : 1 : 1 and 10 : 1 : 2 were obtained at a high monomer conversion (~ 99%) by optimising the curing thermal procedure by the differential scanning calorimeter.

The incorporation of ionic monomers in the p(HEMA) material was assessed with FT-IR spectroscopy. The FT-IR spectrum of p(HEMA) (Fig. 1(a)) revealed two peaks characteristic of alcoholic (OH) groups, respectively at

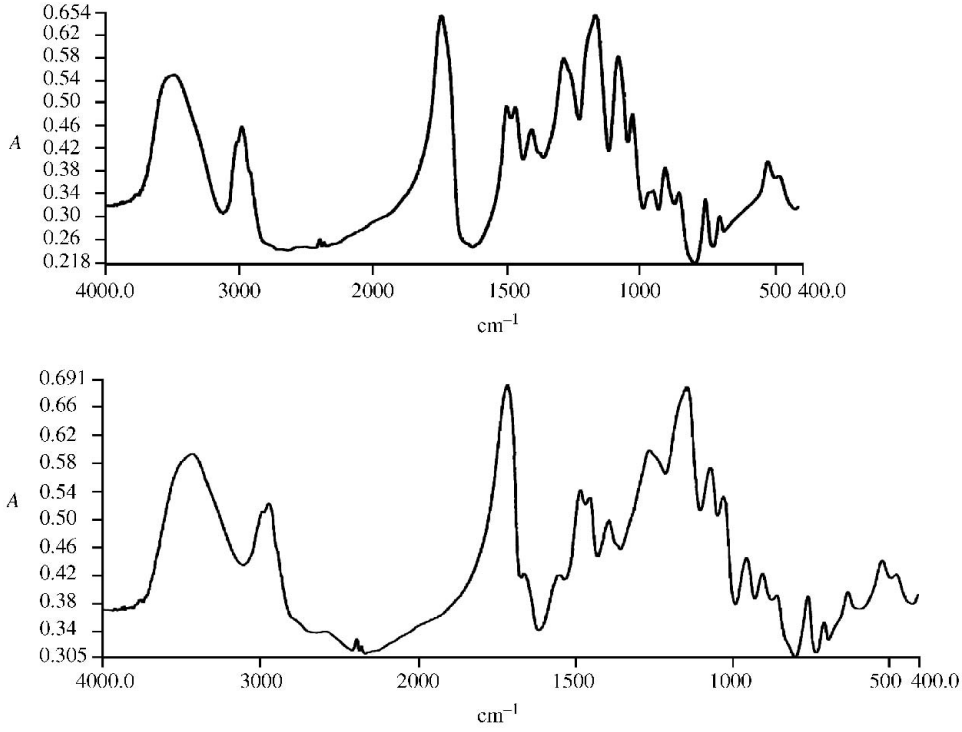

Figure 1 FT-IR spectra of (a) p(HEMA) and (b) 10 : 1 : 1 or 10 : 1 : 2 terpolymers.

3445 cm^{-1} (stretching C–O) and 1022 cm^{-1} (bending O–H). Ester groups were identified by a peak at 1728 cm^{-1} (C=O stretching) and at 1275 cm^{-1} (C–O stretching). Other peaks represent C–C and C–H vibrations of $–CH_3$ and $–CH_2$ groups.

When p(HEMA) and p(HEMA-co-AMPS-co-METAC) 10 : 1 : 1 or 10 : 1 : 2 FT-IR spectra (Fig. 1(b)) were compared, new absorption peaks appeared in the terpolymer spectra, due to the presence of tetralchilic nitrogen of METAC residues (949 cm^{-1}) and to the amide group of AMPS residues (1567 and 1656 cm^{-1}).

XPS surface analysis of HEMA-co-AMPS-co-METAC terpolymers revealed the presence of sulphur atoms (peak at 168.0 eV), chlorine atoms (peak at 199 eV) carbon atoms (peak at 287.0 eV), nitrogen atoms (peak at 401.3 eV) and oxygen atoms (peak at 533.8 eV). Furthermore, when we performed a high resolution analysis with peaks deconvolution, we identified four subpeaks for carbon (Fig. 2(a)) that were attributed, respectively, to carbon in $–\underline{C}H_3$ and $–\underline{C}H_2–$ (subpeak at 285.0 eV), carbon in $–CH_2–(CH_3)\underline{C}–[–C(=O)–]$ (subpeak at 286.3 eV), carbon in $–O–\underline{C}H_2–$ (subpeak at 286.9 eV) and carbonyl carbon $[–\underline{C}(=O)–]$ (subpeak at 289.0 eV). The profile of peaks at high resolution was essentially the same for 10 : 1 : 1 and 10 : 1 : 2 terpolymers (Fig. 2(a)) expected for nitrogen peak. This peak showed two components that identified respectively the organic nitrogen (400.0 eV) and the N^+ ion (402.8 eV). The subpeak at 402.8 eV was about two times more intense in the 10 : 1 : 2 than to 10 : 1 : 1 terpolymer (Fig. 2(b)).

Water swelling of p(HEMA-co-AMPS-co-METAC) 10 : 1 : 1 showed a value two times higher than pHEMA (Table I), while 10 : 1 : 2 terpolymer showed a higher swelling degree due to its presence in the polyelectrolyte complex of an excess of cationic residues.

In NaCl solutions, 10 : 1 : 2 terpolymer showed a drastic decrease in water absorption, proportionally

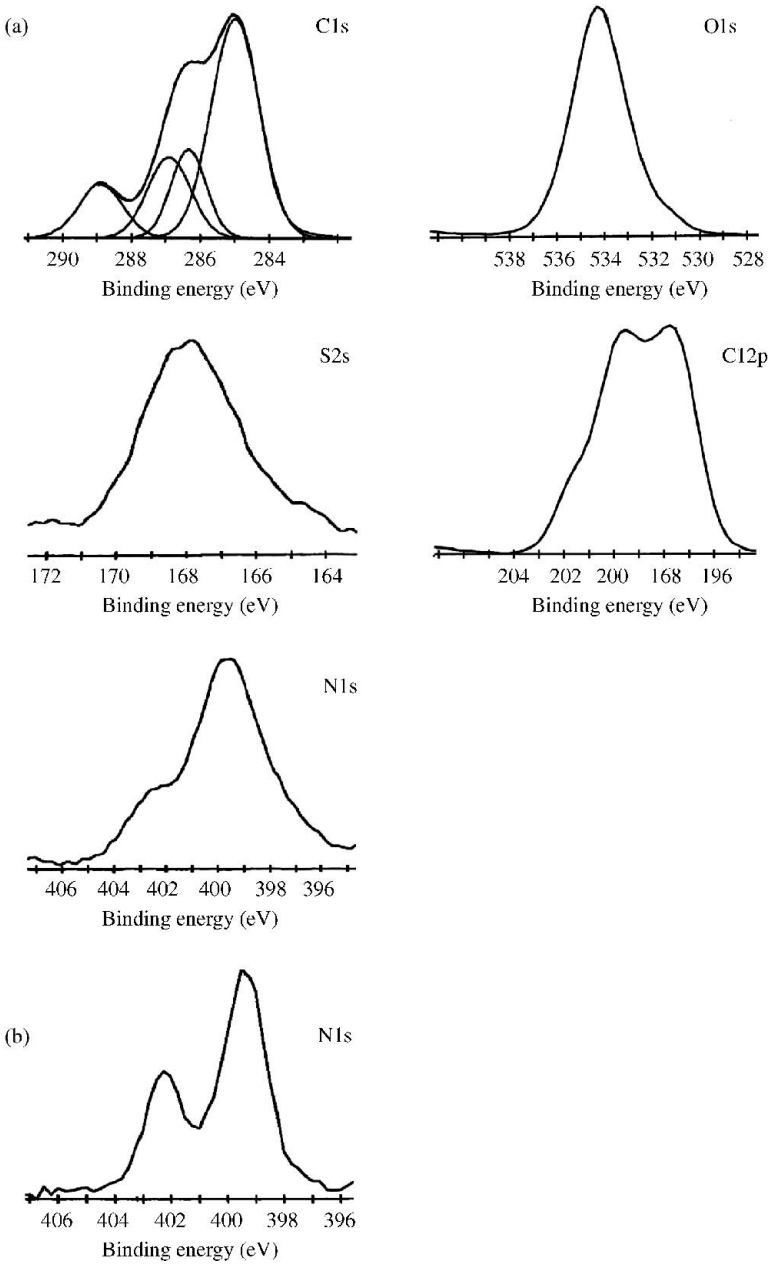

Figure 2 XPS high resolution analysis of p(HEMA-co-AMPS-co-METAC) terpolymers. (a) 10 : 1 : 1 and (b) nitrogen peak of 10 : 1 : 2.

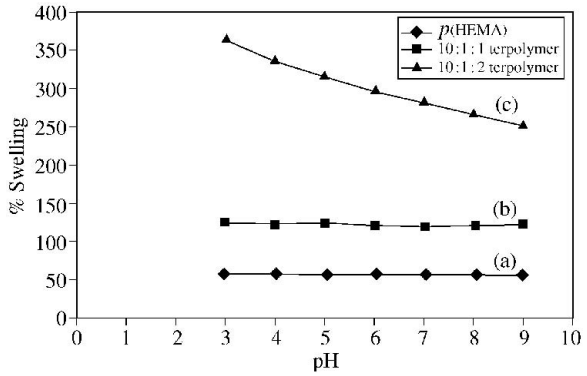

Figure 3 Swelling percentage at the equilibrium of (a) p(HEMA); (b) 10 : 1 : 1 terpolymer; and (c) 10 : 1 : 2 terpolymer, at different pH values.

with the increasing NaCl concentration of equilibrating solutions (Table I), while swelling of p(HEMA) and 10 : 1 : 1 terpolymer were not significantly affected.

For p(HEMA) and 10 : 1 : 1 terpolymer, again, no appreciable volume variations were measured for all the pH studied (Fig. 3(a) and (b)), while the change of pH values from 3.0 to 9.0, 10 : 1 : 2 terpolymer showed a decreasing water absorption at equilibrium (Fig. 3(c)) as function of the increase in pH value, due to the shielding effect exerted by H_3O^+ ions on the polymeric bulk charges.

DSC analysis showed a higher T_g values for synthesised terpolymers (129.9 °C for 10 : 1 : 1 and 135.3 °C for 10 : 1 : 2) with respect to p(HEMA) (87.9 °C), indicating the presence of supplementary forces (ionic interaction) that stabilise terpolymer networks, as results showed more energy was necessary for achieving glass transition.

Mechanical analysis on swollen terpolymers both in bidistilled water and in 1 M NaCl solutions, showed an increase in elastic modulus (E) (about three times for 10 : 1 : 1 terpolymer) associate with a decrease in the stress at break (σ) and strain at break (ε) in comparison with p(HEMA) hydrogel (Table II).

3.2. Cell viability and proliferation

The MTT assay was used to evaluate the effects of polymeric materials on cell viability and proliferation. As determined by MTT cytotoxic assay, cell viability and proliferation were not affected by incubation on polymers tested. Raji cells, that grown in suspension, proliferate perfectly on p(HEMA) and p(HEMA-co-AMPS-co-METAC) 10 : 1 : 1 and 10 : 1 : 2. The samples had final cell counts about equal to the positive control material (TCPS). The results shown in Table III suggest that the polymers tested do not affect the cell growth at 48 h.

3.3. Cell morphology

As shown in Fig. 4(d), fibroblastic cells adhered to p(HEMA-co-AMPS-co-METAC) 10 : 1 : 2 as well as they adhered to the TCPS control substrate after 48 h of undisturbed culture time. There was no effect of polymeric substrate on cell morphology. Phase contrast images revealed that the majority of cells adherent to the polymer and exhibited spreading and flattening. The area covered by the cells was extensive and no apparent cell detachment was observed. In contrast, there were poor adhesion and spreading of fibroblast cells on p(HEMA) and p(HEMA-co-AMPS-co-METAC) 10 : 1 : 1 materials (Fig. 4(b) and (c)). SEM confirmed these observations, showing in details, the flattening and spread morphology of cells seeded on 10 : 1 : 2 terpolymer (Fig. 5(c)), while cells in contact with neutral and 10 : 1 : 1 terpolymer showed, essentially, a round shape (Fig. 5(a) and (b)). The first step of cell adhesion on polymer surfaces is protein adsorption. Probably, as demonstrated in literature, adsorption of plasma proteins (i.e. fibronectin) on cationic surfaces [17, 18] are responsible for human fibroblast adhesion on 10 : 1 : 2 terpolymer.

Further studies are in progress to elucidate the mechanism of plasma protein adsorption on this new cationic surface.

4. Conclusions

Results from swelling, DSC and mechanical experiments confirmed that terpolymer networks were stabilised, in addition to hydrogen bonding and hydrophobic interactions, by co-operative ionic interaction between oppositely charged residues pending from polymeric backbone. Terpolymer 10 : 1 : 1 showed a swelling and biological behaviour overlapping to p(HEMA), indicating that with a 1 : 1 stechiometry of ionic monomers, the opposite charges neutralised themselves, and the terpolymer behave as neutral polymer.

Results from MTT test performed with Raji cells demonstrated the no cytotoxicity of all synthesised materials.

The presence of a cationic charge excess on 10 : 1 : 2 terpolymer induced higher water with absorption respect to 10 : 1 : 1 terpolymer and p(HEMA), and behaviour in salts and in buffer solutions at different pH values overlapping with that of a cationic polyelectrolyte [19]. Moreover, the excess of cationic charges at 10 : 1 : 2 material surface is also responsible for good fibroblast

TABLE II Tensile properties of swollen hydrogels in water solutions

Samples	Water			1 M NaCl solution		
	E (MPa)	σ(MPa)	ε (mm/mm)	E (MPa)	σ(MPa)	ε (mm/mm)
p(HEMA)	0.27 ± 0.03	0.225 ± 0.008	3.125 ± 0.244	0.41 ± 0.05	0.235 ± 0.018	1.414 ± 0.097
10 : 1 : 1 terpolymer	0.88 ± 0.09	0.186 ± 0.012	0.252 ± 0.016	0.77 ± 0.08	0.176 ± 0.010	0.279 ± 0.019
10 : 1 : 2 terpolymer	0.50 ± 0.07	0.088 ± 0.011	0.225 ± 0.012	0.49 ± 0.06	0.098 ± 0.009	0.221 ± 0.017

TABLE III MTT results after 48 h of incubation

Samples	No. cells	% no. cells	OD_{570}	$\%OD_{570}$
Control (polystyrene)	560.000	100	2.349	100
p(HEMA)	490.000	88	2.105	90
p(HEMA/AMPS/METAC) 10 : 1 : 1	520.000	93	2.251	96
p(HEMA/AMPS/METAC) 10 : 1 : 2	560.000	100	2.350	100

No. cells = cell number, % no. cells = percentage of cells with respect to control, OD_{570} = optic density at 570 nm, $\%OD_{570}$ = percentage of optic density with respect to control.

adhesion, in agreement with the literature data that demonstrated the capability of cationic polymers (i.e. polylysine, polyarginine) to promote fibroblast adhesion [20, 21].

In conclusion, the insertion of oppositely ionic charges in polymeric network of neutral p(HEMA) represent a strategy to achieve higher swollen hydrogels via generation of co-operative ionic stabilising interactions in the terpolymer networks. Moreover, the presence of an excess cationic charges confer to 10 : 1 : 2 terpolymer sensitive behaviours in pH solutions, and promote human fibroblast adhesion on its surface.

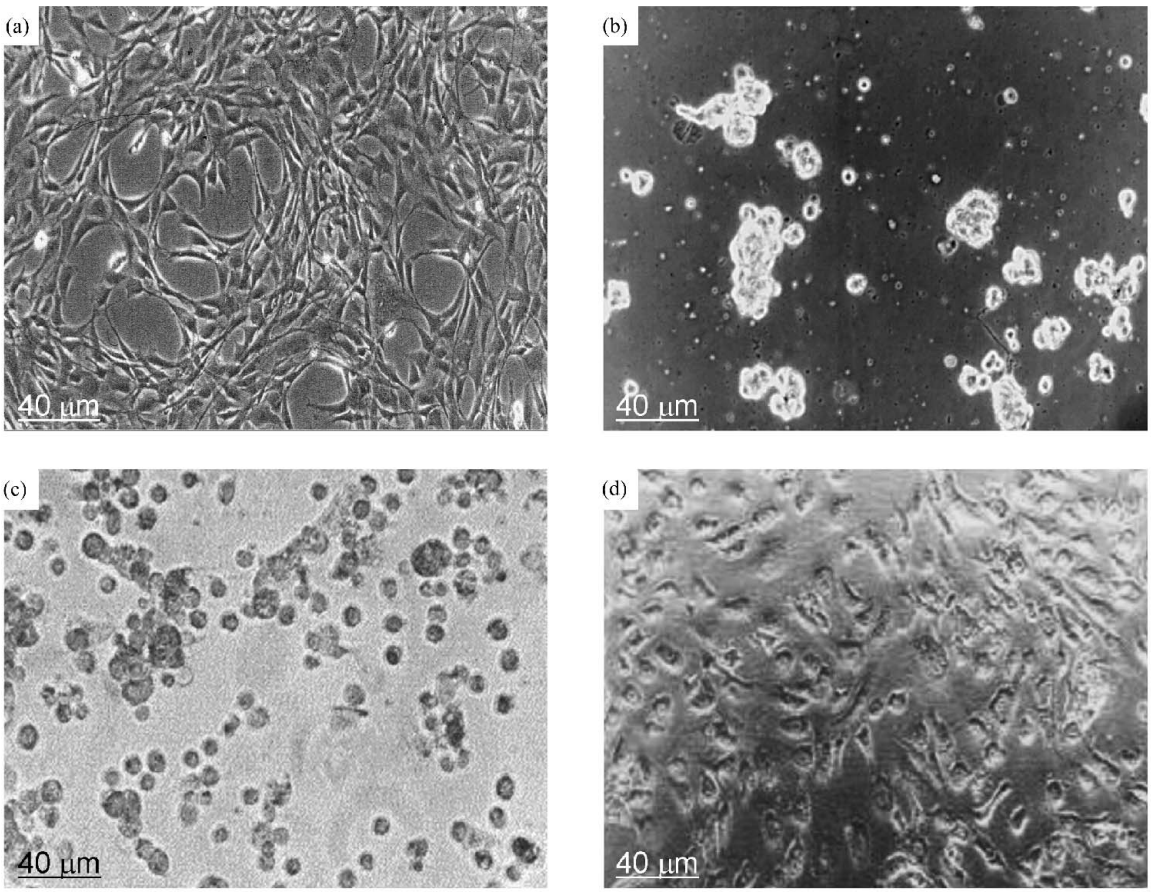

Figure 4 Optical micrographies of human fibroblasts seeded on polystyrene. (a) control; (b) p(HEMA); (c) p(HEMA-co-AMPS-co-METAC) 10 : 1 : 2. (magnification × 400)

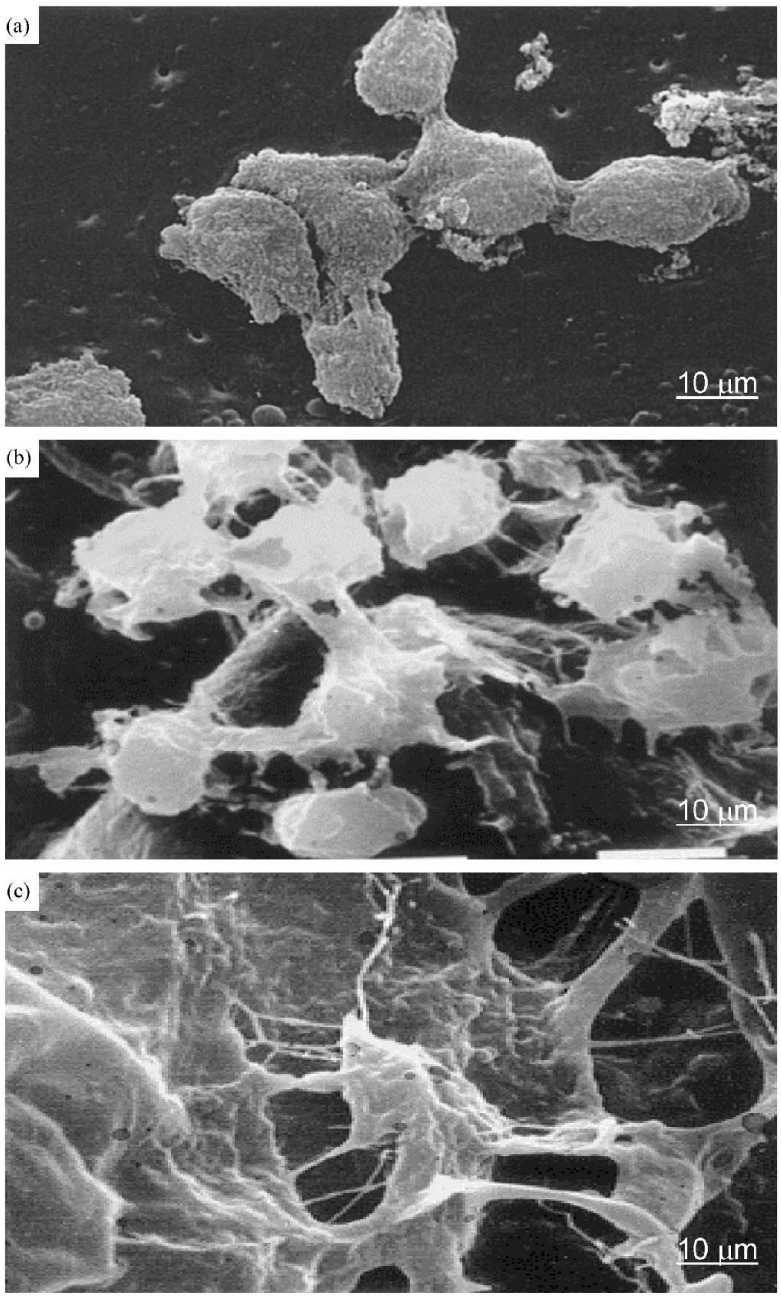

Figure 5 SEM analysis of human fibroblasts seeded on p(HEMA). (a); p(HEMA-co-AMPS-co-METAC) 10 : 1 : 1 (b) and p(HEMA-co-AMPS-co-METAC) 10 : 1 : 2 (c) after 24 h of incubations. (magnification × 4000).

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