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Physicochemical properties of ionic and non-ionic biocompatible hydrogels in water and cell culture conditions: Relation with type of morphologies of bovine fetal fibroblasts in contact with the surfaces

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ABSTRACT

Cationic, anionic and non-ionic hydrogels having acrylamide polymer backbones were synthesized via free radical polymerization with N,N-methylenebisacrylamide (BIS) as crosslinker. The chemical structures of the hydrogels were characterized by Fourier Transform Infrared Spectroscopy (FTIR). Physicochemical properties such as swelling kinetic, maximum swelling capacity, volume phase transition temperature (VPTT) and wettability (static water contact angle) of hydrogels swollen in aqueous and cell culture medium, at room and cell culture temperatures were studied. In order to correlate the surface properties of the hydrogels and cellular adhesivity of bovine fetal fibroblasts (BFFs), cellular behaviour was analyzed by inverted fluorescence optical microscopy and atomic force microscopy (AFM). MTT assay demonstrated that the number of viable cells in contact with hydrogels does not significantly change in comparison to a control surface. Flattened and spindle-shaped cells and cell spheroids were the adopted morphologies during first days of culture on different hydrogels. Cell spheroids were easily obtained during the first 5 days of culture in contact with PNIPAM-co-20%HMA (poly (N-isopropylacrylamide-co-20%N-acryloyl-tris-(hydroxymethyl)aminomethane)) hydrogel surface. After 15 days of culture all hydrogels showed high adhesion and visual proliferation. According to obtained results, non-ionic and hydrophilic surfaces with moderated wettability induce the formation of BFFs cell spheroids. These hydrogel surfaces could be used in clinical and biochemical treatments at laboratory level to cell growth and will allow generating the base for future biotechnologic platform.

1. Introduction

Biomaterials are being used in cell culture technique as scaffolds to create tissue-like structures that simulate the mechanical and physiological features of *in-vivo* tissues. Biomaterials based on hydrogels have great potential for tissue reconstruction as either temporary or stable scaffolds or cell vehicles for tissue engineering [1]. In addition, the cell–cell and cell–extracellular matrix (ECM) interactions play a significant role in cell growth. Hydrogels are crosslinked polymers (covalent or physic bond) with high content of hydrophilic groups allowing high uptake of biological fluids. For that reason, these are one of the most commonly used materials in biomedicine since they could emulate ECM structure providing sites for adhesion, proliferation and even more cell differentiation [2].

Nowadays, synthetic and natural hydrogels are also being widely investigated for biotechnological applications, for example the immobilization of *Saccharomyces cerevisiae* in hydrogel matrix for bioethanol production [3]. In this case, the biocompatibility and protective character of poly-acrylamide hydrogel on yeast were demonstrated. Gelatin is a kind of natural polymer with low cost and good biocompatibility but this could be quickly denatured [4] which may be a disadvantage depending on the applications.

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Poly(N-isopropylacrylamide) (PNIPAM) is the most extensively studied synthetic polymer for therapeutic purposes. PNIPAM has a low critical solution temperature (LCST) value at 32 °C [5,6]. When the environment temperature exceeds the PNIPAM LCST value, the hydrogel undergoes a reversible volume phase transition, collapses and expels the internal liquid. The phase transition produces a drastic change on hydrophilic/hydrophobic properties of the material [7] and consequently this change could be altering the cell adhesion/detachment mechanisms [8,9]. These changes were observed when the chemical composition of scaffold material was modified by copolymerization with hydrophilic or hydrophobic co-monomers [10]. Moreover, interpenetrated or semi-interpenetrated systems based on thermosensitive hydrogel and a second polymer [11], polymeric nanocomposites based on hydrogel matrix [7,12] and thermosensitive surfaces [7] showed great changes on their physicochemical, superficial and mechanical properties.

Citotoxicity, viability and cellular damage have been studied in contact with PNIPAM for Caco-2 and Calu-3 cells [6], mesenchymal stem cells derived from rat bone marrow (BM-MSCs), human adipose tissue (AT-MSCs) [13], smooth muscle (SMC) [14] and other biological systems. However, there is scarce information about physicochemical properties of hydrogels exposed to cell culture medium. We believe that it is important to know the physicochemical behavior of hydrogels under cell culture conditions, in order to understand and predict how the cell will respond to environment changes [15].

In this work, we propose to study the physicochemical properties of polyacrylamide (PAAM), PNIPAM hydrogel and copolymers functionalized with anionic, cationic and non-ionic monomers in both aqueous and cell culture medium. Material properties as swelling kinetics, volume phase transition temperatures (VPTT) and wettability (static contact angle) were characterized. The physicochemical properties of the hydrogel surfaces in culture conditions were related to the adhesion and morphology kinds adopted by bovine fetal fibroblasts (BFFs).

It was demonstrated that flattened and spindle or spheroids cells can be formed on hydrogel surfaces depending on chemical composition. These surfaces kinds will allow applying medical and biomedical *in-vitro* treatments and quickly selecting the best one to be given to the sick patient. The formation of spheroids on these surfaces has the advantage that these are exposed to a direct treatment avoiding that the drug passes through a barrier material. The use of these surfaces for *in-vitro* studies at laboratory level would help to define more quickly which of the medical treatments will be more suitable to apply *in-vivo* (e.g. antibiotic selection, anti-carcinogenic treatments, etc).

Follow-up studies will lead to design scaffolds based on hydrogels for other cell lines and future biomedical applications.

2. Materials and experimental methods

Hydrogels were synthesized via free radical polymerization of acrylamide (AAM), N-isopropylacrylamide (NIPAM) (Scientific Polymer Products) and copolymerized with N-acryloyl-tris-(hydroxymethyl)aminomethane (HMA) (Sigma-Aldrich), (3-acrylamidopropyl)trimethylammonium chloride (APTA) (Sigma-Aldrich) and 2-acrylamido-2-methylpropanesulfonic acid (AMPS) (Scientific Polymer Products). N,N-methylenebisacrylamide (BIS) (Sigma-Aldrich) was used as crosslinker agent. Ammonium persulfate (APS) (Sigma-Aldrich) and N,N,N',N'-tetramethylenediamine (TEMED) (Sigma-Aldrich) were used as the initiator and activator of polymerization, respectively.

2.1. Synthesis of hydrogel scaffolds

PNIPAM were synthesized dissolving 0.5 M NIPAM and 2% moles of BIS (regarded to NIPAM moles) as crosslinker agent in aqueous solution. Next, polymerization initiator system (APS- 0.001 g/mL and TEMED- 10 μL/mL) was added to the precursor solution; O_2 was previously removed by bubbling with N_2 gas. Copolymers with NIPAM were carried out by co-monomer addition (10% moles of AMPS, 10% moles of APTA or 20% moles of HMA) to precursor solution before adding the initiator system.

PAAM hydrogels were synthesized dissolving 1 M of the monomer in aqueous solution and keeping the same proportions of crosslinker and initiator system as indicated in case of PNIPAM. The polymerization reactions were carried out in shallow wells, in order to obtain thin surfaces of hydrogels, at room temperature (22.0 ± 2.0 °C) during 3 h. When the polymerization was completed, the hydrogels were immersed in distilled water at room temperature for 48 h and the water was renewed several times in order to remove unreacted chemicals.

2.2. Fourier transform infrared spectroscopy (FTIR)

The products of the polymerization and copolymerization were monitored by FTIR. Spectra were measured in an Impact 400 spectrometer (Nicolet) by transmission in hydrogel/KBr pills which were previously dried under vacuum. The measurements were made three times to each material. Spectra were recorded in wavenumber range from 4000 to 400 cm^{-1}, at a resolution of 2 cm^{-1}.

2.3. Swelling kinetic measurements

Each dry hydrogel sample, previously washed and weighed, was placed in distilled water or complete cell culture medium – DMEM/FBS (DMEM (Invitrogen, Grand Island, NY, USA with 10% v/v of fetal bovine serum (FBS))- at room temperature (22.0 ± 2.0 °C). The sample was removed from the solution, at certain time intervals, and was superficially dried with tissue paper, weighed on an analytic balance and placed back into the bath. The measurements were repeated until achieving a constant weight. The swelling percentage (%Sw) as a function of time was calculated with the following equation:

$$\%Sw_{(t)} = [(W_{(t)}\text{-}W_{(dry)})/W_{(dry)}]x100 \quad (1)$$

Where $W_{(t)}$ represents the weight of the hydrogel in swollen state at time t and W_{dry} is the weight of the dry hydrogel. Graphics of %Sw *vs* time were used to analyze the swelling kinetic. %Sw results were obtained from five measurements averaged to each hydrogel. Swelling percentage in equilibrium state ($\%Sw_{eq}$) represents the maximum capacity of the hydrogel to absorb solution and corresponds to the plateau of kinetic curve. Nonlinear fitting of the swelling curve with a first order kinetics equation allowed determining the rate constant (*Ks)* of the pseudo-first-order swelling model. The aim of this experience was to evaluate how the kinetic parameters of the hydrogels swelling could be affected by the cell culture medium components in relation to aqueous medium. $\%Sw_{eq}$ was also studied at room (22.0 ± 2.0 °C) and culture temperature (37.0 ± 0.5 °C), being in the last case regulated by a thermostat.

2.4. Volume phase transition temperature (VPTT) by differential scanning calorimetry (DSC)

The DSC measurements were conducted using a TA Instruments DSC 2010 under N_2 flow. The samples were previously swollen in water or DMEM/FBS. The sealed pan with the sample (ca. 100 mg) was quickly cooled inside the DSC chamber at −25 °C by filling the

outer reservoir with a frozen solution of 80% w/w $CaCl_2$ (16 g) in water (20 mL). Several minutes allow the system to attain thermal equilibrium. The sample holder assembly was then heated at a rate of 5 °C/min from −25 to 60 °C, after phase transition and below 100 °C to avoid decomposition of the sample and water evaporation.

2.5. Static contact angle measurements

A digital microscope Intel QX3 with a 60X objective was used for photographing the drop image over flat polymer piece illuminating with a white LED light. Water drop (0.1 mL) was placed over the hydrogel surface by sessile drop method and the internal contact angle was determined between the liquid-air interface line and the hydrogel surface.

The pictures were analyzed using "Drop Analysis" software. Biomedical Imaging Group. (http://bigwww.epfl.ch/demo/dropanalysis/). Experiences were carried out on a heating plate to remain the constant temperature at 20.0 ± 0.5 °C and 37.0 °C ± 0.5 °C.

2.6. In vitro cell culture

BFF cells modified to constitutively express a green fluorescent protein (GFP) (generously provided by Dr. Wilfried Kues, Friedrich Loeffler Institute, Germany), were used to evaluate some parameters of biocompatibility of the hydrogels. The swollen cylindrical hydrogel films (diameter: 0.5 cm – height: 0.2 cm) were dried in an oven at 50 °C during approximately 24 h until removing remaining water and then washed twice during 30 min in phosphate saline buffer (PBS, pH 7.4) supplemented with 10% v/v antibiotic-antimycotic (penicillin-streptomycin-fungizone, Gibco®). To remove traces of antibiotic-antimycotic from matrices, hydrogels were washed three times (30 min/wash) in sterile PBS and then were exposed to UV radiation (λ_{em} = 200–280 nm) for two periods of 15 min to inactive microorganisms. Finally, the surfaces were immersed in complete cell culture medium (DMEM/FBS) during 24 h before cell seeding. Then, cell culture medium was replaced by fresh complete DMEM/FBS and cells were seeded at a density of 10^5 cells/well in a 24-well plate. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.7. Cell morphology evaluation

Cell growth, adhesion, attachment, visual proliferation and cell–cell interaction were evaluated on different hydrogel surfaces by direct microscopy employing an optical inverted fluorescence microscope Nikon model Ti-S 100. Atomic force microscopy (AFM) images were also taken with an Agilent 5420 AFM/STM microscope. A commercial Point Probe-Plus Non-Contact/Tapping Mode-Long Cantilever (PPP NCL) with a constant force of 6 Nm^{-1} and a resonance frequency of 156 Hz was used in the Acoustic AC (AAC) mode.

2.8. Cytotoxicity assay

The number of viable cells in presence of hydrogel was measured by the MTT assay which follows the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a colored insoluble formazan. The obtained formazan was then dissolved in dimethyl sulfoxide (DMSO) and the concentration was expressed as proportional to optical density (OD) at 540 nm.

Briefly, BFFs were seeded in 96-well plates at a density of 5000 cells/well in 200 μL of complete DMEM/FBS. Cells were incubated in presence of a hydrogel piece for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. After 24 h of culture, the hydrogel was removed; the MTT reagent (1 mg/mL) was added to each well and further incubated for 3 h at the same temperature. The reaction mixture was removed from each well and replaced by 100 μL of DMSO. The OD at 540 nm was measured using a microplate reader (Bio-Rad®). Each experimental condition was included a viability control group in which the hydrogel was omitted. The viable cell number after 24 h of culture was expressed as OD of formazan obtained to each hydrogel and control group.

2.9. Statistical analysis

Data were analyzed using ANOVA with INFOSTAT/L program. Post-hoc comparisons were performed using Bonferroniís test. The values are expressed as means ± SD and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. FTIR measurement

Hydrogels were washed and dried to analyze the presence of characteristic functional groups in polymers and copolymers by FTIR. It is known that amide spectra shows particularly the carbonyl stretching vibration typically known as the amide I band (1670–1650 cm^{-1}, state solid) and an intense N—H stretching vibration at 3400 cm^{-1}. In solid state and in the presence of hydrogen bonding, these bands could be shifted to 3350–3200 cm^{-1} because the carbonyl band is dependent on the amount of hydrogen bonding occurring [16].

All materials showed characteristic bands at 3600–3100 cm^{-1} due to N—H bonding presence and —CH_3 and —CH_2 symmetric at 2974 and 2881 cm^{-1} asymmetric stretching (Supplementary information, SI 1).

In addition, primary amides have a weak-to-medium intensity band at 1650–1620 cm^{-1}, which is generally close to the strong carbonyl band. This band is known as the amide II band and is due to a motion combining both the N—H bending and the C—N stretching vibrations of the amide group. PAAM showed a broad band with a maximum of intensity at 1590 cm-1, probably due to overlapping of both amide I and II bands and the presence of hydrogen bonds.

Secondary amides based on PNIPAM showed an amide I band at 1660 cm^{-1}, amide II band at 1570–1515 cm^{-1} and the isopropyl methyl deformation band at 1386 and 1367 cm^{-1} (with strong doublet), according to bibliography [16].

Copolymers derived of NIPAM showed the same bands mentioned. When HMA monomer units were copolymerized with NIPAM, the characteristic band of O—H groups at 3300 cm^{-1} was overlapped with the intense N—H stretching band showing an even wider one. This broad band of O—H stretching indicates intermolecular hydrogen bonding. Moreover, a clear C—O stretching band (1020 cm^{-1}) was observed. In the copolymer containing AMPS monomer units, characteristic bands of the sulfonic groups such as 1192–1040 cm^{-1} (asymmetric and symmetric O = S=O stretching) and 630 cm^{-1} (st. C–S) [17,18] were observed. The quaternary ammonium salt groups (from APTA monomer units) did not present an unique band in the infrared [19]. Therefore, PNIPAM-co-APTA copolymer cannot be differentiated from PNIPAM.

3.2. Swelling kinetic in water and culture medium at room temperature

After verifying the chemical composition of synthesized hydrogels, the physicochemical properties such as swelling capacity,

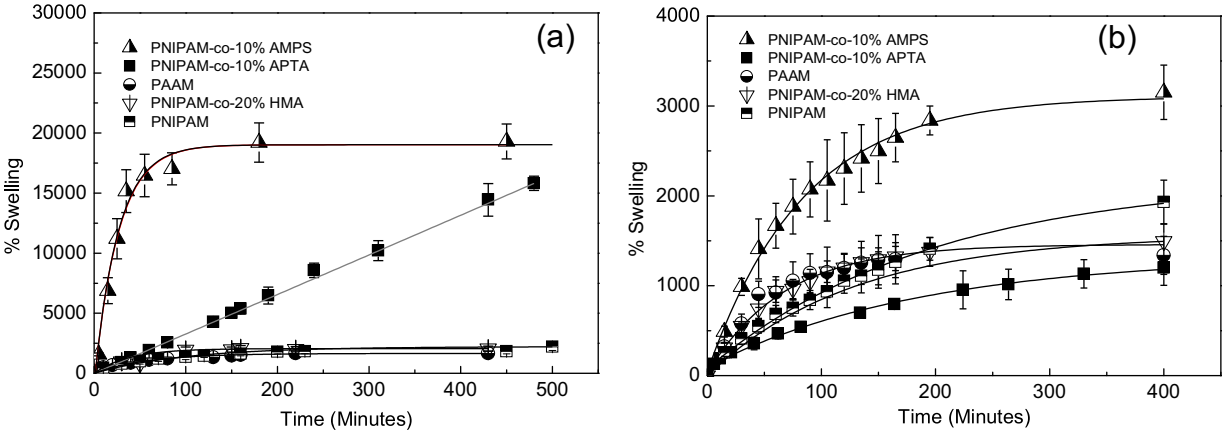

Fig. 1. Swelling kinetics of the different hydrogels in water (a) and DMEM/FBS (b), at 22.0 ± 2.0 °C. PNIPAM-co-10%AMPS and PNIPAM-co-10%APTA (SD, n: 5); PAAM, PNIPAM-co-20%HMA and PNIPAM (SD, n: 5).

Table 1
Rate constant (*Ks*) of swelling of PAAM, PNIPAM and copolymeric hydrogels in DMEM/FBS and water, at 22 ± 2 °C.

Surfaces	Ks (s^{-1}) (SD)		
	Water	DMEM/FBS	Ratio(W/D)
PAAM	0.014 (0.003)	0.020 (0.001)	0.7
PNIPAM	0.013 (0.001)	0.0051 (0.0003)	2.5
PNIPAM-co-20% HMA	0.023 (0.008)	0.015 (0.001)	1.5
PNIPAM-co-10% AMPS	0.037 (0.004)	0.0118 (0.0006)	3.08
PNIPAM-co-10% APTA	0.0003 (0.0002)	0.0054 (0.06)	0.06

volume phase transition temperature and wettability were analyzed considering that these properties depend on the water activity in the solution. Besides the ionomeric hydrogels could also be affected by the activity of counterions in the solution. Therefore, the properties of the hydrogels could be different according to the surrounding environment. To assess this effect, the properties were measured at room temperature in aqueous and complete culture medium (DMEM/FBS). In Fig. 1, the swelling kinetics of hydrogels in both media is shown. It is noteworthy that ionic hydrogels have higher swelling capacity than non-ionic hydrogels in both mediums and the swelling kinetic is considerably affected by cell culture medium composition due to the high concentration of salts, proteins, amino acids and non- specific components of FBS. In the case of PNIPAM-co-10%AMPS, $\%Sw_{eq}$ reaches 19000 in water whereas it only achieves 3100 in DMEM/FBS.

The hydrogel swelling is a process where the solvation of the polymeric chains is counterbalanced by the elastic constant of the crosslinked polymeric matrix. Thus, the rate of swelling is related to the rate of relaxation of the polymeric chains in the hydrogel matrix. In this way, nonlinear fitting of the swelling curve was made with a first order kinetic equation [20]:

$$\%Sw(t) = \%Sw_{eq}(1\text{-}\exp(\text{-}Ks.t)) \quad (2)$$

Where, *Ks* is the rate constant of the pseudo-first-order swelling model. The kinetic values obtained for each hydrogel and ratio of *Ks* in water and DMEM/FBS (Ratio W/D) are shown in Table 1. Cationic hydrogel in water and DMEM/FBS shows a small rate of swelling (*Ks*), whereas the anionic hydrogel has a faster rate. The other hydrogels showed intermediate values.

These results suggest that the swelling kinetic of hydrophilic non-ionic hydrogels (PAAM and PNIPAM-co-20%HMA) are lightly affected by medium change (ratio around 1). However, PNIPAM shows a decrease of rate in DMEM/FBS (ratio >1) due to interaction among medium components.

On the other hand, the swelling rate of ionic hydrogels (PNIPAM-co-10%AMPS and PNIPAM-co-10%APTA) is strongly affected. A swelling rate decrease is observed in DMEM/FBS for PNIPAM-co-10%AMPS (ratio » 1) due to the presence of salts and other components of medium which lowers the driving force of counterion osmotic pressure [15]. A quite large increase in PNIPAM-co-10%APTA (ratio « 1) is observed due to the very small *Ks* in water. Neighboring charges repulsion makes the polymer very rigid in water but DMEM/FBS counterions shield that effect increasing the flexibility of the chains.

3.3. Volume phase transition temperature (VPTT) of hydrogels in water and culture medium

VPTT of hydrogels were determined by DSC. The VPTT of PNIPAM in water [5] is ca. 32 °C and it is known that the introduction of hydrophilic groups in the polymeric chain increases the VPTT in water [19].

Only non-ionic hydrogels, such as PNIPAM and PNIPAM-co-20%HMA, preserved the phase transition temperature below 60 °C (Fig. 2). A little effect of medium properties on VPTT is observed in the case of PNIPAM, being slightly higher in the case of PNIPAM-co-20%HMA due to the presence of hydrophilic groups (-OH).

On the other hand, it is known that PAAM is not thermosensitive and ionic hydrogels such as PNIPAM-co-10%AMPS and PNIPAM-co-10%APTA, did not show VPTT values below 60 °C (the measurement limit to avoid the water boiling point) in both mediums. The high hydrophilic nature and amount of ionic co-monomers effectively increases the VPTT. The results are in agreement with a previous report where it was observed that AMPS presence caused a larger effect on VPTT than HMA [10].

According to DSC results at cell culture conditions (37 °C and DMEM/FBS), PAAM, PNIPAM-co-10%AMPS and PNIPAM-co-10%APTA would be swollen, while PNIPAM and PNIPAM-co-20%HMA would be collapsed.

3.4. Comparison of maximum swelling capacity ($\%Sw_{eq}$) in water and culture conditions

The swelling percentage of hydrogels in the equilibrium state ($\%Sw_{eq}$) was measured at 22.0 ± 2.0 and 37.0 ± 0.5 °C for all materials, in deionized water and DMEM/FBS culture medium. The obtained values are shown in Fig. 3. At 22.0 ± 2.0 °C, higher swelling capacity is observed in water than in DMEM/FBS for ionic hydrogels (PNIPAM-co-10%AMPS and PNIPAM-co-10%APTA). This is due to the presence of ionic moieties ($\text{-}SO_3^-$ and $-N(CH_3)_3^+$), which are

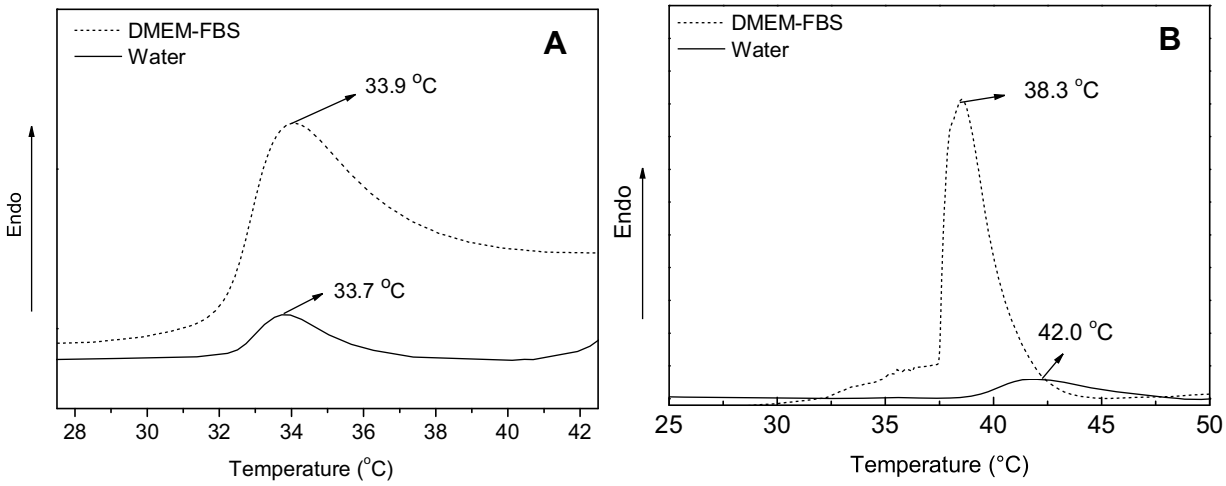

Fig. 2. Thermograms of PNIPAM (A) and PNIPAM-co-20%HMA (B) swelled in both mediums. (SD: 0.3, n: 4).

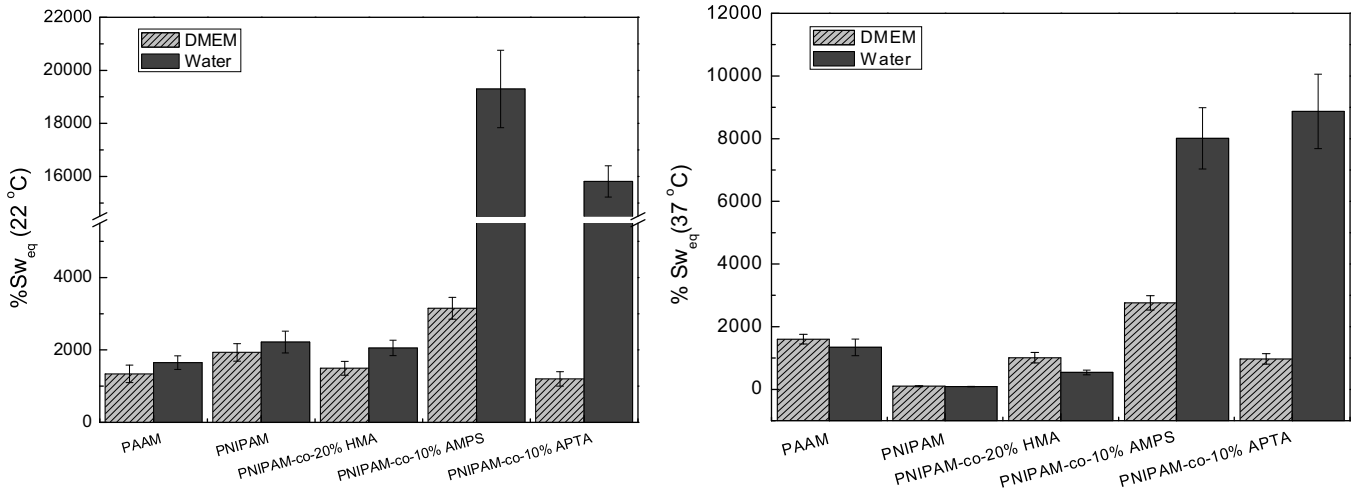

Fig. 3. Equilibrium swelling percentage (%Sw_{eq}) of PAAM, PNIPAM and copolymeric hydrogels, in both DMEM/FBS and aqueous medium at two temperatures: 22.0 ± 2.0 °C and 37.0 ± 0.5 °C. PNIPAM-co-10%AMPS and PNIPAM-co-10%APTA (SD, n: 4); PAAM, PNIPAM-co-20%HMA and PNIPAM (SD, n: 4).

balanced by mobile counterions (H^+ for AMPS and Cl^- for APTA). The swelling of these materials increases due to the osmotic pressure of the mobile counterions solution inside the hydrogel. Such effect is strongly dependent on the ionic force of the external solution. Since DMEM culture medium has large content of salts and proteins, the osmotic pressure is balanced so the hydrogels swell less.

On the other hand, in the case of hydrogels with hydrophilic neutral groups, the difference of%Sw_{eq} between DMEM/FBS and water is small due to probable water activity changes. Similar salt concentration effect was observed in hydrogels based on NIPAM semi-interpenetrated with salecan (higher hydrophilic and non-ionic polymer) [21].

%Sw_{eq} was also analyzed to hydrogels swollen in DMEM and 10% FBS in water. It can be seen that%Sw_{eq} of non-ionic hydrogels in presence of FBS in water or DMEM is similarly affected when both are mixed, while the%Sw_{eq} of ionic hydrogels slightly varies according to nature of ionic charge (Supplementary information, SI 2).

When the swelling measurement is carried out at cell culture temperature (37.0 ± 0.5 °C) in both mediums, the%Sw_{eq} are lower than that at room temperature (22.0 ± 2.0 °C). The temperature effect is significant for PNIPAM and PNIPAM-co-20%HMA due to the fact that these gels are collapsed at 37 °C.

PNIPAM increases 100 times its weight when it is swollen in DMEM/FBS and 88 times when it is swollen in water at 37 °C while PNIPAM-co-20%HMA increases 1010 times its weight when swollen in DMEM/FBS and 540 times in water. Therefore, these hydrogels could absorb enough water or cell culture medium even when they are collapsed.

Clearly, it is demonstrated that the composition of hydrogel, solvent properties and temperature conditions can notably change the physicochemical properties of the materials. In the same way, the superficial properties such as wettability of these materials could also be affected by the mentioned factors. Therefore, it is important to know the physicochemical behavior of hydrogel surfaces at defined working conditions.

3.5. Wettability of hydrogel surfaces in water and culture conditions: static contact angle

The measurement of static contact angle formed between a water drop and a surface could be used to characterize the wettability or hydrophilic/hydrophobic properties of surface. It is known that if the contact angle value is less than 90° the surface is hydrophilic whereas if it is greater than 90° it is hydrophobic. Surfaces with contact angle between 150° and 180° are called superhydrophobics [20].

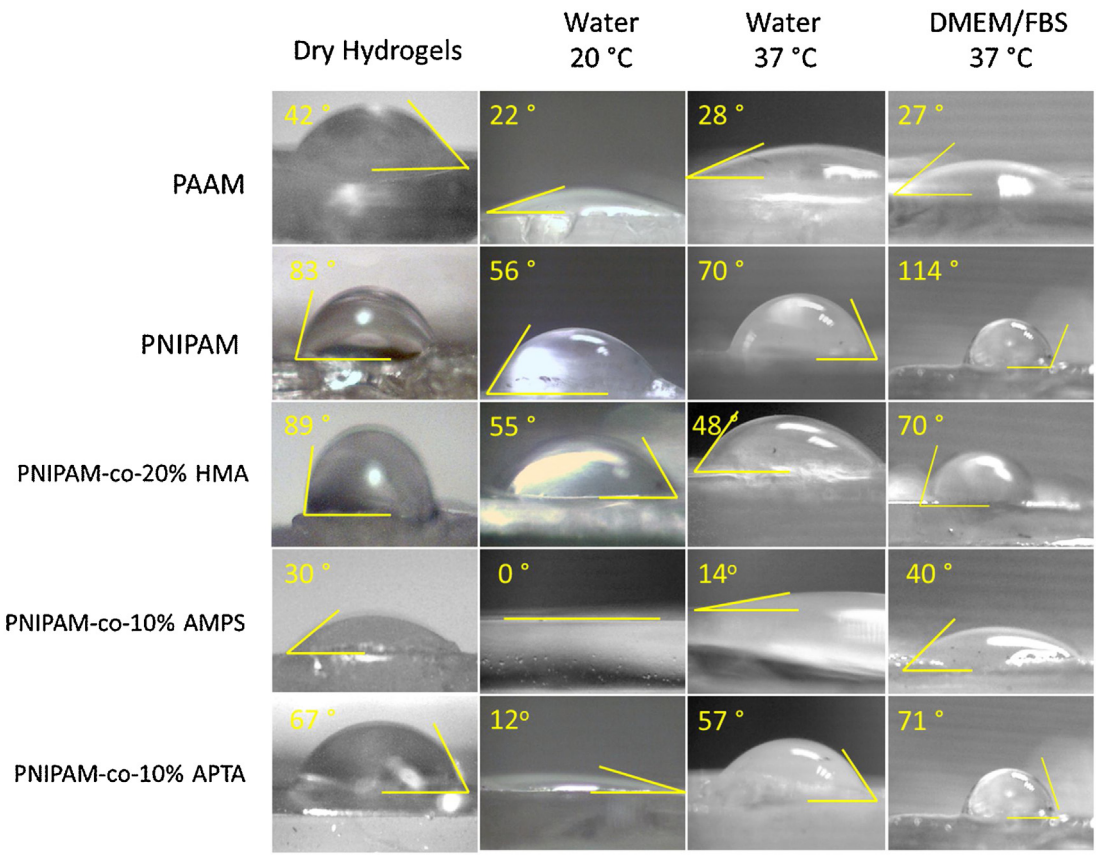

Fig. 4. Photograph of water drops in contact with surfaces of dry and wet hydrogels (at 20.0 ± 0.5 and 37.0 ± 0.5 °C) with the contact angles inserted. (SD= ± 2, n: 5).

In the case of hydrogels, the superficial wettability could be related to the water contained inside hydrogel. Moreover, since the polymeric chains are flexible, the functional groups could be exposed or buried inside the hydrogel depending on the swelling state. Therefore, the wettability of hydrogel surfaces was determined at fully dry and fully swollen conditions. On the other hand, knowing that the volume phase transition temperature in thermosensitive polymers involves a change of hydrophilic (below VPTT) to hydrophobic (above VPTT) states [5], wettability measurements were also performed at both temperature conditions (20.0 ± 0.5 and 37.0 ± 0.5 °C). In Fig. 4 the images of water drop on hydrogels surfaces are shown with the contact angle values inserted.

As expected, at 20.0 ± 0.5 °C swollen hydrogels in water show lower water contact angles than dry hydrogel, indicating high hydrophilicity due to water containing. While, at 37.0 ± 0.5 °C all swollen hydrogels in water at 37.0 ± 0.5 °C show intermediate values due to low water containing (water expulsion by collapse). At cell culture conditions (DMEM/FBS at 37 °C), the contact angles increase respect to water, except to PAAM. The rotation of N-isopropyl moieties in PNIPAM and its copolymers is increased by temperature excluding the water molecules from the vicinity of the amide groups. In that way, the local hydrophobicity increases and long-range interactions lead the hydrogel to collapse. Thus, the surface hydrophobicity could be increased, at least in the areas containing NIPAM monomer units as it is observed in all cases. Nevertheless, the adhesion of proteins and counterions of medium on surfaces also increases hydrophobicity.

An exceptional behavior was observed in PAAM in DMEM/FBS, where the contact angle is not altered by culture conditions. PNIPAM and PNIPAM-co-20%HMA at 37.0 ± 0.5 °C increase the contact angle in DMEM/FBS regarding water tending to be a more hydrophobics surface. In the case of the ionic hydrogels, PNIPAM-co-10% AMPS and PNIPAM-co-10%APTA, the contact angle in DMEM/FBS is even higher than that obtained for dry hydrogels possibly due to strong interaction among ions, proteins, amino acid, etc, present at the culture medium and the ionic moieties existing in the polymeric matrix.

In culture conditions, PNIPAM is extremely hydrophobic while PAAM is very hydrophilic like PNIPAM-co-10%AMPS, while PNIPAM-co-20%HMA and PNIPAM-co-10%APTA seem to be slightly hydrophilic. Similar tendency was observed for swelling capacity in culture conditions (Fig. 3).

3.6. Biocompatibility of the hydrogel surfaces

The viable cell number analyzed by MTT test is one of the used methods to determine the cytotoxic effect of materials. MTT is a yellow tetrazolium salt that is only reduced in metabolically active cell mitochondria. After reduction, a violet formazan dye is formed and can be quantified spectrophotometrically to determine the amount of living cells [6].

MTT assays were performed with BFF cells cultivated during 24 h in contact with the hydrogels already sterilized and using polystyrene multi-well plates surface as control system. In Fig. 5 the optical density (OD) at 540 nm of formazan after each hydrogel was in contact with BFFs are shown. Although, PNIPAM and PNIPAM-co-10%AMPS indicate a slightly increased mitochondrial activity of cells (Fig. 5), the hydrogels do not show any significant difference regarding the control system. A similar case was reported by H. Vihola et al. [6] for Caco-2 cell cultivated on PNIPAM. They observed an increase of mitochondrial activity and considered that this may be a sign of a possible subtoxic effect during the time of exposition. Then, cell damage was not evidenced by LDH test (below 10%).

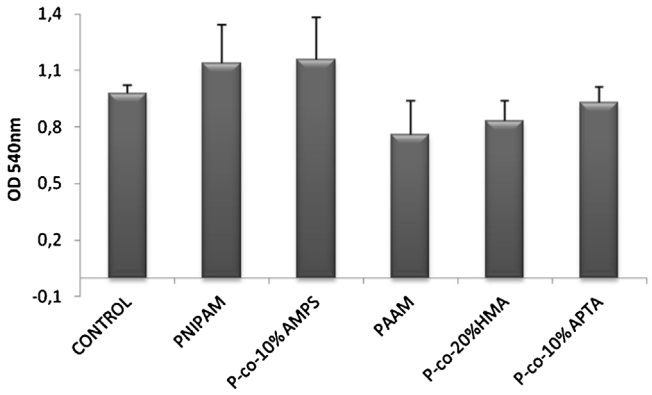

Fig. 5. Optical density (OD) of formazan obtained by MTT assay of BFFs in contact with hydrogels after 24 h of growth (control: BFFs grown on polystyrene multi-well plates without hydrogel). All data are expressed with the mean ± SD at n = 16 and $p < 0.05$.

Being OD proportional to viable cell number and no significant differences with control were observed, these results suggest that the hydrogels under study are biocompatible [9].

3.7. Cellular morphology and adhesion of BFFs in contact with hydrogels

Ocular inspection of BFF cells under a fluorescence microscope revealed that the cells not only adhere over hydrogel surfaces but also apparently would proliferate on them (Fig. 6).

BFFs seeded on PAAM, PNIPAM-co-10%AMPS and PNIPAM-co-20%HMA hydrogels did not spread over the surface; cell spheroids or aggregates were rather formed (Fig. 6Aa, b, c). These tridimensional cell spheroids were remained until 3 days on PAAM and PNIPAM-co-10%AMPS. After 3 days of culture, spheroids disaggregated and spread over the hydrogel surface adopting their typically flattened and spindle-shaped morphology (Fig. 6Af and g). Higher swelling capacity of PAAM and PNIPAM-co-10%AMPS in culture conditions might be one of the factors affecting cell adhesion. Higher swelling capacity could be favorable for the diffusion of nutrients and metabolic waste but if the hydrogel surface is highly hydrated, the cells may consider the surface essentially as water and do not attach on it, which will lead to poor cell adhesion [22].

BFF spheroids on PNIPAM-co-20%HMA were kept for 5 days of culture without observable alterations and then the spheroids started to disaggregate (Fig. 6Ah).

In Fig. 6B, it is clearly observed that although individual cells tend to self-assemble or associate as part of bigger spheroids (red circle) for more than 48 h of seeding a few of them are in contact with PNIPAM-co-20%HMA surface. Even though these spheroids disaggregate after 5 days of growth, there would be enough time to perform *in-vitro* biomedical studies.

Similar spheroids formation was observed for mesenchymal stem cells cultured on transparent membranes of chitosan and chitosan grafted with hyaluronan (HA) [23] and also for normal human dermal fibroblasts (NHDF) cultured on films of alginate [24].

On the other hand, BFF cells seeded on PNIPAM-co-10%APTA and PNIPAM were attached and adopted a typically flattened and spindle-shaped morphology immediately after seeding (Fig. 6A, d-i and e-j). It is known that when cationic groups are present in the hydrogel's matrix, FBS proteins, components of DMEM and/or BFF secreted factors may be easily absorbed by the hydrogel favoring the cell adhesion process on the surface [25]. The hydrophobicity of PNIPAM has always favored the adhesion of several kinds of cells [12,13,26].

After 15 days of culture, cells were fixed with methanol on a glass surface and AFM images were taken to observe possible BFF cells morphology changes in contact with the scaffold materials. Fig. 7A shows an isolated BFF cell on PAAM surface looking as in process of attachment/detachment and showing some points of junction with surface indicated by black arrows. It seems that cell tends to adopt a typically flattened morphology and an extended zone achieving a spindle-shaped. Below the AFM image, the profile graphic shows a cell size around 25×25 μm on plane with a height from 0.4 to 0.5 μm corresponding to a flattened and spindle-shape morphology.

The Fig. 7B shows an isolated BFF cell on the PNIPAM-co-20%HMA surface whose cytoskeleton looks like a condensed or contracted cell without clear points of junction with the surface. The profile graphic shows an approximated size of 6×6 μm on plane and a height from 0.8 to 0.7 μm. In this case, the cell is adopting a contracted form what it would be indicating low adhesion with scaffold material. Clear differences between morphologies adopted by the cells are observed in Fig. 7, concluding that the cell adhesion depends on the physicochemical characteristics of the material surface.

Similar AFM images of contracted cells were also observed in mouse primary cardiac fibroblasts when actin-destabilizing Cytochalsin D was administrated to the fibroblasts, provoking cytoskeletal reorganization as well as a change in adhesion [27]. The observed alterations in the cellular morphology in contact with a material suggest that a link must exist between the scaffold matrix and the extracellular matrix to stimulate cell adhesion.

In addition, fluorescence microphotographs of BFFs after 15 days of culture on hydrogel surfaces were taken (Fig. 8) and the biocompatibility of synthesized materials in contact with BFFs was verified. In this case, partial replace of culture medium (around of 50% of total volume) was made every 48 h to improve cell growth.

Although PNIPAM-co-20%HMA is collapsed at 37 °C its moderated wettability, lightly similar to ionic hydrogels, seems firstly to inhibit the BFFs adhesion and then visible cell proliferation actually happens. The cells may need enough culture time to adapt the surface and to grow on it with typically flattened and spindle-shaped morphology.

According to the obtained results, non-ionic and hydrophilic surfaces with moderated wettability as PNIPAM-co-20%HMA induce the formation of BFFs spheroids during the first culture days. This agrees with many results where different cell lines showed varying adhesion as a function of surface wettability [28]. It has been considered that cells generally adhere better to moderately hydrophilic surfaces compared to hydrophobic or hydrophilic extremes [29,30]. A study about the growth of *Schwann* cells on graphene oxide/polyacrylamide composite hydrogel whose wettability changed in relation to the amount of graphene also showed that the adhesion and proliferation were favored by a surface with moderate wettability [31].

Future studies will allow knowing if the absorption of proteins from FBS, DMEM and/or secreted factors by the cells on surface will favor the cell adhesion/attachment during first days of culture in relation to hydrogel composition. For the time being, the collagen production by BFFs on hydrogels was qualitatively detected after several days of culture on surfaces by Sirius Red staining (supplementary information, SI 3).

Several techniques related to cell spheroids formation have been proposed but many of these are not so versatile [25,32,33]. Oju Jeon et al. built a hydrogel-based microwell platform with methacrylate alginate (OMA)/multi-arm polyethylene glycol (PEG) hydrogels for rapid formation of uniform multicellular hASC (human adipose-derived stem cells) spheroids [34]. Shin et al. designed matrices based on Alginate/marine collagen/agarose composite hydrogels able to generate high yields of multicellular spheroids, with

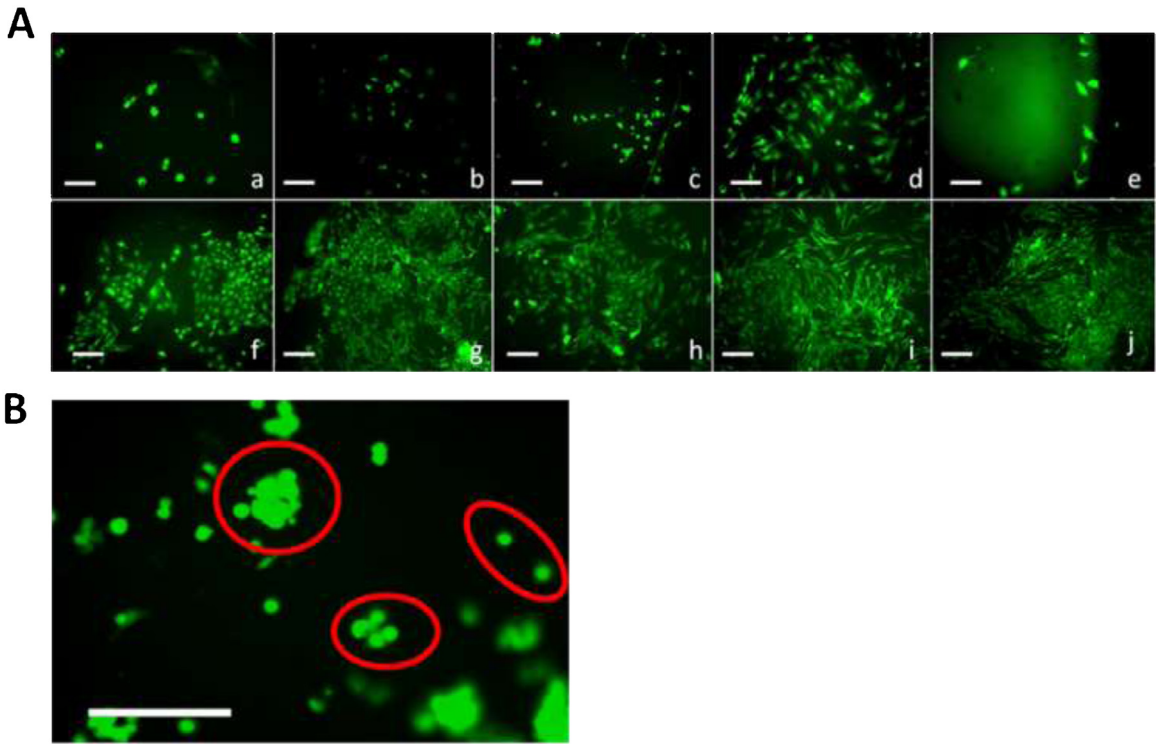

Fig. 6. **A**) Microphotographs of BFF seeded on different hydrogels after 1 day (first line) and 5 days of culture (second line) (magnification: 10X, scale bar: 50 μm). a-f) PAAM; b-g) PNIPAM-co-10%AMPS; c-h) PNIPAM-co-20%HMA; d-i) PNIPAM-co-10%APTA and e-j) PNIPAM. B) Microphotography of BFF on PNIPAM-co-20%HMA hydrogel seeded after 48 h of culture (magnification: 20X, scale bar: 50 μm). Red circles highlight aggregated and isolated cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

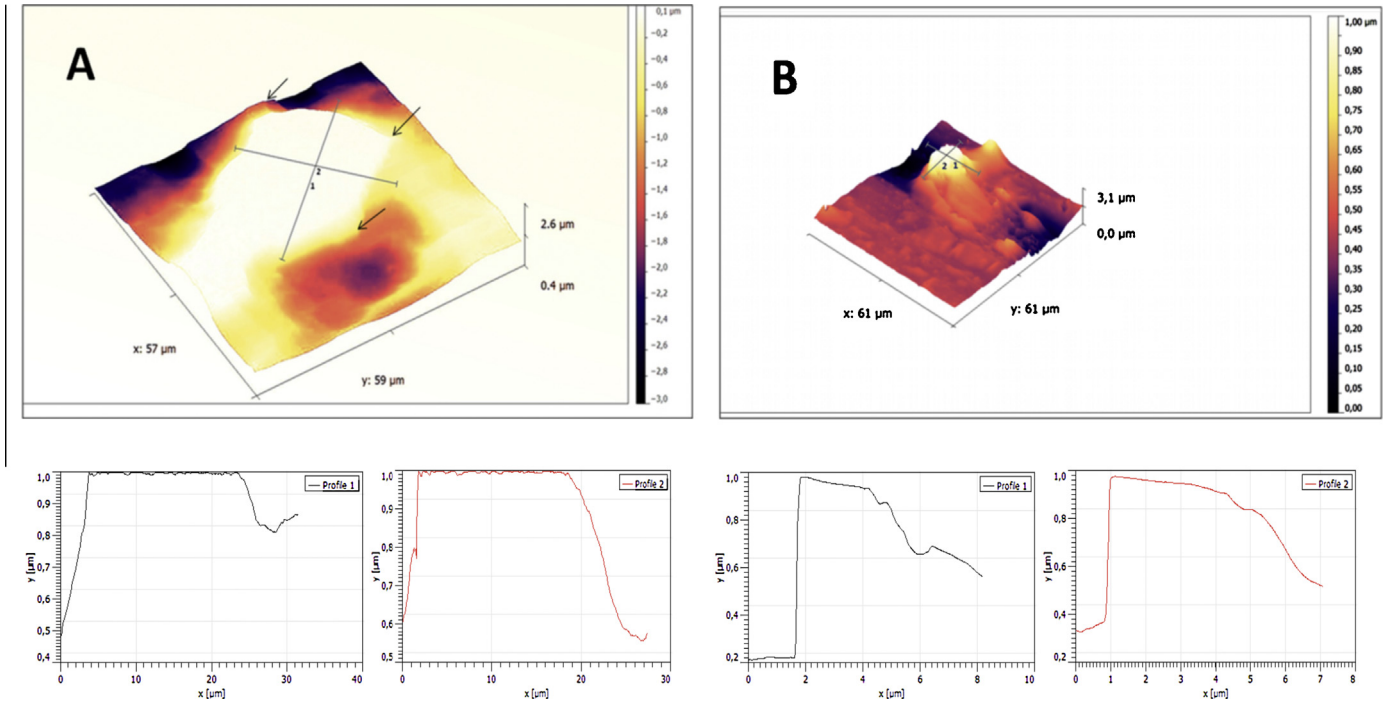

Fig. 7. AFM micrographs of BFF cell after 15 days of culture on hydrogels. A) PAAM and B) PNIPAM-co-20%HMA. Black arrows indicate cell/surface junction points.

high cellular activity and excellent cytocompatibility with various tumor and non-tumor cells [35]. Zhang et al. proposed a thiolated hyaluronic acid derivative (HA-SH) hydrogel as a three-dimensional scaffold to mimic native extracellular matrix (ECM) and encapsulate fibroblasts (L929) and chondrocytes cells by physical crosslinking [36]. However, these hydrogel systems encapsulate or contain the cells forcing to form the aggregates. But application of an *in-vitro* medical treatment to cells could be disadvantageous because the drug should be crossing the scaffold matrix to reach the cells. Whereas in our case, the BFF cell spheroids tend naturally to

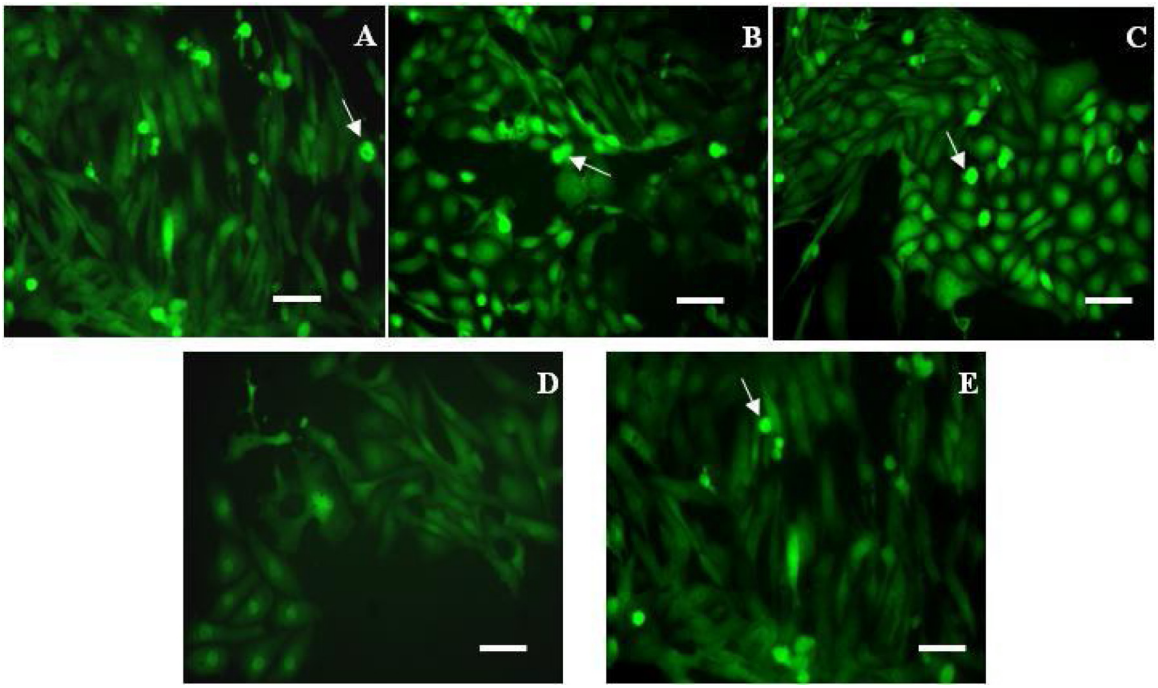

Fig. 8. Microphotographs of BFFs (magnification: 20X, scale bar: 50 μm) after 15 days of culture. A) PAAM. B) PNIPAM-co-10%AMPS. C) PNIPAM-co-20%HMA. D) PNIPAM-co-10% APTA. E) PAAM. Arrows show detached cells.

take that shape on surface by effect of the physicochemical properties of hydrogel, remaining exposed to the environment without a barrier that impairs drug diffusion. The use of these surfaces for *in-vitro* studies at laboratory level would help to define more quickly which of the medical treatments will be more suitable to apply *in-vivo* (e.g. antibiotic selection, anti carcinogenic treatments, etc).

Besides, the bio and physicochemical properties of hydrogel materials, they are easily malleable and moldable, have moderated mechanical resistance, can be stored in dry state and their sterilization process is simple.

4. Conclusions

It has been demonstrated how the physicochemical characteristics of hydrogels change considerably when they are swollen in cell culture conditions (complete DMEM with 10% v/v FBS) regarded to water, being even more notably on thermosensitive hydrogel surface. For the made assays, all materials synthesized proved to be biocompatible and showed high cellular proliferation after 15 days of culture. Furthermore, different cellular morphologies are observed according to superficial properties. High cellular adhesion and proliferation is possible to observe in the case of PNIPAM and PNIPAM-co-10%APTA. Other surfaces tend firstly to inhibit cell adhesion making self-assemble into spheroids but after 3–5 days of culture the adhesion improves because cells produce collagen to adapt surfaces and adopt a typically flattened and spindle-shaped morphology.

Noteworthy, spheroids of BFF cells are easily obtained during the first days of culture (5 days) by growing on surfaces based on PNIPAM-co-20%HMA hydrogels. Therefore, it could be concluded that non-ionic and hydrophilic surfaces with moderate wettability induce the formation of BFF cell spheroids. In addition, the formation of cellular spheroids on hydrogel surface is highly versatile regarding other reported techniques to apply *in-vitro* medical treatments. Complementary researches will allow selecting scaffolds based on hydrogels for other cellular lines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2017.07.032.

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