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Cytotoxicity and bioadhesive properties of poly-*N*-isopropylacrylamide hydrogel

Virginia Capella [a],[1], **Rebeca E. Rivero** [b],[1], **Ana C. Liaudat** [a], **Luis E. Ibarra** [a],
Dardo A. Roma [c], **Fabrisio Alustiza** [d], **Fernando Mañas** [c], **César A. Barbero** [b],
Pablo Bosch [a], **Claudia R. Rivarola** [b],[2], **Nancy Rodriguez** [a],[*],[2]

[a] *Department of Molecular Biology, National University of Río Cuarto-CONICET, National Route N° 36, 601 Km,
Rio Cuarto, X5804ZAB, Argentina*

[b] *Department of Chemistry, National University of Río Cuarto-CONICET, National Route N° 36, 601 Km, Rio
Cuarto, X5804ZAB, Argentina*

[c] *Department of Animal Clinic, National University of Río Cuarto, National Route N° 36, 601 Km, Rio Cuarto,
X5804ZAB, Argentina*

[d] *INTA Marcos Juárez, Province Route N° 12, 3 Km, Marcos Juárez, 2580, Argentina*

[*] Corresponding author.

E-mail address: rodrix.nr@gmail.com (N. Rodriguez).

[1] These authors contributed equally to this work.

[2] These two authors also contributed equally to this work.

Abstract

Several hydrogel surfaces present properties that simulate the mechanical and physicochemical features of extracellular matrix (ECM), providing a platform that mimic the native cellular milieus. Poly-*N*-isopropylacrylamide (PNIPAM) hydrogels are receiving attention in biomedical field due to their thermosensibility and soft texture. However, more extensive biocompatibility and cellular interactions studies with cell lines are needed. Therefore, the aim of this study is focus on evaluating the biocompatibility of PNIPAM through cytotoxicity, genotoxicity, and proliferation tests in murine preadipose cells (3T3-L1), human embryonic kidney cells (HEK293) and human carcinoma-derived cells (A549) in presence of hydrogel surfaces. Bioadhesive capacity above PNIPAM surfaces was also analyzed. MTT and neutral red uptake assays shown non-cytotoxic effect of PNIPAM in the studied cell lines. Genotoxicity was

evaluated by the single-cell gel electrophoresis assay, where DNA damages were not detected. [3H]-thymidine staining allowed to corroborate that cell proliferation had progressed correctly. Adopted morphologies for each cell line over PNIPAM were similar to cell growing observed on polystyrene, indicating that the surfaces favor the cell attachment during 5 days' culture. The good biocompatibility of PNIPAM surfaces make it an interesting scaffold with clinical potential in tissue regeneration engineering, and a possible adipose and kidney tissue-engineered construct.

1. Introduction

In the last decades great advances have been achieved in the development of biocompatible materials that function as scaffolds for the growth and proliferation of various cells types (Liu et al., 2012; Tovar-Carrillo et al., 2013). 2D surfaces would allow cell growth, especially by *in vitro* assays (Caliari and Burdick, 2016; Hess et al., 2017) and if these scaffolds contain high porosity, they would provide a 3D structure for cell adhesion, proliferation, migration and new tissue formation (Ikada, 2006; Mohamad Yunos et al., 2008).

A great potential for biomedical applications has been shown through a cross-linked hydrophilic polymer, called hydrogel (Peppas et al., 2006), mainly due to its innate similarity to the extracellular matrix (ECM) and to its extensive cross-linked network that provides a platform where cells could proliferate and biological flows can diffuse through it (Mendelsohn et al., 2003). The similarity of these materials with respect to ECM is based on two principal features: the chemical structure and the mechanical properties that can mimic elements of the ECM or resemble soft tissues; and the capability of act like a support for cell adhesion and proliferation (Tibbitt and Anseth, 2009).

Hydrogels form a three-dimensional network through covalent or non-covalent bonds in aqueous medium, which can absorb a large amount of liquid (Rogovina et al., 2008) and simulate the properties of ECM. Poly-*N*-isopropylacrylamide (PNIPAM) is one of the most studied synthetic hydrogel, mainly due to its coil-globule phase transition when the temperature increases over the lower critical solution temperature (LCST) of $\sim 32\,°C$ in water (Dainiak et al., 2009; Moran et al., 2007). This phase transition generates a change on hydrophilic/hydrophobic properties of PNIPAM (Molina et al., 2012) and as a result, could alter the cell adhesion/attachment to the surface. These hydrogels are easily synthesized by radical polymerization to obtain variable architectures such as block copolymers, gels, or grafted polymers and when is required biodegradable PNIPAM-based hydrogels could be formed

(Lanzalaco and Armelin, 2017). PNIPAM and its derivate biomaterials are good candidates for biomedical applications not only for the mentioned characteristics but also for the ability to adopt different shapes and morphology (Vedadghavami et al., 2017).

To use a material as cell scaffold it is necessary to evaluate firstly the biocompatibility of the material in contact with cells and then how the cells respond, either by being in contact or growing above it. Biocompatibility is defined by Williams (1999) as "the ability of a material to perform with an appropriate host response in a specific application" and require that it does not induce toxicity allowing a normal cell proliferation and distribution.

PNIPAM hydrogels are widely used in research on tissue engineering. Cells harvested from patients can be grown on PNIPAM or PNIPAM-coated substrates to form viable cell sheets, which can then be layered to form a tissue (Cooperstein and Canavan, 2010; Cooperstein et al., 2017). Some studies have shown that these hydrogels are biocompatible with certain cell types, like bovine fetal fibroblast (FFBs) (Rivero et al., 2015; Rivero et al., 2017), human epithelial colorectal adenocarcinoma cells (Caco-2) and lung cancer cells (Calu-3) (Vihola et al., 2005), mesenchymal stem cells derived from rat bone marrow (BM-MSCs) and human adipose tissue (AT-MSCs) (Yang et al., 2012), showing absence of cytotoxicity and high number of cells adhered to PNIPAM surfaces together with increased rates of cell proliferation. Nevertheless, more studies are necessary to elucidate and confirm the biocompatibility of PNIPAM in a multiple range of tissues and compare the diversity of cell behavior on hydrogel surfaces. Also, it is important to analyze if hydrogel surfaces did not modify cell adhesion/attachment, a crucial parameter in scaffold's development (Li et al., 2018; Slaughter et al., 2009). When cells are seeded over a surface, they take initially a rounded morphology, and after a determined time they adhere to the substrate material, rearranging their cytoplasm to spread out and adopt their normal shape. The occurrence of this process means that the material is bioadhesive, being the proliferation process a necessary parameter to evaluate next (Nash et al., 2012). For this reason, is important to know how the adhesion process occurs and what morphology cells adopt on scaffold.

Due to the fact that cytotoxicity is closely related to the cell type analyzed, it is important to evaluate the effect of hydrogels on several cell lines with different origin and degrees of resistance to cell death in order to verify the biocompatibility (Haghparast et al., 2015). For that reason, the aim of the present study is to compare the behavior of three different cell lines: murine pre-adipose (3T3-L1), human embryonic kidney (HEK293) and adenocarcinoma-derived cells (A549) in contact with poly-*N*-isopropylacrylamide (PNIPAM) hydrogel surfaces.

2. Materials and methods

2.1. Synthesis of PNIPAM hydrogels surfaces

The PNIPAM hydrogels were synthesized by free radical polymerization of *N*-isopropylacrylamide monomer (NIPAM) (Sigma-Aldrich) in distilled water, in the presence of *N,N*-methylenebisacrylamide (BIS) (Sigma-Aldrich). Ammonium persulfate (APS) (Sigma-Aldrich) and *N,N,N′,N′*-tetramethylenediamine (TEMED) (Sigma-Aldrich) were used as initiator system.

A solution of NIPAM 0.5 M was dissolved with 2% moles of cross-linker agent (BIS) based on NIPAM moles. Next 1 mg/mL (APS) and 10 µL/mL (TEMED) were added as initiator system. Quickly, the solution was transferred into a 24-well top cover to form thin surfaces and polymerization was carried out at room temperature (22−25 °C) for 24 h. Then, the hydrogel was washed with distilled water for 5 days at room temperature, replacing the water twice a day to remove unreacted chemicals.

2.2. 3T3-L1, HEK293 and A549 cells culture

Mouse preadipocytes cells (3T3-L1), human embryonic kidney cells 293 (HEK293) and human lung carcinoma epithelial cells (A549) were cultured in complete Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% antibiotic/antimycotic (10,000 U/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL anfotericin) (Gibco) at 37 °C in an atmosphere of 5% CO_2 and high humidity.

Prior to cell culture experiments, hydrogels were sterilized with 70% v/v ethanol/water overnight. Then the hydrogels were collapsed at 37 °C in an oven and washed with sterile phosphate saline solution (PBS, pH 7.3) to remove the ethanol. After that, sterilization with PBS 10% v/v antibiotic/antimycotic was performed. To remove the antibiotic/antimycotic, hydrogels were washed three times (30 min each) in sterile PBS. Then hydrogel was swollen in culture medium for the later cell seeding.

2.3. Cytotoxicity assays

To evaluate if PNIPAM hydrogel has any cytotoxic effect on the studied cell lines, two colorimetric assays were performed: MTT and neutral red uptake. The MTT assay was adapted from the protocol described by Mosmann (1983), and evaluates cellular viability based on mitochondrial function through the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a colored insoluble formazan salt. On the other hand, neutral red uptake assay was developed by

Borenfreund and Puerner (1985) and is based in the ability of viable cells to incorporate and bind the dye in lysosomes.

Cell lines were seeded at 5×10^3 cells per well in a 96-well plate and allowed to grow for 24 h. Then, small pieces of PNIPAM hydrogels (diameter: 5 mm − length: 2 mm) were placed over the culture and incubated for 48 and 96 h. After that, hydrogels were removed and the corresponding test was carried out. A negative control (only DMEM 10% FBS) and a positive control (Dimethyl sulfoxide (DMSO):DMEM 10% FBS, 1:9), adapted from Da Violante et al. (2002), were included to validate the viability protocols.

For the MTT assay, a MTT solution (5 mg/mL) was added to cells to achieve a final concentration of 0.5 mg/mL in DMEM 10% FBS and then incubated for 3 h. The medium was removed and replaced by 100 μL of DMSO to dissolve the formazan crystals.

Concerning to neutral red assay, cells were incubated for 3 h with neutral red dye (1 mg/mL) in DMEM medium. Then the medium was exchange for 100 μL of an elution solution ($C_2H_5OH:CH_3COOH$, 50:1) and the plate was gently shaking in order to achieve a complete dissolution.

The absorbance of both colorants was recorder at 540 nm in a microplate reader (Bio-Rad, Hercules, CA, USA). The viable cell number after 48 and 96 h was expressed as OD (optical density) of formazan or neutral red dye obtained from cell growth in contact with hydrogels and control groups.

2.4. Single-cell gel electrophoresis assay (Comet Assay)

Cellular genotoxicity was determined by the Comet Assay following the protocol proposed by Singh et al. (1988), with slight modifications. This assay is capable of detecting, with high sensitivity, DNA damages in an individual cell (Møller, 2006). Each cell line, seeded at a density of 4×10^4 cells per well in a 24-well plate, was exposed for 96 h to PNIPAM hydrogel (diameter: 10 mm − length: 2 mm). Positive (hydrogen peroxide- H_2O_2 10 μM), adapted from González et al. (2002) and Yang et al. (2003), and negative (only DMEM 10% FBS medium) controls were included. After, cells were collected and 80 μL of cell suspensions were added to 220 μL of 0.75% low melting point (LMP) agarose at 37 °C to be layered onto slides precoated with 0.75% normal melting point (NMP) agarose afterwards. The slides were immersed in an alkaline buffer pH 13 and quickly submersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), supplemented with1% Triton X-100 and 10% DMSO) for 1 h. Electrophoresis was conducted for 30 min, at 30 V and 250 mA. The samples were fixed in absolute ethanol, stained with acridine orange and observed using a fluorescence microscope (Axiophot, Carl Zeiss, Germany). From each treatment, 100 "nucleoids" images were taken with a camera (Powershot

G6, 7.1 megapixels, Canon INC, Japan) attached to the fluorescent microscope and analyzed by the Comet Score 1.5 software. Tail moment (TM) was used as parameter to infer DNA damage (arbitrary units).

2.5. Cell proliferation by [³H] thymidine assay

Cell proliferation of each cell line was determined using the [³H] thymidine assay, which detects the incorporation of the tritium-labeled thymidine nucleotide into DNA strands synthesized *de novo* (Bond et al., 1959). Cells were seeded in 96-well plate (5×10^3 cells per well) and cultured in contact with PNIPAM hydrogel (diameter: 5 mm − length: 2 mm) for 24 and 48 h, in the presence of [³H] thymidine/DMEM (PerkinElmer, Boston, MA 02118 USA). Normal cell growth control (only DMEM-10% FBS medium) and proliferative cell growth control (only DMEM-30% FBS medium) were included. Then, hydrogels were removed and cells were harvested. The samples were diluted in a liquid scintillation cocktail (PerkinElmer, Loughborough Leics, England) and the incorporated [³H] thymidine was measured in a liquid Scintillation β Counter (Beckman LS 60001 C; Fullerton, CA, USA). Cell proliferation after 24 and 48 h of culture was expressed as counts per minute (cpm) of [³H] thymidine obtained to hydrogel and control groups.

2.6. Nuclear and cytoplasmic morphology evaluation

To demonstrate the adhesive/attachment characteristics and cell growth above PNIPAM surfaces and to compare to common growth over polystyrene culture dish, cytoplasmic and nuclear morphology were observed staining with Toluidine Blue and Hoechst 33258, respectively. Cell lines were seeded at 2.5×10^4 cells on each PNIPAM surface (diameter: 15 mm − length: 2 mm), previously swollen in DMEM 10% FBS and cultivated for a period of 5 days inside a 24-well microplate. The same process was following on a polystyrene surface 24-well microplate. Surfaces at 2 and 5 culture days were fixed with methanol at -20 °C and then stained with Hoechst 33258 (1 mg/mL) at final concentration of 20 μL/mL in PBS (Sigma−Aldrich) and Toluidine Blue at 0.05% w/v (Biochem, Buenos Aires), separately. After that, surfaces were washed with PBS and observed in an inverted fluorescence microscopy (Nikon Ti-S 100, Nikon Japan).

2.7. Statistical analysis

Statistical analyses were performed by ANOVA with INFOSTAT/L software for statistical computing. Post-hoc comparisons were performed using Dunnett post-hoc test. The values are expressed as mean ± standar error (S.E.) and were considered significantly different when $p \leq 0.05$. For Comet Assay, mean ± S.E., of the different treatments were calculated by the Graphpad Prism 5 program. The

Shapiro-Wilk normality test was carried out. The means of each treatment were compared using the non-parametric Kruskal-Wallis test to determine differences and Dunns multiple comparisons was used as a posteriori test. In all cases, the values were expressed as mean $\pm$ S.E. and a $p \leq 0.05$ was considered significant.

3. Results

3.1. PNIPAM cytotoxicity

Cytotoxicity assays are basic and essential evaluations carried out to determine the biocompatibility of materials which are intended to be used in the biomedical field. The cytotoxicity of the PNIPAM hydrogel was studied in contact with 3T3-L1, HEK293 and A549 cell lines by MTT and neutral red uptake assays, during 48 and 96 h in culture (Fig. 1), which is important due to the necessity to assess the cyto-toxicity in different cellular organelles in order to demonstrate biocompatibility.

The viability of cells in contact with hydrogel surfaces were compared with cells which were not exposed to hydrogels (control group) in order to determine if PNI-PAM produced any cytotoxic effect. In all cases, no significant alterations in mito-chondrial activity, expressed in optical density (OD), were observed for PNIPAM
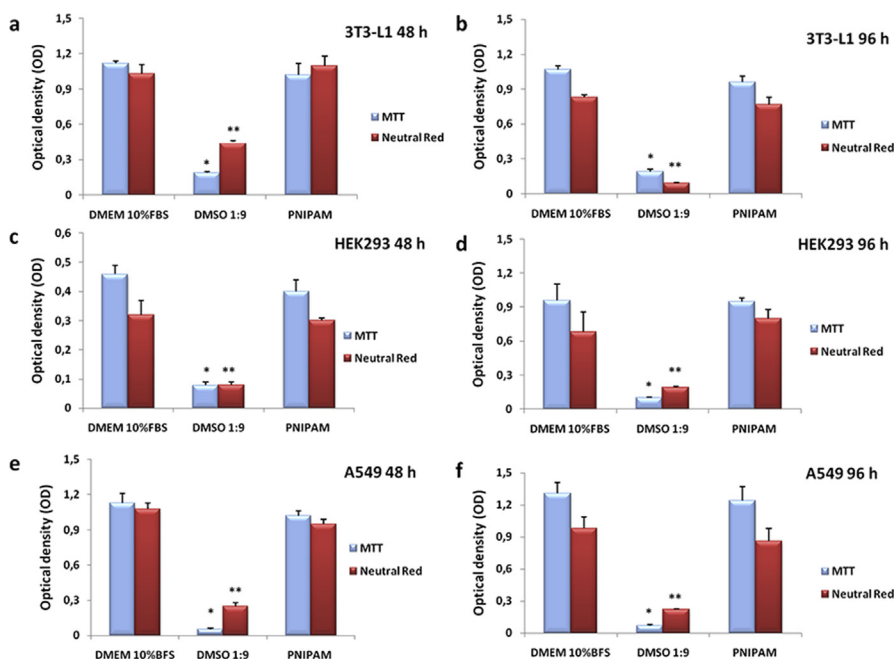

Fig. 1. Cell viability of (a) 3T3-L1 at 48 h, (b) 3T3-L1 at 96 h, (c) HEK293 at 48 h, (d) HEK293 at 96 h, (d) A549 at 48 h and (f) A549 at 96 h exposed to PNIPAM, according to MTT and neutral red uptake assays. Each bar represents the mean of three independent replicates, expressed in optical density $(OD)_{540 \text{ nm}}, \pm$ S.E. *Statistically significant differences between cytotoxicity positive control (DMSO) regarding to the rest of treatments, assessed by MTT. **DMSO was significantly different than all other treatments, by neutral red uptake ($p < 0.05$).

hydrogel (Fig. 1a, c, e), in comparison with the cell grew in DMEM-10% FBS (negative control), regardless of exposure time. In correlation with MTT assay, no cytotoxicity at lysosomal level (OD) was detected in neutral red uptake assay (Fig. 1b, d, f). On the other hand, significant differences were observed in each experiment with 1:9 DMSO: DMEM 10% FBS treatment indicating that the cells did not show any cytotoxic effect when in contact with PNIPAM. Also, the viability values obtained in cells exposed to hydrogels were significantly higher than those obtained with a cytotoxic agent, confirming the biocompatibility of PNIPAM.

It can be seen that both techniques showed the same viability pattern in exposed cells. The slight differences found between them are due to the amount of neutral red dye taken by lysosomes is greater that the amount tetrazolium salt reduce by mitochondria (Borenfreund et al., 1988).

3.2. Comet assay

The single-cell gel electrophoresis assay (Comet assay) is one of the most sensitive techniques to use for the evaluation of genotoxicity, since the assay has the ability to detect damage in individual cells according to their migration in an agarose gel. Cells with increased damage have a greater migration of DNA, indicated by the migration pattern stained with acridine orange, an intercalating agent of DNA. The tail moment was chosen as parameter of DNA damage. This parameter is defined as the product of tail length and the tail DNA percentage.

Cell lines were exposed during 96 h to PNIPAM, according to the largest time that it does not produce effects in cytotoxic assays. The pattern of DNA migration in negative control and PNIPAM treatment was indicative of an absence of chromosomal DNA fragmentation (Fig. 2a). Different patterns were observed in cells treated with H_2O_2 100 μM (positive control), with several levels of migration due to different levels of damage in DNA (Fig. 2b,c).

Fig. 2 also shown the tail moment expressed as arbitrary units corresponding to the treatment implemented for 3T3-L1 (Fig. 2d), HEK293 (Fig. 2e) andA549 cells (Fig. 2f). The obtained results not shown that cells in contact with PNIPAM surfaces had more DNA damage that negative control (DMEM 10% FBS only), claiming the good biocompatibility of this hydrogel with all cells studied as well. Moreover, a positive control was added in order to compare the effect of each treatment with a well-known genotoxic agent as H_2O_2. In all cases, the cell exposed to both, the negative control and polymer showed significant difference with this agent.

3.3. [³H] thymidine assay

The most reliable assays evaluating cell proliferation are based on direct following of the DNA synthesis. [³H]-thymidine is a tritium-labeled thymidine nucleotide which
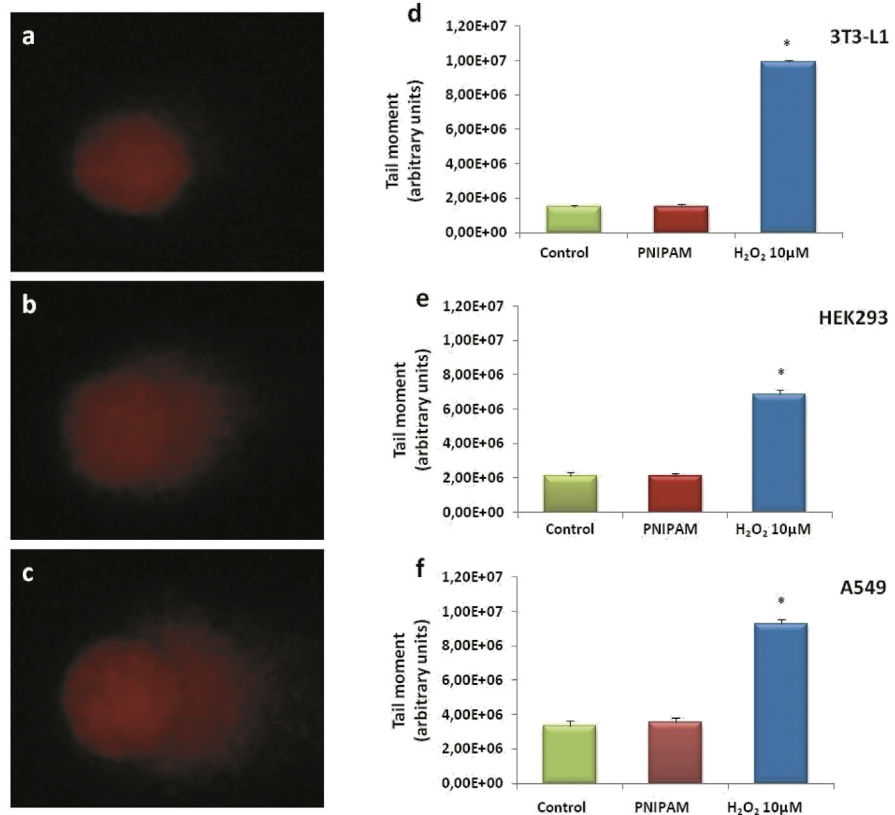
Fig. 2. Microphotograph of cells stained with acridine orange (magnification 400 X). Representative picture of cells with not DNA damage, found in the exposed PNIPAM 3T3-L1 culture (a); representative picture of cell with presence of cloud, indicative of DNA damage, found in the H_2O_2 3T3-L1 exposed cells (b-c). DNA migration of (d) 3T3-L1, (e) HEK293 and (f) A549 cells exposed to PNIPAM hydrogels during 96 h, compared with control of not exposed cells and a genotoxic control (H_2O_2). Each bar represents the mean of the tail moment of two independent replicates ± S.E.*H_2O_2 was significantly different than all other experiments ($p < 0.05$).

is incorporated into the new DNA strand in each mitotic cell division. Cells were exposed to PNIPAM hydrogels for 24 and 48 h in the presence of [^{3}H]-thymidine and then the brands were quantified in a Scintillation Counter.

As it is shown in Fig. 3, the analysis of exposition times showed no statistical differences between cells in contact with PNIPAM compared to the corresponding control cells (DMEM 10% FBS only). The incorporation of tritium-labeled thymidine indicates that 3T3-L1, HEK293 and A549 have the capability to proliferate on the PNIPAM surface, without affecting the S phase of the cell cycle.

The cells grown with DMEM 30% FBS were used as a control of proliferation stimulation, to corroborate that hydrogel does not alter the normal division of cells. 3T3-L1 and HEK293 lines showed increases levels of [^{3}H]-thymidine labels with DMEM 30% FBS compared to negative control and cells exposed to polymer. The results,
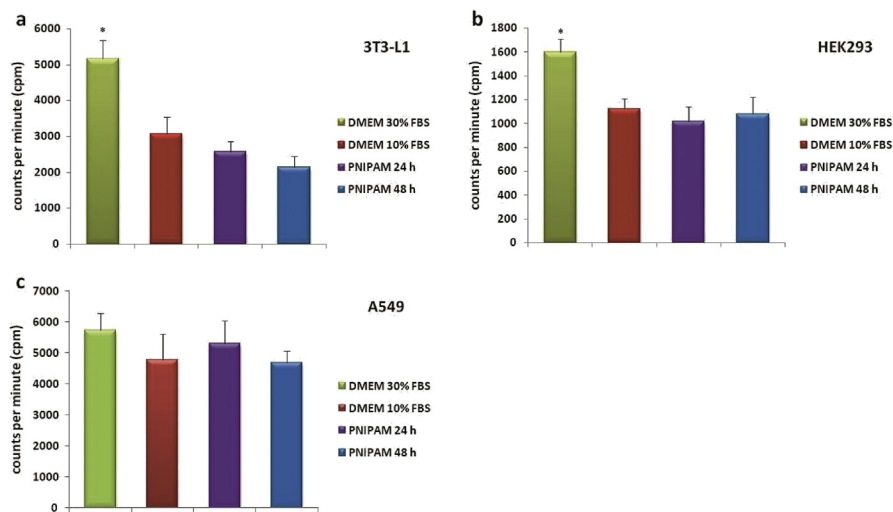
Fig. 3. Effect of PNIPAM hydrogels exposition during 24 and 48 h in (a) 3T3-L1, (b) HEK293 and (c) A549 cell lines proliferation. Each bar represents the mean of three independent replicates, expressed in counts per minute (cpm) ± S.E. *Statistically significant differences between proliferation control (DMEM 30% FBS) and treatments ($p < 0.05$).

indicate that PNIPAM does not increased cell proliferation like DMEM 30% FBS. No difference was found in A549 line by any of the treatments.

3.4. Morphology of cells adhered to PNIPAM surfaces

Evaluation of cellular interaction between 3T3-L1, HEK293 and A549 cells with PNIPAM is important to understand and define the bio-adhesive property of this hydrogel. To observe if cells modify their behavior when grow on hydrogel surfaces, they were seeded over PNIPAM and polystyrene surfaces during 5 days and the adopted morphology of stained cells was observed by optical and fluorescence microscope, through Toluidine Blue and Hoechst 33258 stain.

In Fig. 4, at day 2, it can be seen that a high number of cells grown over PNIPAM surfaces with the typical flat shape morphology. During 5 days of culture the cellular shape remains equal to that adopted over polystyrene plates indicating that there are not changes in cytoplasmic morphology. Also, an increase in number of adhered cells was observed throughout the culture days, revealing that cells adhered easily to PNIPAM.

HEK293 cells normally are more rounded, often appearing polygonal at confluence, which suggests an epithelial character. This morphology can be seen on the control (polystyrene plate) as PNIPAM at 2 and 5 culture days and still have an intense cell-matrix adhesion that allows presenting a uniformly distribution. Also, HEK293 normally has a stronger cell-cell cohesion which could be
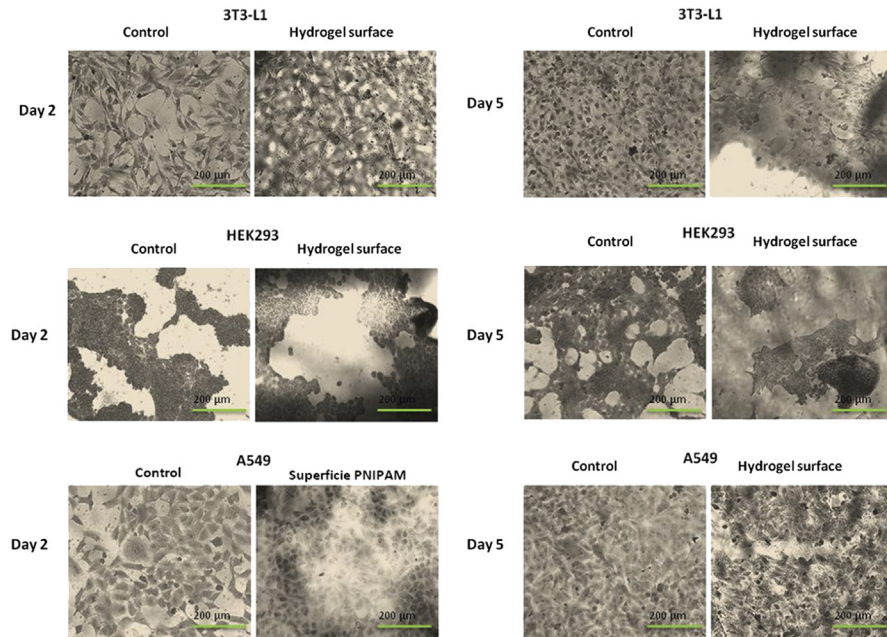
Fig. 4. Microphotographs of 3T3-L1, HEK293 and A549 cytoplasmic morphology stained with Tolui-dine Blue, after 2 days (left panel) and 5 days of culture (right panel) of seeding on polystyrene and PNI-PAM surfaces (magnification: 20X).

seen in polystyrene plate as on PNIPAM which explains the like-cluster growth, different to the other two cell lines.

The number of 3T3-L1 over PNIPAM surface at 5 days of culture seem to be slightly smaller that cells cultured over polystyrene since they adopt a more sharpened cell morphology, probably due to the lower contact between cells, compared to that morphology when they were closer to one another.

Nuclear morphology changes (Fig. 5) were not founded in any of the three cultivated cell lines. Cell behavior on PNIPAM hydrogel and polystyrene was similar in all cells, showing a uniform distribution through the whole surface in both periods of times. These results are indicative of a good cell-matrix adhesion.

4. Discussion

Hydrogels have been studied and applied in investigations as a scaffolding system since they provide a structural and mechanical base, with properties similar to ECM, allowing that whether the cells are attached to its surface (2D) or inside of three-dimensional network (3D) (Hess et al., 2017; Hersel et al., 2003; Johnstone et al., 2013; Peppas et al., 2006; Rivero et al., 2015, 2017). The evaluation of cell growing behavior in contact with a biomaterial is necessary for future application in tissue engineering. This material should be compatible
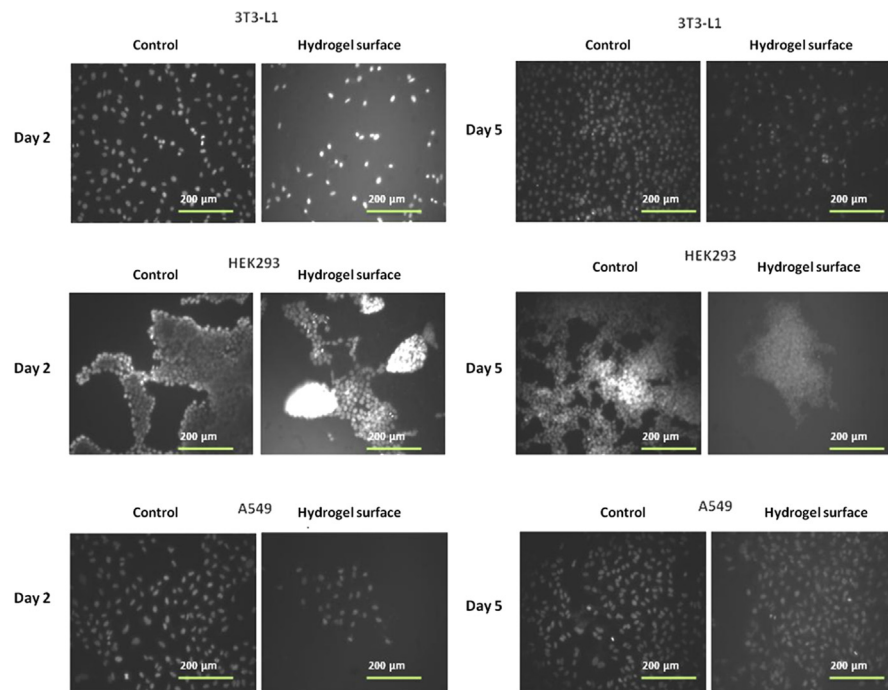
Fig. 5. Microphotographs of 3T3-L1, HEK293 and A549 nuclear morphology stained with Hoechst 33258, after 2 days (left panel) and 5 days of culture (right panel) of seeding on polystyrene and PNIPAM surfaces (magnification: 20X).

with the tissue of interest and at the same time with other nearby cells, that is why research with several cell lines is important. In this study, poly-*N*-isopropylacrylamide (PNIPAM) surface was proposed as 2D scaffold system for different cell lines growth.

Although PNIPAM is a highly studied hydrogel in biomedical research, there is not enough information about its biocompatibility in a widespread of tissues or cell lines. The high biocompatibility of this material was demonstrated for murine pre-adipose (3T3-L1), human embryonic kidney (HEK293) and human lung carcinoma epithelial cells (A549), indicating that PNIPAM hydrogel could be a good candidate for 2D scaffold.

Cytotoxicity is a basic assessment to define biocompatibility of materials and an important parameter to take into account to think in a biomaterial like scaffold (Slaughter et al., 2009), therefore it is necessary to determine it by more than one assay with the aim of screening accuracy values that certify the safety of the agent. Tetrazolium MTT and Neutral Red uptake are two well reported assays that evaluate the mitochondrial activity and lysosomes integrity, respectively. Results are consistent with those obtained by Rivero et al. (2017), which indicated that PNIPAM hydrogel does not produce damage at mitochondrial activity level on bovine fetal fibroblasts (BFFs). In addition, intestinal Caco-2 and bronchial Calu-3 cultivated

on PNIPAM, reported by Vihola et al. (2005), did not demonstrate evidence of cytotoxicity by MTT and lactate dehydrogenase (LDH) tests. They observed an increase in mitochondrial activity in Caco-2 cells at the time of exposition regarding the other polymers studied and control, which might be a sign of subtoxic effect, but was not significant to consider it cytotoxic. Comparing previous data with the results obtained in our lab, it could indicate that PNIPAM would not only be innocuous for primary cultures but also for different established animal cells.

Mechanical forces and changes in matrix stiffness in cell environment can activate signaling pathways mediated by the cytoskeleton that influence cell division, differentiation and migration, an event called mechanotransduction. Internals signals pathways are convergent in the nucleus in order to regulate events like DNA replication, transcription and cell cycle progression. The presence of a matrix or scaffold could activate these pathways, even if those are not nanoparticles internalized within the cells. In this way, effects on nuclear morphology can be evidenced (Graham and Burridge, 2016). Since that PNIPAM hydrogels could have physicochemical and mechanical properties similar but not equals to ECM, others tests that asses the genotoxicity and cell proliferation are necessary to verify the potentiality of PNIPAM 2D surfaces to cell growth. Absence of genotoxicity is a great indicator of cell-matrix biocompatibility. Our results indicate that PNIPAM hydrogel does not generate genotoxic response in 3T3-L1, HEK293 and A549 after 96 h of exposition. Previous research has also demonstrated the absence of genotoxicity for PNIPAM internalized nanoparticles in different cell lines. Naha et al. (2010) found no genotoxic response of PNIPAM nanoparticles in human keratinocyte (HaCaT) and colon cells (SW 480) at concentrations of 12.5−800 mg/L, assessed by alkaline Comet assay. A similar case was also reported by Guo et al. (2017) for human bronchial epithelial (HBE), A549, macrophages RAW264.7 and human umbilical vein endothelial cells (HUVEC), where concentrations up to 1 mg/mL PNIPAM nanoparticles did not induce early and late apoptosis. Kawecki et al. (2016) showed that fibroblast sheets cultured over PNIPAM/poly(tri(ethyleneglycol)monoethyl-ethermethacrylate) during 24 hours do not produce genotoxicity by Comet assay. Nevertheless, there are few data that evaluates the genotoxic effect of PNIPAM surfaces, and considering the evidence about mechanotransduction, so this information takes relevance in biomaterial field.

Cell proliferative activity was studied *in vitro* after 24 and 48 h of exposition to PNIPAM and incubation with tritiated thymidine. The tritium-labeled thymidine nucleotide is only incorporated during synthesis stage into DNA strand. The labeling in cell lines at different exposition time evaluated in this work indicates an active DNA synthesis (S phase) by the presence of the hydrogel. The same result was observed in normal cell growth over polystyrene surfaces.

Cell adhesion on hydrogel surfaces, assessed by stain using Toluidine Blue and fluorescence of Hoechst 33258 microscopies, revealed that cells easily adhere on PNIPAM and the attachment morphology immediately after seeding and during the 5 days of culture is similar to that adopted by cell growth over polystyrene plates. During culture days, an increase in number of 3T3-L1, HEK293 and A549 cells adhered/attached to PNIPAM surface and were observed the same morphological and nuclear characteristics in the attached cells to PNIPAM surfaces and polystyrene. It is known that components of culture medium, and FBS proteins among others, could be adsorbed by PNIPAM matrix stimulating cell adhesion (Fukuda et al., 2006). The possibility of PNIPAM to adsorbing vital substances, like growth factors that are part of the cellular microenvironment, is of great importance to take under consideration in future research due to its activity in cellular growth and proliferation (Slaughter et al., 2009). Also, several research groups have shown that the hydrophobicity of hydrogel surface could be affecting the adhesion of cells obtained from different tissues (Vihola et al., 2005; Yang et al., 2012; Rivero et al., 2017). Our results are similar with those obtained by Rivero et al. (2017), where bovine fetal fibroblast (FFB) adopted their typical flattened morphology on PNIPAM surfaces while adopted a contracted morphology on PNIPAM-co-20%HMA surface (HMA: *N*-acryloyl-tris-(hydroxymethyl) aminomethane) (slightly hydrophilic). This demonstrates that the interaction cell-matrix is not only dependent of matrix surface properties but also of the natural behavior of each cell line.

The findings of this study suggested that PNIPAM has potential to be a 2D scaffold with good biocompatibility and adhesive properties for adipose and kidney tissue cells. Further studies with macroporus PNIPAM are necessary to elucidate the behavior of these cells growing into a 3D environment.

5. Conclusion

Biocompatibility studies of PNIPAM hydrogel in contact with tree cell lines verified by different techniques show that the material is non-cytotoxic and non-genotoxic. Assays with tritiated thymidine indicate the presence of proliferative activity.

Cell adhesion on PNIPAM surface depends not only on physicochemical properties of scaffold but also of the characteristic behavior of the cell lines. Here, it was verified PNIPAM is a potential 2D scaffold of normal and cancerous cancer cell lines.

Therefore, PNIPAM surfaces can be proposed as 2D scaffold for a potential application in clinical and tissue engineering field being a promise tool to be included in adipose and kidney tissue-constructs development. In the future, PNIPAM scaffold could be also applied as 3D scaffold of these or other cell lines.

Declarations

Author contribution statement

Virginia Capella, Rebeca E. Rivero: Performed the experiments, Analyzed and interpreted the data, Wrote the paper.

Ana C. Liaudat, Dardo A. Roma: Performed the experiments, Analyzed and interpreted the data.

Luis E. Ibarra: Performed the experiments, Wrote the paper.

Fabrisio Alustiza: Analyzed and interpreted the data, Contributed reagents, materials, analysis tools or data.

Fernando Mañas: Analyzed and interpreted the data.

César A. Barbero, Pablo Bosch: Contributed reagents, materials, analysis tools or data.

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The authors declare no conflict of interest.

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