

Cytotoxicity of thermo-responsive polymeric nanoparticles based on N-isopropylacrylamide for potential application as a bioscaffold

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Polymeric nanoparticles based on poly-N-isopropylacrylamide (pNiPAM NPs) and their bio-medical applications have been widely investigated in recent years. These tunable nanoparticles are considered to be great candidates for drug delivery systems, biosensors and bioanalytical devices. Thus, the biocompatibility and toxicity of these nanoparticles is clearly a crucial issue. In this work, the cytotoxicity of thermo-responsive pNiPAM nanoparticles was studied, followed by a detailed analysis of the NPs morphology in growing cell cultures and their 3D structure. Cytotoxic examination was conducted for two cell cultures — HeLa (cervical cancer cell line) and HeK293 (human embryonic kidney cell line), employing MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay and viability tests. We used Cryo-SEM (scanning electron microscopy) and fluorescence microscopy (IN Cell Analyzer) in order to investigate the morphological structure of the polymer network. We show that pNiPAM nanoparticles do not exhibit any cytotoxicity effects on the investigated cell lines. Additionally, we report that the pNiPAM nanoparticle based scaffold promotes cell growth.

Key words: nanoparticles, toxicology, poly-N-isopropylacrylamide, drug delivery system, cytotoxicity, polymers

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INTRODUCTION

Poly-N-isopropylacrylamide (pNiPAM) hydrogel is a well-known example of a new class of stimuli responsive materials. PNIPAM hydrogel is formed by a polymeric nanoparticle network which offers an important parameter for tuning its properties, with its lower critical solution temperature (LCST) at 33°C (Hoffman, 1995). Considering their nanometric size and tunable properties this so-called ‘intelligent’ polymer, due to its sensitivity to variations within the temperature range of the human body, is extensively studied with a wide range of applications (Gil & Hudson, 2004; Akiyama *et al.*, 2004; Xu *et al.*, 2011; Kanazawa *et al.*, 1996). Since pNiPAM is forming a hydrogel and the temperature dependent transition is taking place by releasing water from hydrophobic interior of a nanoparticle, it is a promising tool for drug delivery systems, biosensors and bioanalytical devices with a wide range of applications (Huber *et al.*, 2003; Ravichandran, 2009; Alaghemandi & Spohr, 2013). Recent

studies have shown a number of improvements especially in employing hydrogels (Mansfield *et al.*, 2014) for bio-medical applications that have led scientists to study new applications of pNiPAM NPs (Naha *et al.*, 2010; Lien *et al.*, 2011; Meenach *et al.*, 2009). One of these potential applications is the usage of pNiPAM NPs coated with substrates that can positively influence cell growth patterns (Mandal *et al.*, 2012; Abu Samah & Heard, 2014). Potential cytotoxic effects are crucial for investigation of any bio-material and require more detailed tests, that can determine: (i) biocompatibility of pNiPAM nanoparticles, (ii) cell line viability and proliferation capabilities after introducing nanoparticles and (iii) cell capability for growth directly on the pNiPAM NPs surface (Vihola *et al.*, 2005; Hussien *et al.*, 2013). In this work we confirm the biocompatibility of pNiPAM NPs and we focus on a new application of pNiPAM NPs coated surfaces for cell growth, as examined with fluorescent microscopy techniques and cryo-scanning electron microscopy.

MATERIALS AND METHODS

pNiPAM hydrogel preparation. Four different thermoresponsive pNiPAM nanoparticles (named td-1, td-2, td-3 and td-4) with different degrees of crosslinking, size and volume fraction were synthesized (see Table 1). The synthesis took place by free-radical emulsion copolymerization in water (Shimizu *et al.*, 2009). As a result four different pNiPAM nanoparticles were obtained as presented in Table 1. The size distribution of the samples, expressed as the polydispersity index defined as $PDI = \text{square of (standard deviation/mean diameter)}$, is also given in Table 1.

Morphological evaluation. The hydrodynamic radius of the polymeric nanoparticles in dilute regime (nanomoles) in water was measured by dynamic light scattering (DLS) using the green laser at a wavelength of 532 nm as the light source in dilute regime in water. The

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Abbreviations: DLS, Dynamic Light Scattering; DMEM, Dulbecco/Vogt modified Eagle's minimal essential medium; DMSO, Dimethyl sulfoxide; DNA, Deoxyribonucleic acid; EDTA, Ethylenediaminetetraacetic acid; FITC, Fluorescein isothiocyanate; HeK293, Human Embryonic Kidney 293 cells; LCST, low critical solution temperature; MTT, metabolic Toxicity test; NPs, nanoparticles; OD, optical density; PDI, polydispersity index; pNiPAM, poly-N-isopropylacrylamide; SEM, Scanning Electron Microscope

Table 1. Characteristics of pNiPAM nanoparticles synthesized.

Sample	Cross-linker BIS	Concentration (mg/g)	Polydispersity	Size R_h [nm]	
				20°C	35°C
TD-1	1 mol%	12.7	0.133	72	53
TD-2	3 mol%	13.6	0.195	61	44
TD-3	1 mol%	14.5	0.247	94	52
TD-4	3 mol%	10	0.182	78	46

SDS, sodium dodecyl sulfate; BIS, N,N-methylenebisacrylamide

photon correlation was performed by an ALV-5000/E digital correlator.

The morphology of the synthesized samples was assessed by performing cryo-scanning electron microscopy, Cryo-SEM (Jeol, JSM 7001F TTLS). For scanning electron microscopy imaging of micrographs, the accelerating voltage of 15 kV and secondary electron (SEI) mode was used. Samples of a high concentration (1 g of stock solution, see Table 1, in 1 ml of water) were deposited on a glass plate and rapidly frozen in liquid nitrogen and coated with platinum using a sputtering system (Quorum Technologies PP3000I) for 60 seconds to provide an electrically conductive thin film to reduce thermal damage and charging of the samples.

Cell cultures and cytotoxicity evaluation. Examination of cytotoxicity was conducted for HeLa (cervical cancer cell line) and HEK293 (human embryonic kidney cell line) cell cultures, which were cultured in DMEM (Dulbecco's modified Eagle's — Sigma) containing 10% FBS (fetal bovine serum — Gibco Invitrogen) supplemented with antibiotics (penicillin 100 µl/ml, streptomycin 100 µg/ml — Gibco Invitrogen) and incubated in a humid atmosphere, 5% CO₂ at 37°C. When the culture reached the required confluence, cells were trypsinized (trypsin — Gibco Invitrogen) and seeded on culture plates. For the tests, first, HeLa cancer cell line was selected mainly due to its durability and fast proliferation. Secondly, HEK293 cell line was employed since their proliferation is fast but they do not possess the cancer cell line features. For the next step of evaluation, fibroblast cell lines will be used. To assess cytotoxicity and cell viability two tests were performed:

a) MTT (dimethylthiazol diphenyl tetrazolium bromide) — colorimetric assay (from Roche) for assessing cell viability by measuring the absorption of formazan at a wavelength of 572 nm. It reflects the number of metabolically active cells and is based on cleavage of the yellow tetrazolium dye (MTT) to form purple formazan by metabolically active cells. Cells were seeded in a 96-well plate under conditions described above, and then were cultured to 70% confluence before adding the nanogel. In order to get different pNiPAM hydrogel solutions, 50 to 2000 µg of the stock solutions were dissolved in 1 ml of ultrapure (type 1) water (Merck Millipore Milli-Q® Water), see Table 1 (50, 100, 200, 500, 1000, 2000; µg/ml). Each solution was added to 3 wells for cell viability test plus negative probe with dimethyl sulfoxide (DMSO) and, respectively, three positive probes (cells with no NPs added) were analyzed each time. Cells were cultured in an incubator at 37°C and 5% CO₂ in a humid atmosphere for 24 and 48 h in the presence of

pNiPAM nanoparticles. After the incubation time, 10 µl of MTT (0.5 mg/ml) were added. The formazan crystals were then dissolved in DMSO and the plate was incubated at 37°C for 4 h. The optical density (OD) was read with a multiwell microplate reader (Zenyth) at 570 nm.

b) Cell viability test — measured by Muse® Cell Analyzer: Mini, Affordable Flow Cytometry (Muse Count & Viability Assay Kit Merck Millipore). Cells were seeded on a 12-well plate at conditions described above, and then were cultured to 70% confluence before adding the nanogel. In order to obtain different pNiPAM hydrogel solutions, 50 to 2000 µg of the stock solutions were dissolved in 1 ml of ultrapure (type 1) water (Merck Millipore Milli-Q® Water), see Table 1 (50, 100, 200, 500, 1000, 2000; µg/ml). Each solution was added to 3 wells for: cell viability test plus negative probe with dimethyl sulfoxide (DMSO-10%) and, respectively, three positive probes (cells with no NPs added) were analyzed. Cells were cultured in an incubator at 37°C and 5% CO₂ in humid atmosphere for 24 and 48 h in the presence of pNiPAM nanoparticles. Then, the medium was removed and the cells were harvested in 0.2 ml trypsin-EDTA solution (0.25% trypsin, 0.02% EDTA). Then, the cells were treated with Muse Count & Viability Assay Kit according to the manufacturer's description (Merck Millipore).

Statistical analysis. To obtain statistically relevant data from both MTT and cell viability test, three independent probes were taken into account each time. Additionally independent t-test for each probe with differences established at $p < 0.05$ ($p = 0.0408$, standard deviation ~5%) was used to verify statistical significance.

Bioimaging. To confirm direct cell growth on pNiPAM, first we coated 96-well plates by adding 50 µl of pNiPAM NPs (doped with FITC dye) solutions which were added to each well at a given concentration. After one hour of incubation (enabling drying) HeLa and HEK293 cell lines were seeded for 24 h under conditions described above. Medium was then removed and cells were stained with Hoechst33258 blue fluorescent dye for visualization of the DNA present in the nuclei of HeLa and HEK293 cells. Cells were examined using IN Cell Analyzer 2000 automated cellular and subcellular imaging system.

RESULTS

It can be clearly seen in Figs. 1–4 that in comparison to the negative probe all of the examined pNiPAM concentrations did not affect cell lines substantially. Since the MTT assay is considered to be a standard cytotoxicity test we can report no cytotoxicity effects in the tested cell lines at the concentration used for several different pNiPAM nanoparticles (Figs. 1–4), in agreement with previous reports (Wadajkar *et al.*, 2009). Additionally, in the presence of pNiPAM NPs, cellular metabolic activity is clearly higher than in the presence of the reference probe (Pos. — positive) for both HeLa and for HEK293 cell cultures seeded for 48 h. Considering the reference (Pos. — positive) probe as fully supplied in terms of nutrition, samples exhibiting more than 100% of the reference activity should be considered as containing an additional viability promoting factor. To double check these data, a proliferation test was performed.

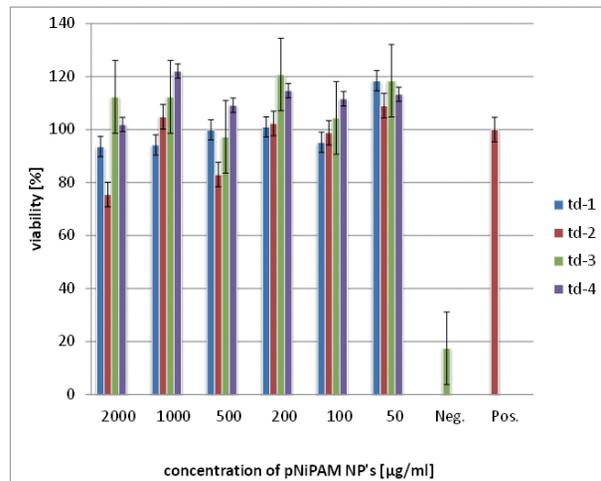


Figure 1. Percentage of absorbance as a result of MTT assay for HeLa cells examined after 24 h with added pNiPAM NPs at the indicated concentration range.

A higher proliferation activity of cells was also observed upon addition of pNiPAM polymer in comparison to the positive control, for majority of nanoparticle concentrations used (Figs. 5, 6). These results indicate that cells proliferate better in the presence of pNiPAM NPs, especially in the case of cell cultures seeded for 48 h, which suggests that the presence of pNiPAM NPs could be an essential factor for longer cell culture growth. As expected, the most significant increase in proliferation percentage was observed for lower concentrations of NPs. One cannot detect any significant decrease in cell viability for any of the pNiPAM systems in the range of the examined concentrations which might be interpreted as a lack of cytotoxicity. Additionally, the proliferation tests were performed for the same range of concentrations. For the TD-3 sample (the sample with the largest NPs size, see Table 1) the result clearly shows that there is no decrease in cell proliferation capability in the presence of pNiPAM nanoparticles (Figs. 5, 6). Based on previous studies (Cooperstein and Canavan 2013) and considering our experimental results — lack of cytotoxicity and additionally an increase of viability due to the pNiPAM NPs presence, we suggest that pNiPAM is forming the so-called bio-scaffold which is promoting cell growth and might be used for cell cultivation. To investigate this hypothesis, Cryo-SEM pictures of pNiPAM NPs networks were analyzed. This morphological evaluation of the pNiPAM layer structure on glass was performed using Cryo-SEM and it is shown in Figs. 7, 8 and 9 with increasing magnification. It can be clearly seen in these figures that pNiPAM nanoparticles used at high concentrations form a 3D scaffold which includes porous layers with water trapped inside. These images give clear information how the scaffold can be formed. Clusters of nanoparticles are aggregating into a sub-micron porous structure. The fact that the scaffolds can be formed creating a way for efficient distribution of the nutrients within its pores is responsible for the observed increase in proliferation and cell viability. Additionally, images of HeLa and HEK293 cell cultures grown on a pNiPAM coated surface were obtained by means of the IN Cell Analyzer 2000 automated cellular and subcellular imaging system (Figs. 10–11). Finally we used the same concentration of pNiPAM NPs (1 g of stock solution, see Table 1, in 1 ml of water) as for the Cryo-SEM in-

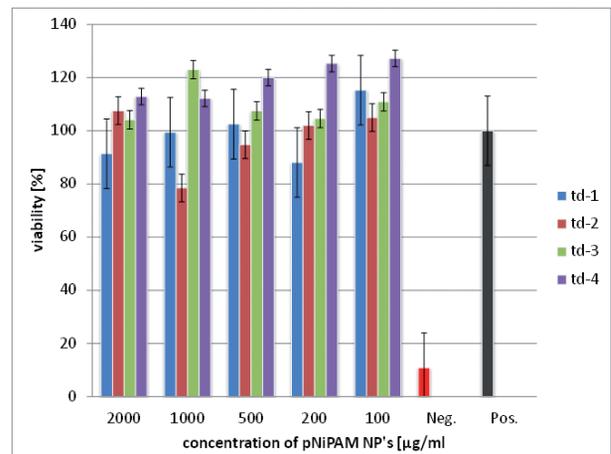


Figure 2. Percentage of absorbance as a result of MTT assay for HEK293 cells examined after 24 h with added pNiPAM NPs at the indicated concentration range.

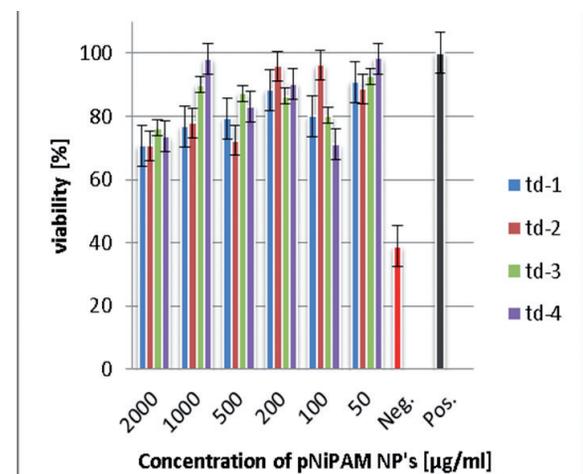


Figure 3. Percentage of absorbance as a result of MTT assay for HeLa cells examined after 48 h with added pNiPAM NPs at the indicated concentration range.

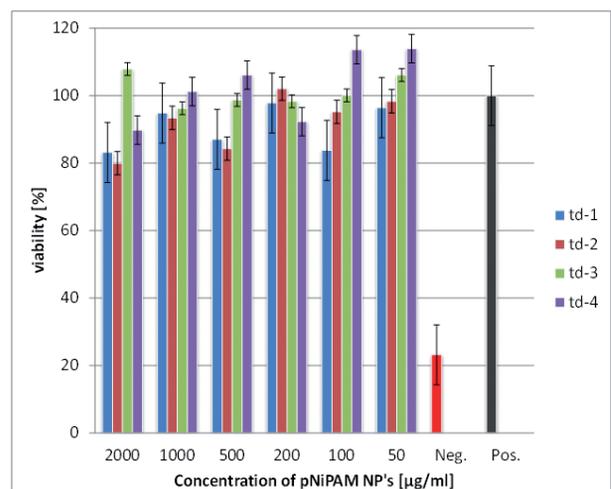


Figure 4. Percentage of absorbance as a result of MTT assay for HEK293 cells examined after 48 h with added pNiPAM NPs at the indicated concentration range.

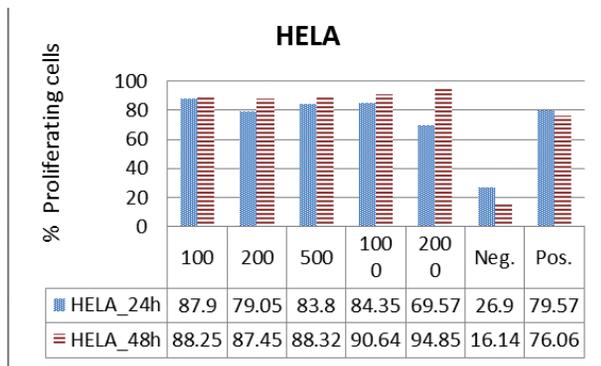


Figure 5. Percentage of proliferating HeLa cells, versus added concentration of NPs ($\mu\text{g/ml}$). Neg and Pos is negative probe with DMSO added and positive one with no additional factors.

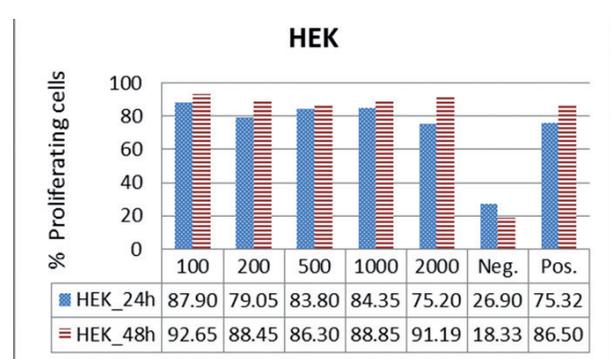


Figure 6. Percentage of proliferating HEK293 cells, versus added concentration of NPs ($\mu\text{g/ml}$). Neg and Pos is negative probe with DMSO added and positive one with no additional factors.

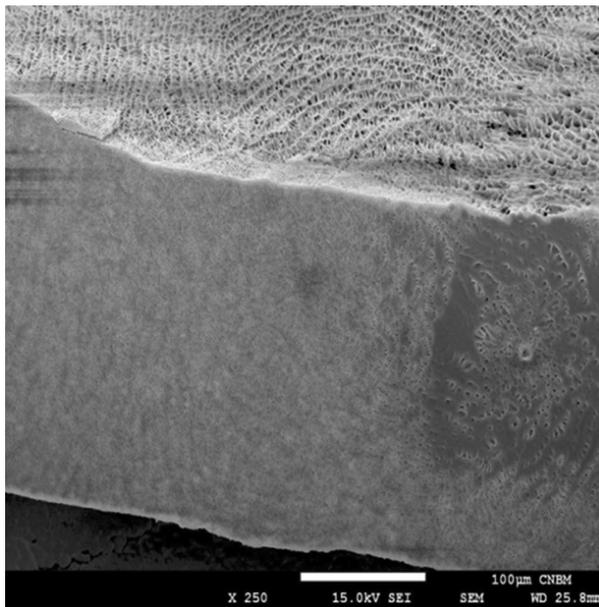


Figure 7. CRYO-SEM pictures of td-3 pNiPAM thermo-responsive nanogel showing the surface and the 'interior'.

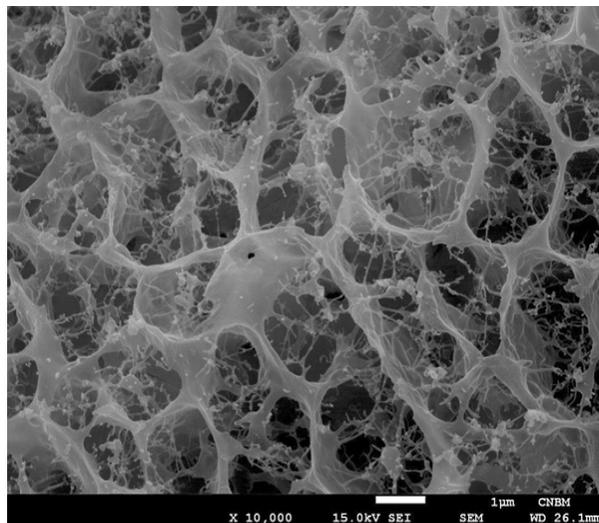


Figure 9. CRYO-SEM pictures of pNiPAM thermo-responsive nanogel; Close-up of the porous interior.

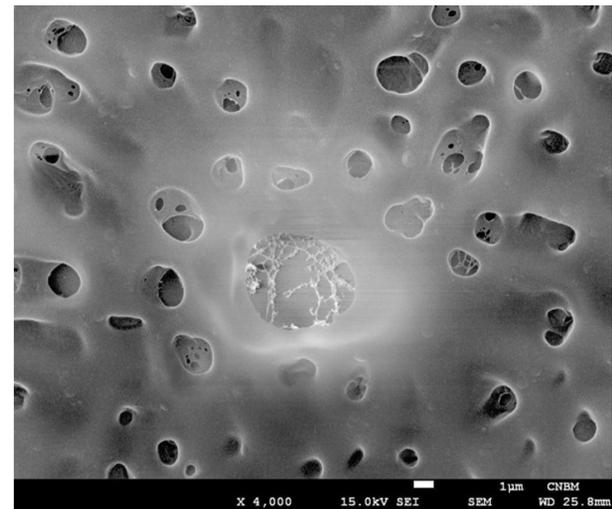


Figure 8. CRYO-SEM pictures of pNiPAM thermo-responsive nanogel; Close-up of the porous surface

vestigation and we can confirm that the nanoparticles are uniformly distributed on the surface and they do not aggregate. Moreover, the pNiPAM layer does not negatively affect the cell cultures.

DISCUSSION

The cytotoxicity tests of the thermo-responsive pNiPAM hydrogel nanoparticles have clearly demonstrated lack of cytotoxicity in the HeLa and HEK293 cell lines in the range of the concentrations used. Moreover, in the presence of NPs, cells tend to exhibit a higher metabolic activity (viability — more than 100%, meaning higher than the positive control probe); the maximum of a metabolic activity for TD-4 and TD-3 can be observed at higher NPs concentrations, especially 200 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ (see Figs. 1–4). We suggest that this phenomenon may be related to the presence of pNiPAM NPs. The pNiPAM NPs form a three dimensional structure which was observed in Cryo-SEM (see Figs. 9) and therefore the distribution of nutritional factors in the medium is more efficient. Moreover, the metabolic activity measured is higher than the control probe (cells with no NPs added) for all four types of pNiPAM almost over the entire concentration range in the HeLa cell line. We correlate this with the presence of pNiPAM based scaffolds whose efficiency might differ for differ-

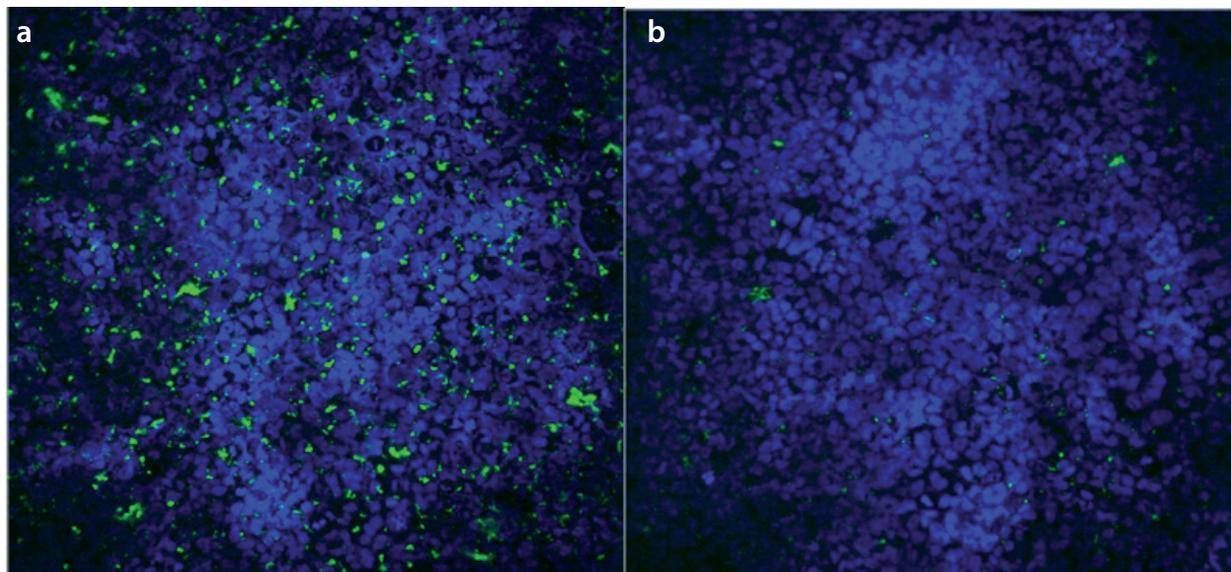


Figure 10. (a, b) HeLa growing on pNiPAM TD-3 coated with FITC (green). Concentration of pNiPAM hydrogel used was 1 mg/ml (a), 50 µg/ml (b).

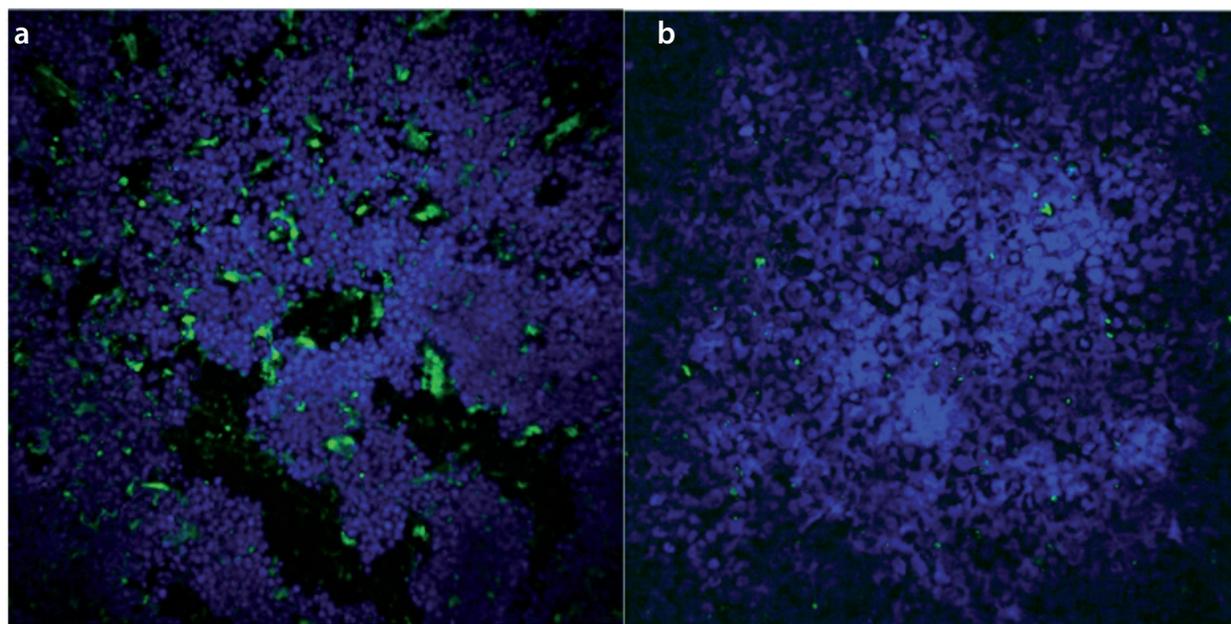


Figure 11. (a, b); HEK293 cells growing on pNiPAM TD-3 coated with FITC (green). Concentration of pNiPAM hydrogel used was 1 mg/ml (a), 50 µg/ml (b).

ent pNiPAM NPs size. For HEK293 cell line, the TD-3 and TD-4 nanoparticle influence seems to be the most significant and promising. Additionally, by employing Cryo-SEM and IN Cell Analyzer we have shown that there is a porous surface formed by the pNiPAM nanoparticles which form a bio-scaffold for efficient cell culture maintenance. Moreover, based on the MTT assays and proliferation test results we can confirm that the cell growth promotion occurs as a result of introduction of pNiPAM NPs.

CONCLUSIONS

Our studies confirm that no toxic effects can be seen in the studied cell lines due to the presence of the pNi-

PAM NPs, in agreement with previous reports (Wadajkar *et al.*, 2009). Additionally, an increase of the viability of the cells in the presence of the pNiPAM NPs was observed. This effect was assigned to the formation of the bio-scaffold by the pNiPAM NPs which promotes efficient distribution of the nutrients and thus enhances cell growth. Therefore it can be concluded that a scaffold based growth mechanism is introduced by the presence of pNiPAM nanoparticles and can positively influence the growth pattern of the cell lines used, at the range of NP concentrations applied. The biocompatibility of the pNiPAM nanoparticles opens further possibilities of applications for cell-based research and for the non-destructive release of the biological cells (Cooperstein & Canavan, 2010; 2013). We used HeLa and HEK293 cell lines for investigation and based on the positive results

obtained in this work it would be recommended to perform a similar cellular sensitivity test on other cell types in the future, such as human skin fibroblasts and stem cells.

Conflict of interests

The authors declare that they have no conflict of interest.

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