

Smart magnetic poly(*N*-isopropylacrylamide) to control the release of bio-active molecules

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Abstract Thermo switchable magnetic hydrogels undoubtedly have a great potential for medical applications since they can behave as smart carriers able to transport bioactive molecules to a chosen part of the body and release them on demand via magneto-thermal activation. We report on the ability to modify the lower critical solution temperature (LCST) of poly(*N*-isopropylacrylamide) (PNIPAM) on demand from 32 °C to LCST ≥ 37 °C. This was achieved by the absorption of controlled amounts of magnetite nanoparticles on the polymer chains. We show, through the effect on cell viability, that the resulting magnetic PNIPAM is able to trap and to release bio-active molecules, such as cell growth factors. The activities of the released bio molecule are tested on human umbilical vein endothelial cells culture. We demonstrate that the LCST of the magnetic PNIPAM can be reached remotely via inductive heating with an alternating magnetic field. This approach on magnetic PNIPAM clearly supports appealing applications in safe biomedicine.

1 Introduction

Biomedical strategies on drug carriers are strongly concentrated on sub-micrometric scale, where carriers have the ability to “move” and “eject” bio-active molecules on demand in a selected part of the body reducing thus the risk for the health. Along this trend, magnetic composite materials have been intensively investigated in the last few years, with a particular attention to the poly(*N*-isopropylacrylamide) (PNIPAM) polymer and magnetite nanoparticles (MNPs) [1–9].

Indeed, PNIPAM is able to generate biocompatible hydrogels and belongs to the class of the so called smart polymers. When it is heated in water above 32 °C, that is the lower critical solution temperature (LCST), it shrinks via a reversible phase transition from a swollen hydrated state to a shrunken dehydrated state. During this process PNIPAM contracts and behaves like a hydrostatic pump, ejecting outside the inner water with its solute [9–12]. Some examples of bio-active molecules expelled by PNIPAM hydrogels at the LCST are reported in the literature. Among them the release of myoglobin (a protein), vitamin B₁₂ (a vitamin) and methylene blue (a heterocyclic aromatic chemical compound) was reported [9, 12, 13]. They all have a different degree of hydrophilic attitude and even in the case of myoglobin, which has a hydrophobic core, PNIPAM is able to release the protein at the LCST.

It was recently shown that the adsorption of MNPs on PNIPAM chains can increase the LCST of PNIPAM which can be controlled in the range from 32 to 50 °C. To each desired LCST corresponds a specific amount of MNP absorbed on the PNIPAM [2]. This magnetization process leads to the formation of magnetic hydrogels, where the presence of MNPs provides a mechanism for the remote temperature control via inductive heating. The energy

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which is transferred from an external alternating magnetic field to the MNPs [14–24] heats up the embedding polymer.

Embedding polymers featuring a LCST >37 °C, that is greater than body temperature, prevent spontaneous drug release upon the injection into the body, while the magnetization of PNIPAM enables the release on demand by a remotely controlled inductive heating.

Moreover, magnetic PNIPAM can also provide a versatile method to guide the material to a chosen part of the body by using an external magnetic field. The LCST of magnetic PNIPAM below 38 °C and above 37 °C was selected to demonstrate the absorption of vascular endothelial growth factor (VEGF) in magnetic PNIPAM and the release at LCST. The chosen magnetic hydrogel had 49 % w/w of MNP.

The effect of released VEGF on viability and proliferation of human umbilical vein endothelial cells (HUVEC) was used to deduce both the effective absorption/release of the growth factor and the preservation of its biological activity after the release.

2 Materials and methods

2.1 Materials

Bare MNPs of about ~ 10 nm diameter [25] were obtained by chemical co precipitation of a stoichiometric mixture of $\text{Fe}[2]^+$ and $\text{Fe}[3]^+$ salts (FeSO_4 and FeCl_3), respectively with ammonium hydroxide [26]. All the reagents were purchased from Aldrich and used without further purification.

The PNIPAM hydrogels were prepared by surfactant free radical polymerization [8, 13] under nitrogen atmosphere in 50 mL of boiled Milli-Q water using an aqueous solution of *N*-isopropyl-acrylamide 0.1 M, (550 mg) and the cross-linker *N,N*-methylenebis-acrylamide, 0.007 M, (50 mg). First the temperature was raised to 80 °C and 250 mg of ammonium persulfate were added to start the

polymerization. After 10 min the temperature was decreased to 70 °C. After 15 min from the start of the polymerization, sodium acrylate, (40 mg) was added to generate a shell enriched with carboxylic groups.

The mixture was refluxed for 4 h in a N_2 atmosphere, and subsequently, the warm hydrogels were purified by centrifugation at a Relative Centrifugal Force of $5,591 \times g$ for 3 min. After the centrifugation, the tube used for centrifugation was heated back to 70 °C to make the PNIPAM deposit visible. The deposit was separated and re-dispersed at room temperature in 50 mL of Milli-Q water.

PNIPAM/MNP hydrogels were obtained by adsorbing MNPs on the shrunken dehydrated state of PNIPAM. The MNP suspension is characterized by a pH value of about 10. In order to shrink PNIPAM, the hydrogel was heated to 50 ± 1 °C in a N_2 atmosphere and different amounts of MNPs in water were added to form the composite. The amount of added MNPs, ranging from 0.4 to 1.5 mL (Samples A and G, respectively in Table 1) was diluted in 50 mL of freshly synthesized PNIPAM solution. Thus the final pH of PNIPAM/MNP suspensions varied between 5 and 6 depending on the added volume of the MNP suspension. All the other parameters were kept constant in all syntheses. The magnetic hydrogels in the shrunken state at 50 ± 1 °C allowed the magnetic separation of PNIPAM/MNP by leaving it overnight ($\cong 12$ h) at 50 ± 1 °C in close proximity of a NdFeB magnet with a surface magnetic field of 0.48 T. The removal of the supernatant liquid left the PNIPAM/MNP deposit ready to be tested.

In particular, the synthesis of magnetized PNIPAM with 49 % of MNP w/w corresponding to $37 < \text{LCST} < 38$ °C, was carried out by adding 0.64 mL of aqueous MNP suspension (21 mg/mL) to the standard PNIPAM synthesis [2].

2.2 Characterization methods

Thermogravimetric analyses (TGA) were performed on magnetic hydrogels dried in air at 70 °C. A STA1500

Table 1 Properties of magnetic PNIPAM hydrogels

Samples	Added MNP ^a (mg)	Added MNP ^a (mL)	% Fe_3O_4 in PNIPAM (w/w ^b)	M_s (emu/g)	LCST (°C)	Hydrodynamic radius at 25 °C (nm)	Hydrodynamic radius at LCST (nm)
O	0	0	0	0	32	430	261
A	8.4	0.4	40	9.44	32	609	453
B	10.5	0.5	30		30	284	264
C	12.6	0.6	27		35	466	387
D	14.7	0.7	49.4	29.77	44	573	484
E	16.8	0.8	65.14	35.28	50	235	205
F	21.3	1.0	67.5		49	244	198
G	31.5	1.5	70.76	45.80	52	290	199

^a Aqueous MNP suspension 21 mg/mL, pH 10

^b Concentration measured by TGA

System (Simultaneous Thermal Analyzer) was used in air. A temperature ramp of 5 °C/min was used.

Photon correlation spectroscopy (PCS) was carried out using a Malvern zeta sizer 3000 HS system (Malvern Instruments Ltd., Malvern, UK) equipped with a 10 mW He–Ne 633 nm wavelength laser with a beam diameter of 200 µm working at a fixed scattering angle of 90° at temperatures from 25 to 55 °C with 5° increment. This means that no local heating on the magnetic nanoparticles caused by the absorption of the laser light should be expected.

A delay time of 10 min was used at each temperature to ensure that the sample viscosity reached equilibrium before the measurements were taken. The suspension of microgels was diluted to a concentration of 0.02 % (w/v) with a NaCl solution of concentration 10 mM to prevent coagulation and to enhance heat dissipation.

UV–Vis analyses were performed by Jasco V-550 UV–Vis Spectrophotometer in room conditions.

Scanning electron microscopy (SEM) observations on dried samples were performed using a Zeiss FEG-SEM LEO 1530 electron microscope at 5.00 kV equipped with an energy dispersive X-ray spectrometer (EDX). To collect good quality images, SEM was performed on samples coated with gold films about 15 nm thick and deposited by Ar sputtering.

Magnetic characterizations were performed by using a vibrating sample magnetometer (VSM). Magnetic hysteresis loops were obtained by cycling the field between –1 and 1 T at room temperature.

Hyperthermia characterizations were performed by using an adiabatic inductive heating equipment, working with alternating magnetic field of strength 30 mT and frequency 293 KH. The sample holder was filled always with 140 µL of ferrofluid and placed in the same position in the middle point of the magnetic field in order to minimize errors coming from the measurement procedure, so that the results are due only to the magnetic characteristics of the samples.

2.3 Biological experiments

2.3.1 Cell culture

The Human Umbilical Vein Endothelial Cell line HUVEC-C (ATCC, Manassas, VA, USA) was cultured in the incubator (New Brunswick Galaxy 48 R, by Eppendorf) at 37 °C, 5 % CO₂, RH 95 % and maintained in continuous culture in EndoGRO™ LS Basal Medium supplemented with EndoGRO™ LS Supplement Kit (Millipore, Billerica, MA, USA): rh EGF 5 ng/ml, ascorbic acid 50 µg/ml, L-glutamine 10 mM, hydrocortisone hemisuccinate 1.0 µg/ml, heparine sulfate 0.75 U/ml, FBS 2 %.

2.3.2 Cell seeding

HUVEC were detached from T-75 culture flask by incubating them with a solution of trypsin–EDTA 0.5 %/0.2 % 10× in PBS for 1 min at 37 °C. The dissociated cells were collected and centrifuged at 800 rpm for 5 min, then the cells were seeded into two 24 wells plate, at a concentration of 10,000 cells/well. Before being used for cell culture, PNIPAM was sterilized by UV for 20 min. Two different kinds of PNIPAM were used: PNIPAM 32 °C, with LCST at 32 °C, and PNIPAM/38, with predicted LCST at 37–38 °C, both of them with or without VEGF (Abcam, Active VEGF full length protein) added at a concentration of 10 ng/mL. To obtain the swollen form of PNIPAM, it was incubated at 4 °C o/n after VEGF addition.

2.3.3 PNIPAM: loading and release of proteins

The ability of PNIPAM to adsorb and release proteins was first tested using fetal bovine serum (FBS) that is a heterogeneous peptide mixture containing up to 100 mg of proteins per ml. We mixed 200 µL of PNIPAM hydrogel (concentration in water 10 mg/mL) with 1–100 µL of FBS (concentration in water 20 mg/L) and with 0.01 % trypan blue. The samples were incubated in 4 °C for 3 h to loading the proteins inside PNIPAM. The mixture was further mixed with Alginate inside 24-multiwell and polymerized using 50 nM CaCl₂ in order to block the free proteins from the fraction loaded by PNIPAM. After careful washing with PBS, the supernatant was investigated using a spectrophotometer (in absorbance mode at 230, 330 and 600 nm wavelength). It was found that the PNIPAM is able to load most of the proteins even at room temperature. After only 1 h of loading, the FBS proteins loaded by PNIPAM were more than 68.3 % of the initial amount of FBS proteins as determined by spectrophotometric analysis using alginate/FBS as control. The maximum loading of proteins into PNIPAM was achieved with 40 µL of serum. From spectrophotometric analyses it resulted that in these conditions, 20 mg of proteins per ml of PNIPAM hydrogel solution was loaded/trapped into PNIPAM hydrogel. This proteins amount corresponded to double the weight of the PNIPAM hydrogel. In this case, the level of FBS proteins released at 38 °C during PNIPAM thermal phase transition, corresponded to 31.3 % of the maximum loading. As a first approximation, we expect this to hold for VEGF too. In this case, the maximum amount of released VEGF would be sufficiently high to play an active role in cell proliferation.

2.3.4 Cell viability applications: Resazurin based assays

VEGF was diluted in PNIPAM (100 ng/mL); after that the solution was incubated at 4 °C for 3 h in order to provide the accumulation of VEGF inside the PNIPAM. Then

100 μL of PNIPAM with VEGF were mixed with 900 μL of growth medium and added in each of the 24-multiwells in a plate, with 20,000 cells per well.

Cells of the second multiwell were treated as the first one, but after PNIPAM addition, the plate was incubated for 40 min at 37–38 $^{\circ}\text{C}$, 5 % CO_2 , RH 95 %.

After this period, we remove medium and add new one to each plate. Then plates were incubated for 24 h. After that period, 100 μL of sterile filtered resazurin sodium salt (Sigma-Aldrich) was added to each well, at a concentration of 3 mg/mL. Plates were incubate in incubator for 3 h and then the supernatant was taken, and tested using Jasco spectrophotometer.

The wells with standard cultural medium with and without VEGF (10 ng/ml) were used as controls. In order to reach the right volume, 1 mL of sterile PBS was added to each cuvette, and absorbance values were read at a wavelength of 570, 600 and 690 nm.

3 Results and discussion

3.1 Magnetic PNIPAM

PNIPAM doped with acrylate was used as a template to adsorb MNPs [8]. As already reported elsewhere [2], the acrylate strongly interacts with Fe atoms at the MNP surface providing a binding mechanism for the MNP-PNIPAM composite formation [1]. TGA was used on dried hydrogels to measure the amount of magnetite adsorbed on PNIPAM. PCS was used to estimate the mean size of the Hydrodynamic Radius of the hydrogel aggregates [27, 28]. In the table the hydrodynamic radius of the aggregate are reported at room temperature and at the LCST. The variability of the radius could be due to the effect of the MNP on PNIPAM aggregation in water. All the experimental details on a selected set of magnetic PNIPAM and the characterization results are reported in Table 1. From the graph of these results it was possible to infer the data reported in [2] Fig. 1a, the composition of magnetic PNIPAM with $37 < \text{LCST} < 38^{\circ}\text{C}$ suitable for medical applications and cell culture conditions. We selected this range of temperatures with the intention to preserve the standard conditions of human cell culture protocols.

The magnetization of the selected set of samples of magnetic PNIPAM hydrogels (A, E, D, G) was measured with a VSM by applying a cyclic magnetic field up to 1 T at room temperature. Results have been normalized to the total sample mass in order to obtain comparable results.

Magnetization curves are shown in Fig. 2 as a function of the applied magnetic field. The MNPs used in the present work [26], had a diameter of ~ 10 nm (well below the single to multidomain limit) and were initially super

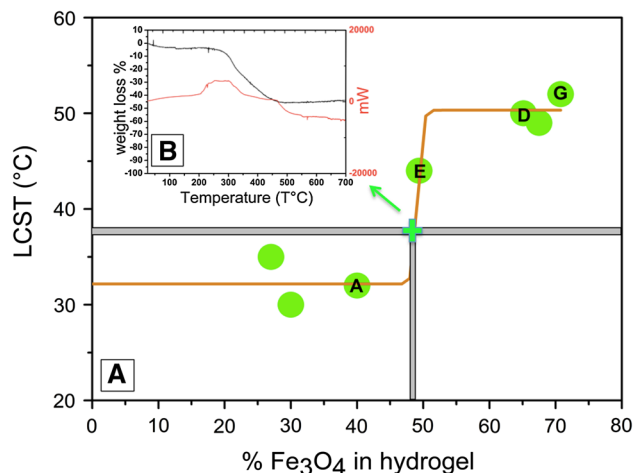


Fig. 1 a LCST of magnetic PNIPAM hydrogels (samples A, E, D, G) at different magnetite concentrations in the hydrogels. The orange line is a guide for the eye, green cross represents the composition of magnetic PNIPAM/37 showing $37 < \text{LCST} < 38$; b TGA of the sample composed by 49 % of MNP with $37 < \text{LCST} < 38^{\circ}\text{C}$ (Color figure online)

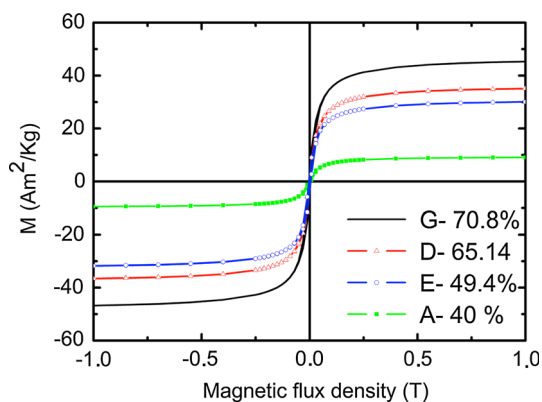


Fig. 2 Magnetization cycles of PNIPAM/MNP hydrogels at different % weight concentrations of MNPs in PNIPAM

paramagnetic (no hysteresis in the magnetization cycles). During storage, they aggregated slightly and some hysteresis in the magnetization cycles was observed. For this reason, the PNIPAM nanocomposite showed also a slight hysteresis of the magnetization cycles [2].

The samples reached different saturation magnetizations. Sample A presented the lowest saturation magnetization, followed by samples E and D which had similar saturation magnetizations and then by G, which had a slightly higher magnetization. The large gap of ~ 25 A m^2/Kg between the magnetization of sample A and all the rest suggested that it contained less magnetite than the others.

After having magnetically characterized the synthesized materials, we move to the hyperthermia experiments to investigate the fine control of the thermal response achieved in magnetic PNIPAM hydrogels. Magnetic hyperthermia experiments have been performed by

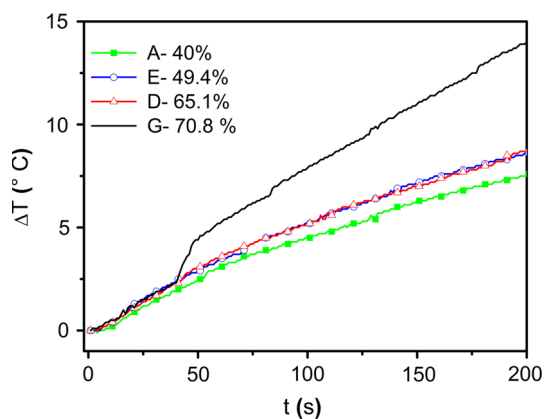


Fig. 3 Magnetic hyperthermia experiments performed by applying an alternating magnetic field (frequency 293 kHz, field amplitude $H = 30$ mT) to fluid samples at room temperature (25 °C) made of the PNIPAM-MNP hydrogels at different magnetite concentrations (% w/w)

applying an alternating magnetic field of $f = 293$ kHz and field amplitude of $B = 30$ mT to fluid samples of the magnetic PNIPAM at room temperature ($T_r \sim 25$ °C).

In Fig. 3 the increase in temperature of samples A, E, D, G, is shown as a function of the duration of the magnetic field exposure. It can be observed that the maximum temperatures obtained were directly correlated to the amount of magnetite on the hydrogel as determined by TGA characterization and by the magnetization cycles.

These curves also showed the ability of magnetic hyperthermia to produce heating not only in the ferrofluids, in which Brown and Néel relaxation process are assumed to contribute [25], but also in media with fewer mechanical degrees of freedom as PNIPAM matrices.

In addition, these results showed that by controlling the amount of adsorbed magnetite on the PNIPAM matrix, one could precisely achieve the desired thermal response by the application of an external field, allowing the use of this nano-composite for safe biomedical therapies [1]. In this context, it is particularly important to validate the expulsion of bio-active molecules from magnetic PNIPAM at a selected LCST compatible with the stability of the bio-active molecule. We chose VEGF as the bio-active molecule, and we tested the activity of the expelled molecule by studying its effect on the proliferation test on HUVEC.

4 Biological applications of magnetized PNIPAM

Two batches of both bare and magnetized PNIPAM were loaded with VEGF, following the reported methodology for protein loading [13], to test the ability to release VEGF at different temperatures. The PNIPAM hydrogels were prepared by surfactant free radical polymerization as described in the experimental section [1]. PNIPAM

showing $37 < LCST < 38$ °C will be referred to as PNIPAM/38 in the remainder of this work, while bare PNIPAM will be referred to as PNIPAM.

4.1 Investigation of HUVEC proliferation after VEGF release from PNIPAM

VEGF, also known as vasculotropin, is a potential mitogen for vascular endothelial cells that induces angiogenesis. VEGF induces gene expression, directs cell migration, new blood vessels formation and endothelial cell proliferation. HUVEC were utilized for in vitro PNIPAM release capability test. Indeed, HUVEC cells are very well characterized, thoroughly accepted, easily obtained and reproducible cell types that can be used to demonstrate angiogenic effects [29]. Because of their increased sensitivity to VEGF, which stimulates HUVEC growth, the proliferation test on HUVEC can be considered an indirect method to detect the VEGF release by PNIPAM.

The absorption and the release of VEGF by PNIPAM were determined indirectly through HUVEC proliferation bioassay (Resazurin assay) in solutions incubated with VEGF-loaded PNIPAM. Results of this assay are reported in Fig. 4. The results demonstrated significant increase of cell proliferation at LCST in both PNIPAM and PNIPAM/38 loaded with VEGF. In bare PNIPAM and magnetic PNIPAM, the LCST is the temperature at which a contraction of the hydrogel occurs. This contraction causes the release of the water and of its solute contained in the hydrogel. According to the result of two independent experimental series, the level of VEGF released at cell culture temperature (38 °C) from “PNIPAM/38” was reliably higher than in case of PNIPAM and definitively higher in comparison with the control. In some experimental series, PNIPAM without VEGF provided stimulating effects on cell proliferation comparable with VEGF, but it was sporadically observed and can be attributed to the variability of the system. Indeed, it should be taken into account that the effects on cell proliferation were observed as a direct consequence of a multicomponent system where released VEGF strongly conditioned the cell culture, but the effects due to the presence of PNIPAM hydrogel, MNP, derivate of PNIPAM with adsorbed VEGF and PNIPAM phase transition should also be considered. At this first level of investigation, from a comparative analysis of the data, we can assess a no-negligible change of HUVEC proliferation under the influence of VEGF released by PNIPAM and PNIPAM/38 in the range 37 – 38 °C.

5 Conclusions

In this article we explored the possibility of delivering a cell growth factor, such as VEGF, under cell-friendly

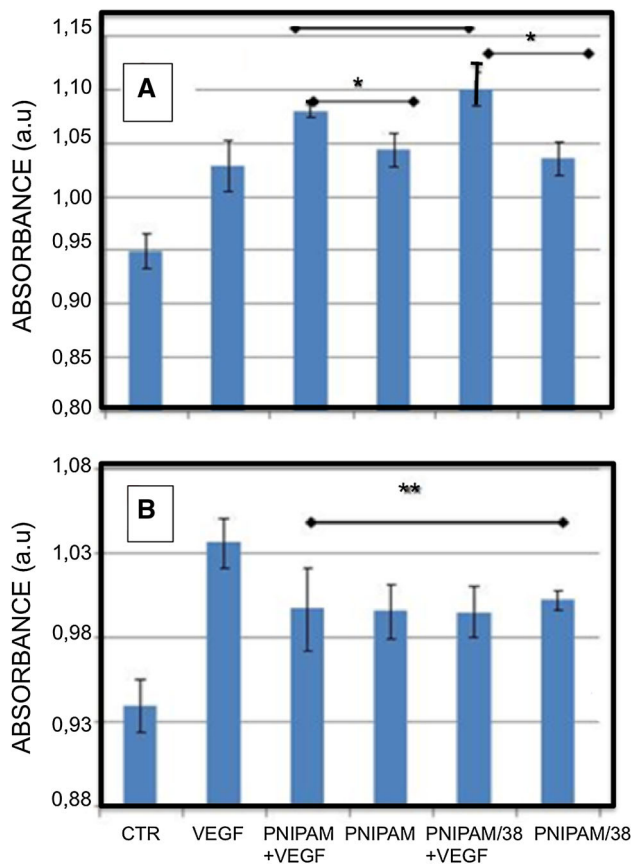


Fig. 4 Resazurin assay ($\lambda = 570$ nm) at different temperatures (**a** -38 °C and **b** -20 °C) after incubation of HUVEC with PNIPAM loaded with VEGF. The wells with standard cultural medium with and without VEGF (10 ng/mL) were used as controls. ** Values do not have a significant difference ($P > 0.05$); * values have significant difference ($P < 0.05$); error bars represent mean \pm SD

conditions to assure a high level of cell viability of HUVEC, a well known class of human cells. Therefore, we reported the synthesis of magnetic PNIPAM (PNIPAM/38) having a LCST of 38 °C. Indeed, for medical applications, a LCST of 38 °C is slightly over the body temperature and can be easily achieved by inductive heating, which allows controlled release of the drug. Both bare PNIPAM and PNIPAM/38 incubated with HUVEC and loaded with VEGF, demonstrated the release of the latter at 37–38 °C. The effect of the release of VEGF on the proliferation of cultivated HUVEC demonstrated both the loading and the preservation of the biological characteristics of the released VEGF. No adverse effect on cell proliferation was detected from the presence of MNPs. Magnetic PNIPAM can be therefore considered a promising material to be used for a controlled release of VEGF or other proteins able to stimulate vascular cells inside a scaffold.

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