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Polyisocyanopeptide hydrogels: A novel thermo-responsive hydrogel supporting pre-vascularization and the development of organotypic structures

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ABSTRACT

Molecular and mechanical interactions with the 3D extracellular matrix are essential for cell functions such as survival, proliferation, migration, and differentiation. Thermo-responsive biomimetic polyisocyanopeptide (PIC) hydrogels are promising new candidates for 3D cell, tissue, and organ cultures. This is a synthetic, thermo-responsive and stress-stiffening material synthesized via polymerization of the corresponding monomers using a nickel perchlorate as a catalyst. It can be tailored to meet various demands of cells by modulating its stiffness and through the decoration of the polymer with short GRGDS peptides using copper free click chemistry. These peptides make the hydrogels biocompatible by mimicking the binding sites of certain integrins.

This study focuses on the optimization of the PIC polymer properties for efficient cell, tissue and organ development. Screening for the optimal stiffness of the hydrogel and the ideal concentration of the GRGDS ligand conjugated with the polymer, enabled cell proliferation, migration and differentiation of various primary cell types of human origin. We demonstrate that fibroblasts, endothelial cells, adipose-derived stem cells and melanoma cells, do survive, thrive and differentiate in optimized PIC hydrogels. Importantly, these hydrogels support the spontaneous formation of complex structures like blood capillaries *in vitro*. Additionally, we utilized the thermo-responsive properties of the hydrogels for a rapid and gentle recovery of viable cells. Finally, we show that organotypic structures of human origin grown in PIC hydrogels can be successfully transplanted subcutaneously onto immune-compromised rats, on which they survive and integrate into the surrounding tissue.

Statement of Significance

Molecular and mechanical interactions with the surrounding environment are essential for cell functions. Although 2D culture systems greatly contributed to our understanding of complex biological phenomena, they cannot substitute for crucial interaction that take place in 3D. 3D culture systems aim to overcome limitations of the 2D cultures and answer new questions about cell functions.
Thermo-responsive biomimetic polyisocyanopeptide (PIC) hydrogels are promising new candidates for 3D cell, tissue, and organ cultures. They are synthetic and can be tailor to meet certain experimental demands. Additionally, they are characterized by strain-stiffening, a feature crucial for cell behaviour, but rare in hydrogels. Their thermos-responsive properties enable quick recovery of the cells by a simple procedure of lowering the temperature.

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1. Introduction

Classic two-dimensional (2D) cell culture platforms contributed greatly to our understanding of biological phenomena on the cellular level. Studies with cells cultured on cell culture plastics or comparable surfaces have brought comprehension to complex molecular processes, such as the cell cycle, cell-cell interactions, stem cell self-renewal and differentiation. However, those experiments removed cells from their native 3D environments and forced them to adhere and proliferate mostly as a completely artificial monolayer. The disadvantages of this simplicity and versatility are abnormal polarization of epithelial cells, flattened shape, altered responses to stimuli, and dramatic changes in phenotype [1,2].

Present knowledge on cell and tissue biology gives prominence to the tremendous difference between (2D) and three-dimensional (3D) culture systems. Various 3D systems were developed to mimic more physiological cell-cell and cell-ECM interactions [3]. Among many approaches to achieve this feat, hydrogels are considered as most promising. These water-swollen networks of polymers maintain a distinct 3D structure. The high water content of these materials facilitates their biocompatibility. Moreover, hydrogels mimic native properties of extra-cellular matrices in terms of adhesion and mechanical properties. Utilization of hydrogels for cell culture has revealed some basic phenomena orchestrating cell behaviour and organization. One of the most striking examples disclosing superiority of 3D cell culture systems based on hydrogels is the work of Reichmann et al, who showed already in 1989 that mammary epithelial cells formed doming monolayers in 2D culture, whereas they developed into organotypic structures, like mammary ducts and endbuds, in collagen type I hydrogels [4].

3D cell culture systems from natural sources (e.g., Matrigel [5] and Collagen [6]) provide excellent cell viability and fast enzymatic degradation profiles. In addition to these natural 3D matrices, there are synthetic materials that can form hydrogels. In contrast to natural hydrogels, synthetic polymers have a clearly defined and constant chemical composition. Their disadvantage is frequently a lower biocompatibility, which is due to a lack of integrin-like cell binding sites. To compensate for this disadvantage biomolecules, such as GRGDS peptides, may be coupled to the polymer. This will allow efficient cell binding to the corresponding extracellular structures of the hydrogel.

In this paper, we tested and optimized a novel, fully synthetic, biocompatible, 3D cell environment, namely a hydrogel formed by the water soluble polymer polyisociano peptide (PIC) [7]. This material is synthesized via polymerization of the corresponding monomers using a nickel perchlorate as a catalyst. In addition, the decoration of the polymer with short GRGDS peptides is performed using copper free click chemistry. These peptides make the hydrogels biocompatible by mimicking the binding sites of certain integrins.

Notably, these hydrogels exhibit reversible thermo-responsive behaviour. At temperatures below 16° the polymer solution is a free flowing liquid, whereas above 16° the viscosity dramatically and rapidly increases whereby the polymer solution comprises a hydrogel. This flexible behaviour is advantageous for cell recovery and allows removal of the gel after it served as a (transient) carrier for bio-engineered cell tissues. One of the most interesting characteristics of PIC hydrogel is its strain-stiffening property [8]. This stiffness, wide-spread in nature's but rarely observed in synthetic scaffolds, is not constant but increases when the gel is strained.

The above mentioned characteristics of the PIC hydrogel make it a versatile and tuneable system for 3D cell culture and experimental *in vivo* applications. Here we demonstrate that indeed complex biological structures, such as a network of capillaries, can develop in PIC hydrogels under the appropriate conditions. The cornerstone of our research was successful *in vivo* transplantations of cellular PIC hydrogels.

1.1. Experimental design

First, we optimized the PIC concentration that is the most suitable for cell culture. Then, using knowing the best PIC concentration we screened for the GRDGS concentration on the PIC backbone that gives the best results in terms of cell proliferation. For these two steps we used primary human dermal fibroblasts. We checked whether thermo-responsive properties of PIC hydrogel can be used for quick and harmless cell retrieval from hydrogels. Subsequently, we checked whether optimized PIC hydrogels support formation of blood capillaries *in vitro* by HDMECs, adipogenic differentiation of ASCs and whether PIC supports growth of melanoma cells. Finally, using PDMS molds we subcutaneously transplanted PIC hydrogels in immunocompromised rats.

2. Materials and methods

2.1. Synthesis of the polymers

PIC polymers were synthesized via copolymerization of a triethylene glycol functionalized isocyano-(D)-alanyl-(L)-alanine monomer (M1) and the corresponding azide-terminal monomer (M2) according to a previous report from our laboratory [9]. Nickel perchlorate hexahydrate was used as catalyst in 1:4000 M ratio with respect to the 3OEG monomer M1. The molar ratios used between monomer M1 and M2 were 1:30, 1:50, 1:70, 1:100 giving and statistical spacing between each azide group of 3, 5, 7 and 10 nm. By using different ratios, the concentration of GRGDS functions along the polymer chain changes. The polymers with the lowest ratios (1:30) are able to accommodate higher amounts of GRGDS on the chain. On the contrary, polymers with the highest ratio (e.g. 1:70) contain less azide functional groups and, hence, are able to accommodate less GRGDS molecules on the polymer chain. The resulting polymers were precipitated from diisopropyl ether. The molecular weight of the final polymers was determined by viscosity to be 500 Kg/mol.

2.2. Conjugation of the polymers with GRGDS

GRGDS peptide was reacted with dibenzocyclooctyne-N-hydro xysuccinimidyl ester (DBCO-NHS ester) to obtain the complex DBCO-GRGDS. Secondly, the complex DBCO-GRGDS was reacted to M2 monomers present on the PIC polymer backbone via copper free click chemistry according to a previous report from our laboratory [9]. Finally, the polymers decorated with cell-adhering peptide were purified by precipitation from diispropyl ether.

2.3. Hydrogel formation and characterization

Hydrogel samples of different PIC-GRGDS concentration (2, 2.5, 3 and 5 mg/mL) were prepared for each molar ratio M1:M2. PIC-GRGDS polymers were dissolved directly in the cell culture media in a way that the final concentration of the polymer in a hydrogel in a transwell equalled 2, 2.5, 3 or 5 mg/ml. Solutions were gently stirred at 4 °C over 12 h in order to facilitate the complete dissolution of the material. Gels were formed on warming the solution above 16 °C. The gelation temperature and mechanical properties of the gels decorated with GRGDS peptide were studied by rheology. Gels were subjected to temperature swift 5–50 °C. All the gels showed similar gelation temperatures (16 °C) and similar elastic modulus (G' = 100–300 Pa) at 37 °C.

2.4. Isolation and culturing of the primary cells

Human skin samples and adipose tissue samples were obtained from patients after approval was obtained from the Ethics Committee of the Canton Zurich and informed consent was given by parents or patients. Fibroblasts and HDMECs were isolated and cultured from skin samples as described before [10,11]. ASCs were isolated and expanded following the before-described procedure [12]. Cultured human melanoma cells were used from before isolated and maintained cells [13].

2.5. Cell culture in hydrogels

All PIC hydrogels used in this study were prepared according to the following the protocol. The respective PIC polymer was weighted on an analytical balance (Kern&Sohn, Balingen, Germany). Next, the PIC polymer was transferred to sterile tubes and dissolved in cold culture medium. Subsequently, cells were seeded into the PIC solution in a concentration of 100,000 cells/ml. The solution was then gently pipetted into inserts of Transwell-Clear plates (Corning, Kaiserslautern, Germany). After 30 min in incubator gels were covered with medium above and below. Hydrogels with human dermal fibroblasts and melanoma cells were grown in DMEM containing 10% fetal calf serum (FCS), 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, and 5 mg/ml gentamycin (all from Invitrogen, Paisley, UK). PIC gels for capillary formation studies were prepared with the 1:1 mixture of HDMECs and fibroblasts, and subsequently cultured in EGM-2MV medium (Lonza, Basel, Switzerland). ASCs in hydrogels were differentiated into adipocytes in MesenCult Adipogenic Differentiation Medium (Stem Cell, Cologne, Germany).

Collagen hydrogels were prepared as described previously [14]. PIC and collagen hydrogels were imaged using a confocal microscope after staining with specific antibodies. The number of blood capillaries and branching points in pre-vascularized hydrogels, and the number of differentiated adipocytes in the hydrogels with ASCs were calculated per mm^2 of a hydrogel (n = 4 per condition).

2.6. Proliferation assay

Cell proliferation rate was determined using a Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol (Sigma-Aldrich, Buchs, Switzerland). Briefly, PIC and collagen type I hydrogels with human dermal fibroblasts (100,000 cells/ml) were prepared in triplicate. For 4 consecutive days 2 ml of accordingly diluted CCK-8 solution was added to each well with a hydrogel and incubated for 2 h. Afterwards, the solution was collected and the resulting color was assayed at 450 nm using a microplate absorbance reader (Biotek Epoch, Luzern, Switzerland). Each measurement was carried out in triplicate.

2.7. Recovery of the cells

For the recovery of cells cellularised hydrogels were removed from culture inserts and transferred into a tube where cold 4 °C sterile PBS was added. The solution was then centrifuged for 3 min at 400*g*. After removal of the supernatant the cells were suspended in a culture medium and seeded on a cell culture plastic and cultured in an appropriate medium. To check the percentage of recovered cells that attached to plastic 4 h after the seeding, culture medium was collected and unattached cells were counted.

2.8. Animal studies

The surgical protocol was approved by the local Committee for Experimental Animal Research (permission number 252/2013). Molds were made of poly(dimethylsiloxane) (PDMS) formed from a pre-polymer (Sylgard 184) at a ratio of 10:1 base to curing agent. The mold dimensions were 4 mm in diameter and 1 mm in depth. Hydrogels with molds were transplanted subcutaneously on the back area of 10-week-old, female, athymic Nu/Nu rats (Charles River Laboratories, Germany). All animals were sedated with 15 mg/kg ketamine s.c. (Company) prior to surgery. For anaesthesia, isoflurane (Baxter, Volketswil, Switzerland) and as post-operative analgesia 0.5 mg/kg buprenorphine s.c. (Temgesic, Essex) was provided. The skin was sutured with non-resorbable polyester sutures (Ethicon, Norderstedt, Germany) and the wound dressed by a polyurethane sponge (Ligasano, Ligamed, Austria), and tape (Leukoplast, BSN medical, Germany). Animals were sacrificed 7 days later and hydrogels were removed and processed for sections.

2.9. Immunofluorescence staining

Immunofluorescence staining was performed as described in Pontiggia *et al.* [15]. The following antibodies were used for immunofluorescence: HMB-45 (1:50, Dako, Baar, Switzerland), CD90 conjugated with FITC (1:20, dianova, Hamburg, Germany), HIS48 (1:50, Santa Cruz, Heidelberg, Germany). AlexaFluor 488 and 555-conjugated immunoglobulins were used as secondary antibodies (Invitrogen, Paisley, UK). Photos of immunofluorescence staining were taken with DXM1200F digital camera connected to Nikon Eclipse TE2000-U inverted microscope. The device was equipped with Hoechst 33342-, FITC-, and TRITC-filter sets (Nikon AG, Switzerland; Software: Nikon ACT-1 vers. 2.70). Images were processed with Photoshop 10.0 (Adobe Systems Inc., Basel, Switzerland).

2.10. Whole-mount staining

PIC hydrogels with cultured cells were washed with PBS for 2 h, with PBS exchanged every 30′. Gels were blocked for unspecific binding and permeabilised with 0.1% Triton X-100/10% FBS for 30 min and incubated with HMB-45, CD31 (both 1:50, Dako, Baar, Switzerland), FABP4 (1:200, clone 9B8D, abcam, Cambridge, UK) antibodies for 2 h. Subsequently, gels were washed with PBS for another 1 h and incubated with secondary antibodies: AlexaFluor 488 and 555-conjugated immunoglobulins were used as secondary antibodies (Invitrogen, Paisley, UK) or with BODIPY FL (1:1000, Life Technologies, Paisley, UK) for 2 h. Finally, constructs were washed for 2 h with PBS. Tissues were mounted in Dako mounting solution (Dako, Baar, Switzerland) for confocal imaging using a Leica SP1 confocal laser scanning microscope (Leica, Heerbrugg, Switzerland). Images were processed with Imaris 5.0.1 (Bitplane AG, Zurich, Switzerland).

2.11. Fluorescein diacetate (FdA) vital cell staining

In order to visualize cell viability in the hydrogels and cell culture plastic, FdA staining was performed as published [16]. Briefly, from an acetone 5 mM stock solution, FdA (Sigma, Buchs, Switzerland) was added to the culture medium in the lower and upper chambers to a final concentration of 5 μM. After 2 min, FdA was removed by washing twice in PBS before fresh culture medium was applied. The substitutes were analysed by fluorescence microscopy.

2.12. Statistical analysis

Group data is presented as mean ± standard deviation (SD). Data analysis was performed using GraphPad Prism 5.0 (La Jolla, California USA). Statistical significance was determined using one-way analysis of variance (ANOVA) with Dunnett's Multiple

Comparison Test after assumptions of normal distribution and homogeneity of variances were verified. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Optimizing the biological properties of PIC hydrogels

To unravel the biological potential of PIC hydrogels for a complex 3D cell culture, we had to optimize two parameters that are crucial to cell survival and viability: (1) the polymer concentration in a hydrogel and (2) the concentration of the GRGDS peptide conjugated to the polymer backbone. The former is especially important as the properties of the PIC polymer, like strain-stiffening, change as a function of concertation, the latter enables cells to interact with the material.

As the optimization of both above mentioned properties was not possible at the same time, we first focused on determining the optimal concentration of the PIC polymer. For these experiments the concentration of 1:100 PIC:GRGDS polymer was used as previously reported [17]. GRGDS peptide was prepared for in a form of DBCO-GRGDS complex (Fig. 1A). The PIC polymer is built from two monomers: M1 and M2, differing only in the azide group, present only at the M2 (Fig. 2B). This group is used for the decoration of the PIC polymer with GRGDS peptides, thus a ratio between the two monomers translates directly to the GRGDS concentration on the PIC polymer (Fig. 1C). For instance, the 1:100 PIC:GRGDS means that for 1 part of M1 monomer, 100 parts of M2 monomers were used and their azide groups were fully saturated with the GRGDS peptide. Freshly obtained polymer was in a form of flakes (Fig. 1D), that easily dissolved in a culture medium to form a hydrogel (Fig. 1E).

Human dermal fibroblasts isolated obtained from foreskin were mixed with the PIC polymer and their proliferation was monitored by observation in bright field as well with use Fluorescein Diacetate (FdA) dye to stain living cells. The most suitable condition supporting cell proliferation became obvious already after 48 h of culture. In hydrogels consisting of 2.0 mg/ml PIC, fibroblasts showed their usual stretched appearance, colonizing the hydrogel in three dimensions (Fig. 2A). In contrast, fibroblasts in the gels consisting of higher PIC concentrations (2.5, 3.0, and 5.0 mg/ml) did not acquire the typical spindle-shaped morphology remained round in shape, and did not stretch out (Fig. 2B–D). Cells cultured in higher concentrations of the polymer, 3.0 mg/ml and 5.0 mg/ml, although did not change their round morphology stayed viable after 5 days in culture (Fig. 2A).

Having determined the optimal concentration of the PIC polymer, we investigated whether increase of the concentration of the GRGDS peptide on the polymer backbone would further improve the environment and culture conditions for dermal fibroblasts. The PIC polymer in a concentration of 2.0 mg/ml was combined with the GRGDS peptide in the following ratios– 1:30, 1:50, 1:75, 1:100. The 1:30 ratio is the highest ratio when the polymer still retains its physico-chemical properties, such as gelation and stress-stiffening characteristics.

The PIC to peptide ration of 1:30 turned out to be the optimal ratio at which almost all cells showed positive FDA staining, and exhibited their typical spindle morphology already 18–24 h after submerging them in the hydrogel. The proliferation of fibroblast was similar to the one observed in control collagen type I hydrogels (Fig. 2E–G). In addition, they displayed optimal growth characteristics, as revealed by a proliferation assay (Fig. 2A–H). What is more, the proliferation rate of fibroblasts in these conditions was similar to the proliferation in collagen type I hydrogels (Fig. 2H).

3.1.1. PIC hydrogels support formation of blood capillaries, adipogenic differentiation and growth of cancer cells

Culturing fibroblasts, although being important for establishing the optimal PIC features for cell growth, could not reveal the full potential of the tested material. Hence, we decided to analyse whether the optimized environment would allow complex cell interactions and migration of both fibroblasts and endothelial cells that foster formation of a capillary plexus in the hydrogel. For these experiments we combined freshly isolated human dermal microvascular endothelial cells (HDMECs) and human dermal fibroblasts mixed in a 1:1 ratio. The cells were submerged within the PIC polymer (2.0 mg/ml, PIC:GRGDS 1:30) forming a functional hydrogel, and cultured in endothelial growth medium for 3 weeks. Immunofluorescent staining of hCD31, a marker for human endothelial cells, revealed the presence of a network of blood capillaries in the hydrogels that was similar to the ones observed in collagen type I hydrogels (Fig. 3A–C). After 3 weeks of culture we observed 52 blood capillaries per mm^2 (SD = 9) with 79 branching points (SD = 14), which corresponds to results observed in control collagen hydrogels (number of capillaries = 55, SD = 8; branching points = 85, SD = 12). Obtained results were not statistically significant ($p > 0.05$).

In a further attempt we investigated the differentiation of human adipose-derived stem cells (ASCs) into adipocytes. Freshly isolated (primary) ASCs of human origin were mixed with the PIC polymer (2.0 mg/ml, PIC:GRGDS 1:30). Hydrogels were cultured in ASCs expansion medium until the cells occupied almost the entire volume of the gel. Subsequently, the medium was changed to adipogenic differentiation medium for 10 days. During this time the approximately 76% (SD = 11) of ASCs underwent differentiation into adipocytes. The changes in appearance were clearly visible in bright field microscope (Fig. 3E). The cells were no longer stretched, but embraced a more relaxed form and became filled with lipid vesicles. The presence of lipids in these vesicles was confirmed by the retention of BODIPY dye. Additionally, the differentiated cells were positive for widely-used adipocyte markers, such as FABP4 (Fig. 3D) and Perilipin (not shown). Importantly the adipogenic differentiation in PIC hydrogels was similar to the differentiation in collagen hydrogel (Fig. 3F).

Finally, we determined whether and how cancer cells would grow in PIC hydrogels. We submerged melanoma cells isolated from cancer patients in PIC polymer (2.0 mg/ml, PIC:GRGDS 1:30). Confocal imaging of melanoma cells stained for the HMB45 marker confirmed that the cell proliferated and clustered into spheres (Fig. 3G). Similarly, to collagen hydrogels (Fig. 3I), spontaneous condensation into multicellular spheres was observed under the bright field microscope (Fig. 3H).

3.2. Recovery of cells

The thermo-responsive properties of the PIC polymer make it a perfect system for biologically efficient and transparent 3D culture and effortless extraction of cultured cells. The polymer forms stable hydrogels at room temperature and 37 °C, whereas it liquefies at 15 °C and lower. To test whether viable cells could be extracted from PIC hydrogels we cultured human dermal fibroblasts for 5 days in PIC hydrogels until the cells uniformly colonized the gel (Fig. 4A). The hydrogels were then transferred into falcon tubes and ice cold PBS was added. As a consequence, the hydrogels turned from the gel into a liquid state (Fig. 4B–D). After centrifugation and removal of supernatant, cells were re-suspended in culture medium and plated on cell culture plastic. Recovered fibroblasts attached to the plate already after 5 h post-plating (Fig. 4E). The FDA staining performed 24 h later confirmed their viability (Fig. 4F). No influence of the PIC polymer on cell attach-

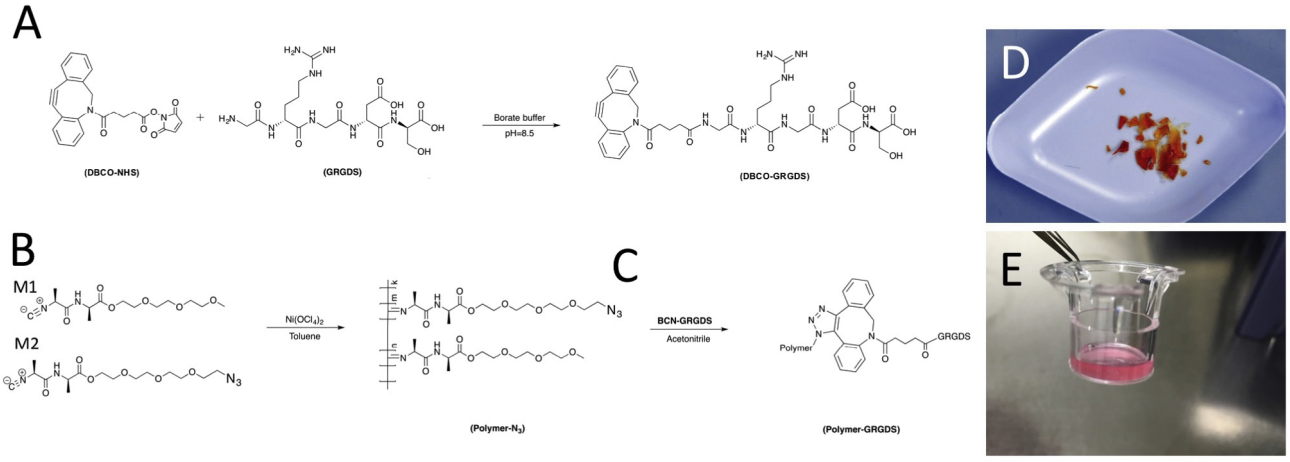

Fig. 1. Synthesis of the polymer and decoration with short peptide GRGDS. A. Synthesis of the complex DBCO-GRGDS. B. Co-polymerization of acize functionalized and unfuctionalized monòmers. C. Decoration of azide fuctionalized polymers with DBCO-GRGDS complex. D. PIC polymer flakes before dissolving in a cell culture medium. E. A PIC hydrogel in cell culture insert.

ment was observed. The number of cells that did not attach to plastic after 4 h did not exceed 2% of the number of seeded cells.

3.3. Transplantation of PIC hydrogel-based pre-tissues

To transplant cellularized PIC hydrogels we employed poly (dimethylsiloxane) (PDMS) molds . These small devices were designed to be flexible and to have a small well for a gel with one face open to enable cells interactions with the surrounding tissue (Fig. 5A). We used them to first culture cells and then transplant subcutaneously hydrogels with molds into Nu/Nu immunocompromised rats.

Two types of cells were submerged in the hydrogels, namely dermal fibroblast in one set of experiments and melanoma cells in a second one. All hydrogels were prepared using optimal PIC conditions (2.0 mg/ml, PIC:GRGDS 1:30. Cell proliferation was not negatively influenced by culturing in molds (Fig. 5B). As soon as cells colonized the volume of a hydrogel in 3D, molds were transplanted subcutaneously.

Immunofluorescence images of tissue excised after one or two weeks showed the presence of the hydrogels in both instances. The PIC hydrogels supported by molds served well as a source for cell delivery. Staining for human CD90 molecule present on the surface of fibroblasts allowed to clearly distinguish these cells from the rat tissue (Fig. 5C). To characterize the granulocyte infiltration in transplanted PIC hydrogels, we analysed the expression of the granulocyte marker HIS48 present on basophil, eosinophil, and neutrophil granulocytes (Fig. 5C, D). Seven days after transplantation the majority of granulocytes resided in the interface between the hydrogel and rat tissue (Fig. 5B, C)

Immunofluorescence staining of excised tissue with melanoma samples revealed that most of the melanoma cells remained within the transplanted hydrogel, but some cells migrated out of it and colonized the neighbouring tissue (Fig. 5E, F).

4. Discussion

Hydrogels have become a workhorse for a wide range of cell behaviour and physiology studies, allowing experiments that could not be performed with traditional 2D culture systems [2]. We have proven that after the optimization of the stiffness of the hydrogel and the concentration of the GRGDS peptide, PIC hydrogels are suitable for cell culture. Among a few of the distinct characteristics of the hydrogel, the mechanical properties of the material are the most crucial for robust cell growth. They are not only important for the stability of a construct during the culture period, but, even more importantly, also for an appropriate cell mechanotransduction, which translates directly to cell migration, spreading, and differentiation [1,2,18].

Another characteristic of the PIC hydrogels directly related to their concentration is the property of strain-stiffening. Many natural extracellular matrices become stiffer when deformed, what is defined as an increase in a material's elastic modulus with applied strain [19]. Understanding of the various physiological processes suggest a high relevance of this mechanism for cell responses, differentiation and proliferation, but also a mean to prevent damage from exposure to large deformations [19–21]. Although, the strain-stiffening response is of a primary importance when designing artificial biomaterials, this characteristic is missing in most of the synthetic hydrogels. Therefore, the PIC polymer is the first synthetic biomaterial fully resembling responses of natural filaments.

Our results on the optimization of PIC concentration, which translates directly into the stiffness of the material, showed how small changes in concentration may induce dramatic changes in the behaviour of cells. It has been shown that tailoring the stiffness of a material may drive the differentiation of stem cells into a desired direction [8,22].

In the same way as the vast majority of synthetic hydrogels the PIC hydrogel has to be functionalized to enable cell attachment and interactions. To facilitate cell adhesion, we functionalized the PIC polymer with integrin GRGDS motifs, which have become a golden standard in the field. It has been long known that not only the presence but also the concentration of the motifs is crucial for cell growth [23,24]. We observed the same behaviour in our experiments when we fine-tuned the amount of the GRGDS peptide on the backbone of the polymer. Higher concentrations of the peptide allowed cells to rapidly adopt physiological morphology.

The exact mechanism through which PIC and GRGDS concentrations influence cell behaviour needs to studied in detail. At the moment we speculate that higher concentrations of the polymer inhibit physiological behaviours such as spindle-shape morphology, stretching and migration. It is plausible to think that with increasing concentration at some point the stiffness of the hydrogel becomes so high that cells are unable to move in the polymer's network. We also believe that higher concentration of the GRGDS peptide facilitate interactions of cells with the polymer easier, as they can attach to a higher number of ligands.

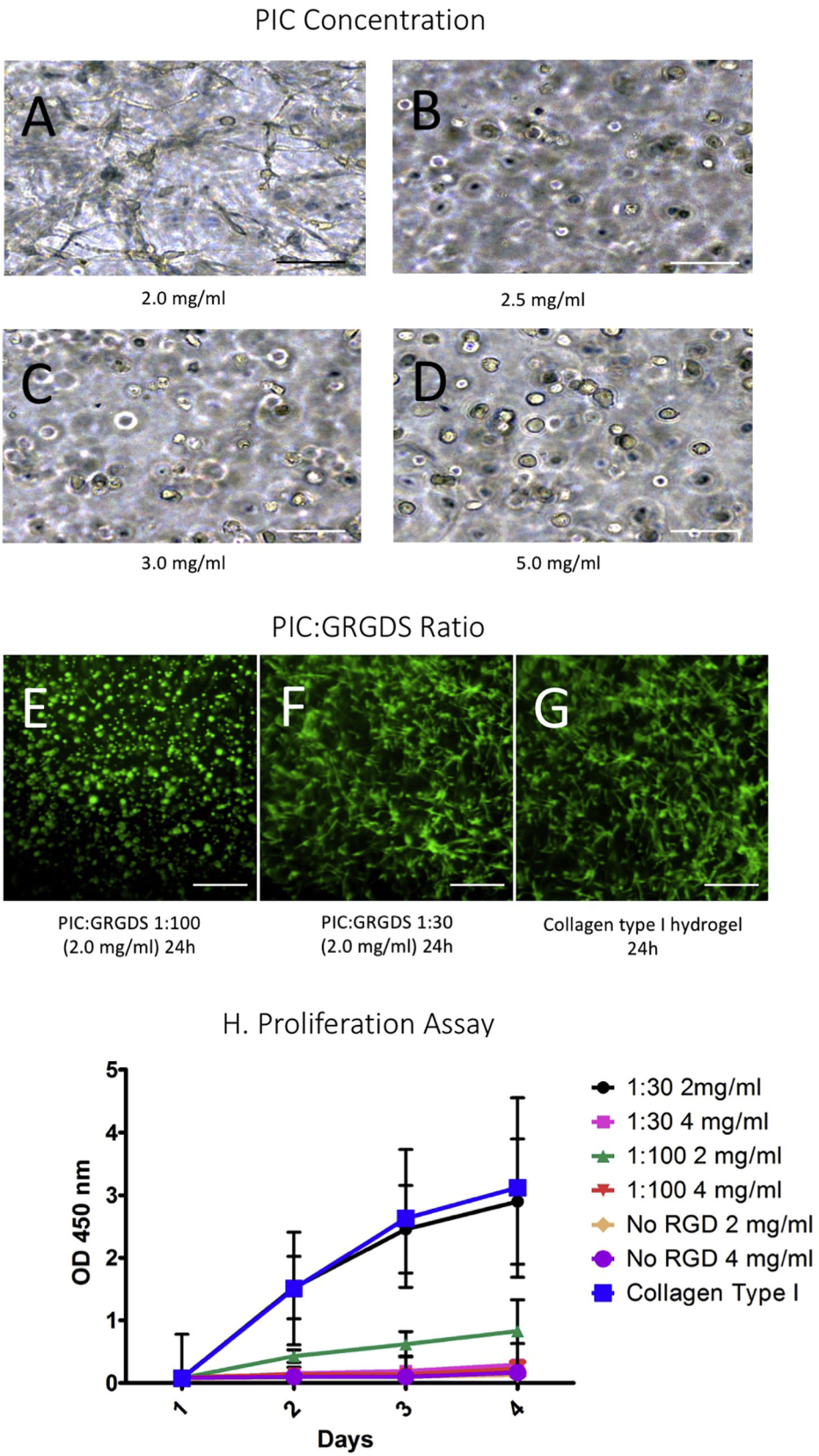

Fig. 2. Optimization of PIC concentration and functionalization for cell culture. A–D. Human dermal fibroblasts cultured for 48 h in 4 different PIC hydrogel concentrations as seen in bright field microscopy (scale bar = 100 μm) E and F. Human dermal fibroblasts were cultured for 24 h in optimal 2.0 mg/ml PIC polymer hydrogel with various concentrations of GRGDS ligands conjugated with polymer's backbone. FDA staining. G. Representative fibroblast culture in collagen type I hydrogel. FDA staining (scale bar = 200 μm). H. Optimal features of PIC polymer hydrogel for cell culture were confirmed in proliferation assay. Human dermal fibroblasts were cultured for 4 days and the assay was performed every 24 h. Results are presented as mean ± SD (Collagen Type I vs 1:30 2 mg/ml, Collagen Type I vs 1:100 2 mg/ml $p > 0.05$; Collagen Type I vs 1:30 4 mg/ml, Collagen Type I vs 1:100 4 mg/ml, Collagen Type I vs No RGD 2 mg/ml, Collagen Type I vs No RGD 4 mg/ml $p < 0.05$).

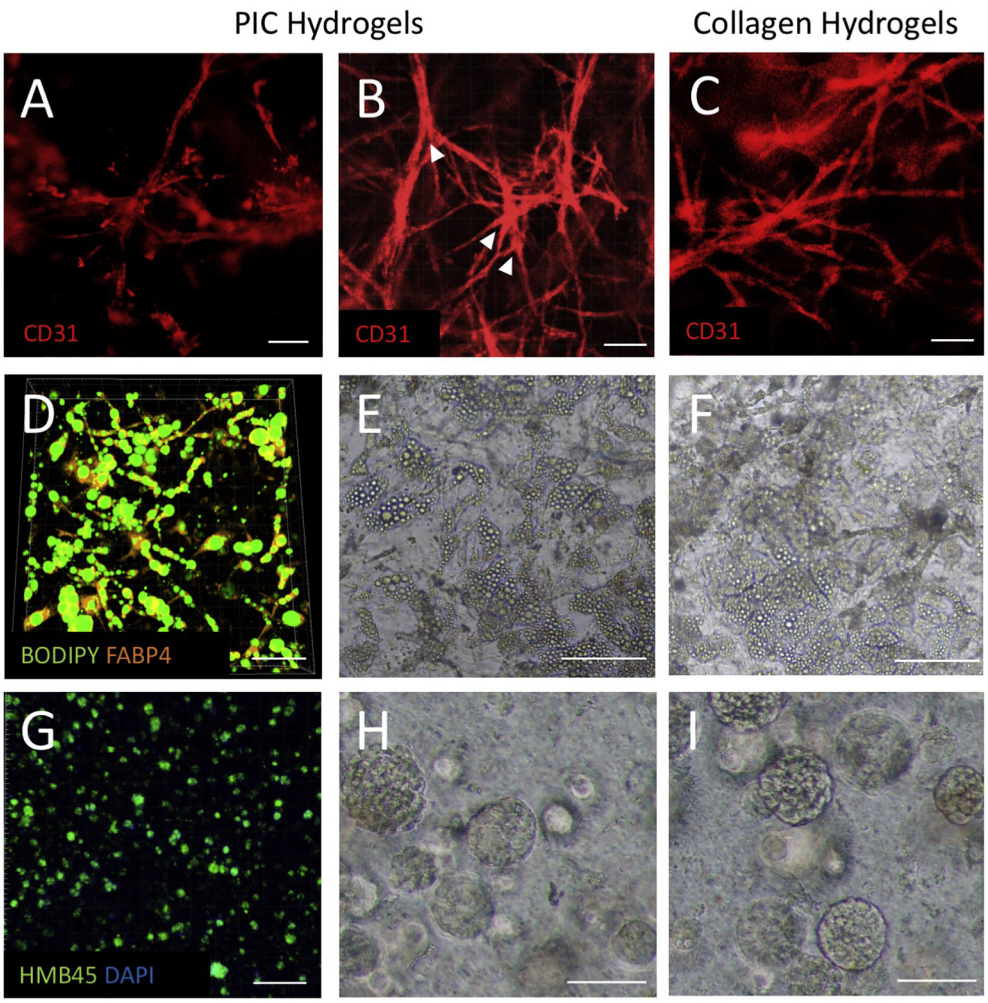

Fig. 3. Suitability of PIC functionalized polymer hydrogels for growth and differentiation of various cell types. A. Capillary network in PIC polymer hydrogel spontaneously formed after 10 days by 1:1 mixture of HDMECs and fibroblasts (scale bar = 50 μm). B. Exemplary branching points of HDMECs capillary network after 3 weeks in PIC hydrogel was marked with arrows (scale bar = 300 μm). C. Exemplary blood capillaries network in collaged type I hydrogel after 3 weeks of culture. D. Adipocytes differentiated from ADSCs in PIC hydrogel with lipid depots stained with BODIPY dye and cell membrane stained with FABP4 adipocyte marker (scale bar = 300 μm). E. Differentiated adipocytes in PIC hydrogel as seen in bright field microscope (scale bar = 50 μm). F. Differentiated adipocytes in collagen type I hydrogel as seen in bright field microscope (scale bar = 50 μm). G. Spontaneously formed melanoma cell spheres stained for HMB45 marker in PIC polymer hydrogel (scale bar = 200 μm). H. Melanoma cell spheres as visible in bright field microscope in PIC hydrogel and I. in collagen type I hydrogel (scale bars = 100 μm).

The flexibility of the PIC functionalization gives an interesting outlook for the future. One could imagine that studies involving differences in GRGDS concentrations or even functionalization with mixtures of various mimetic peptides could increase our understanding of ligand density on biological processes. High tunability of the PIC hydrogels may allow the creation of synthetic environments that closely mimic the natural niche [25,26]. The most important factors determining stem cell fate is cell-cell interaction and cell interaction with the surrounding microenvironment [27]. The growing number of clinically applied cell therapies alone demands exceptional biomaterials for *in vitro* studies to better understand the fate of the stem cells after transplantation into a patient [28,29]. Bio-engineered synthetic and fully controllable hydrogels are more likely to provide cells with rational cues for diagnostic and therapeutic studies [30].

Because of the novelty of the PIC hydrogels and the various possible ways of their utilization we decided to test some of these utilizations, including adipogenic differentiation and spontaneous blood capillary formation.

The perception of adipose tissue changed dramatically in the recent years: from a an energy reservoir and insulation it transformed into a source of stem cells and paracrine signals [31]. ASCs potential was extensively studied in plastic and reconstructive surgery, where their employment has resulted in exceptionally good outcomes [32–34]. Thus, understanding of the adipogenic differentiation, but also the interactions of adipocytes with other cell types and tissues has become an important area in regenerative medicine [35,36]. It has already been shown that PIC hydrogels can facilitate stem cells differentiation into adipocytes [8]. Our observations confirmed these results and proved that adipogenic differentiation of human ASCs into adipocyte is robust in the soft network of the PIC polymer.

In contrast to adipogenic differentiation, spontaneous blood capillary formation involves simultaneous interactions of multiple cell types. Vascularization is one of the most important obstacles when engineering thick tissue constructs as they are unable to survive after *in vivo* implantation without sufficient oxygen and nutrients supply [37]. However, the migration and proliferation of endothelial cells may be influenced by distinct biological parameters of the scaffold such as stiffness, concentration or pore size [38,39]. The minimum porosity necessary for the growth of a blood vessel and to enable the metabolic exchange and endothelial cell

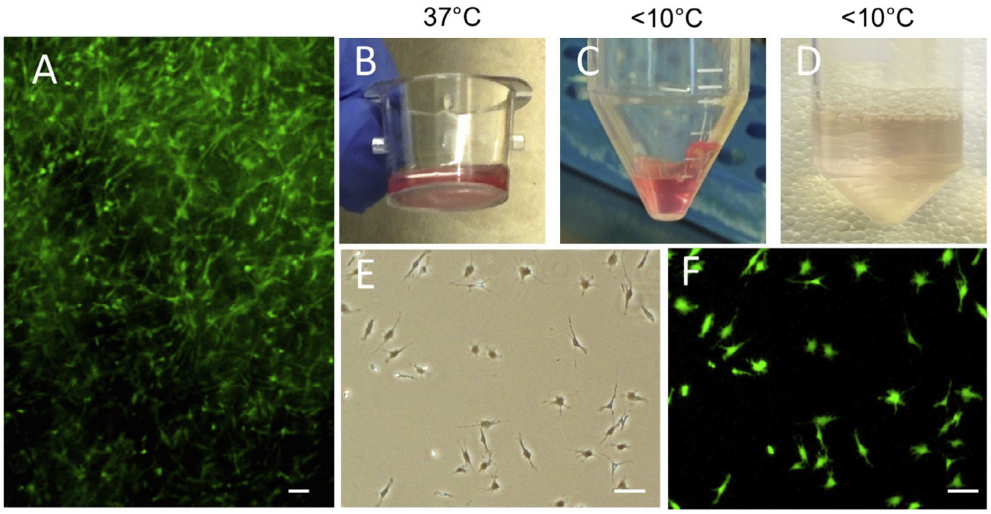

Fig. 4. Recovery of viable cells from PIC hydrogels. A. Human dermal fibroblasts cultured for 5 days in PIC hydrogel (2.0 mg/ml, PIC:GRGDS 1:30); FDA staining (scale bar = 100 μm). B. Insert in which PIC hydrogel was kept in culture with cells for 5 days. C. The hydrogel was cooled down causing its transition to liquid state. D. For centrifugation the solution of PIC and cells was further diluted with cooled PBS. E. Recovered fibroblast 5 h after plating on cell culture plastic (scale bar = 100 μm). F. FDA staining of recovered fibroblasts.

infiltration is approximately 30–40 μm [40,41]. Therefore, we tested the suitability of PIC hydrogels to support the development of a complex microvascular network in vitro via the incorporation of human dermal microvascular endothelial cells (HDMEC) with supporting human dermal fibroblasts. Interestingly, the cells developed spontaneously into a dense capillary plexus when integrated at a ratio of 1:1 into PIC hydrogels. These results confirm that PIC hydrogels with pore diameters of 100–150 μm provide a stimulating 3D microenvironment for microvascular growth and can be used as a vascularized template for various tissue-engineered organs [7]. To our best knowledge this is the first time that vascularization was observed in synthetic thermo-responsive hydrogels.

Thanks to its adjustable properties, PIC hydrogels present a valuable model for clinical application, including cancer research and drug screening. Cell cultures in 2D were used to investigate the biology of cancer cells and their responses to anti-cancer drugs. However, they often have little value in predicting the clinical efficacy of anti-cancer drugs for clinical therapies [42].

Tumour cells grown in 3D display physiologically relevant cell-cell and cell-extracellular matrix interactions, resulting in a gene expression that is more similar to that *in vivo* tumours [43]. Such characteristics make 3D systems physiologically relevant models to study tumour dynamics and responses to anti-cancer drugs for therapies [44,45]. As the properties of the PIC hydrogels can be tuned, they can be designed to mimic stiffness and other mechanical characteristics of tumours in order to understand the impact of these properties on tumour invasiveness and metastatic potential.

Whereas harvesting cells from 2D cultures does not present any problem, isolation of cells grown inside hydrogels usually becomes a technical challenge. Widely used approaches of cell isolation from hydrogels include enzymatic digestion and mechanical disruption of a hydrogel. However these approaches can damage cells and change their gene expression profiles [3]. In great contrast to the methods just described, the isolation of cells from PIC hydrogels is rapid and harmless for the cells. The whole procedure of liberating cells consumes the same amount of time as to isolate cells from a monolayer and generates high yields too. What is more, the cells can be liberated without the use of any chemical or biological agents, just with a small decrease of the temperature. This characteristic is extremely advantageous for genomic and proteomic studies where quick recovery of unaltered cells is crucial for valid results. Additionally, during this procedure the polymer is virtually removed from the solution due to dilutions during cooling down the gel, centrifugation and finally seeding and culturing cells. Thus it seems not to influence cell attachment after recovery.

The PIC hydrogels belong to the category of soft hydrogels and are fragile, making that it is hard to handle them outside culture vessels and to apply them *in vivo*. This was overcome by the utilization of molds made of PDMS, a material that is biocompatible and widely used in *in vivo* studies [46,47]. To our best knowledge this is the first case where PIC hydrogels are transplanted and used *in vivo*, and afterwards characterised. Our observations of migration of melanoma cells out of a hydrogel into the rat tissue strongly suggest that cells delivered in PIC hydrogels can freely interact with surrounding tissues. This observation was additionally supported by the influx of rat granulocytes into hydrogels at the border between the construct and animal tissue. Mild infiltration of the hydrogels by granulocytes supports their usefulness for animal studies. Materials characterized by a high influx of granulocytes and by granuloma formation are considered not to be suitable for *in vivo* applications [48,49]. The fact that cellular PIC hydrogels survive after transplantation and may interact with recipient tissue make them promising systems for *in vivo* studies.

However, we have not observed any cytotoxic effects of PIC polymers on cells, it is crucial to perform detailed study on the polymers' effects on cell viability. We are aware of one parallel study focused on this subject. Most importantly, the data included in the manuscript in preparation confirm our observations about lack of cytotoxicity of PIC polymers. The possibility of *in vivo* transplantations of PIC poses also an interesting question on pathways contributing to PIC degradation in a living organism. Strain stiffening is the most interesting perspective for the future studies involving PIC hydrogels. PIC hydrogels offer a platform for studying how mechanical stimuli and gel composition influence cell proliferation and which parameters are crucial for the best cell growth. Further, as shown previously this feature of PIC polymers can influence the path of stem cell differentiation [8]. In the work of Das et al. altering the strain stiffening, while keeping all other conditions constant, was the sufficient trigger to govern the fate of MSCs towards either osteogenesis or adipogenesis. However, as the exact mechanism of this phenomenon is not yet fully understood, further

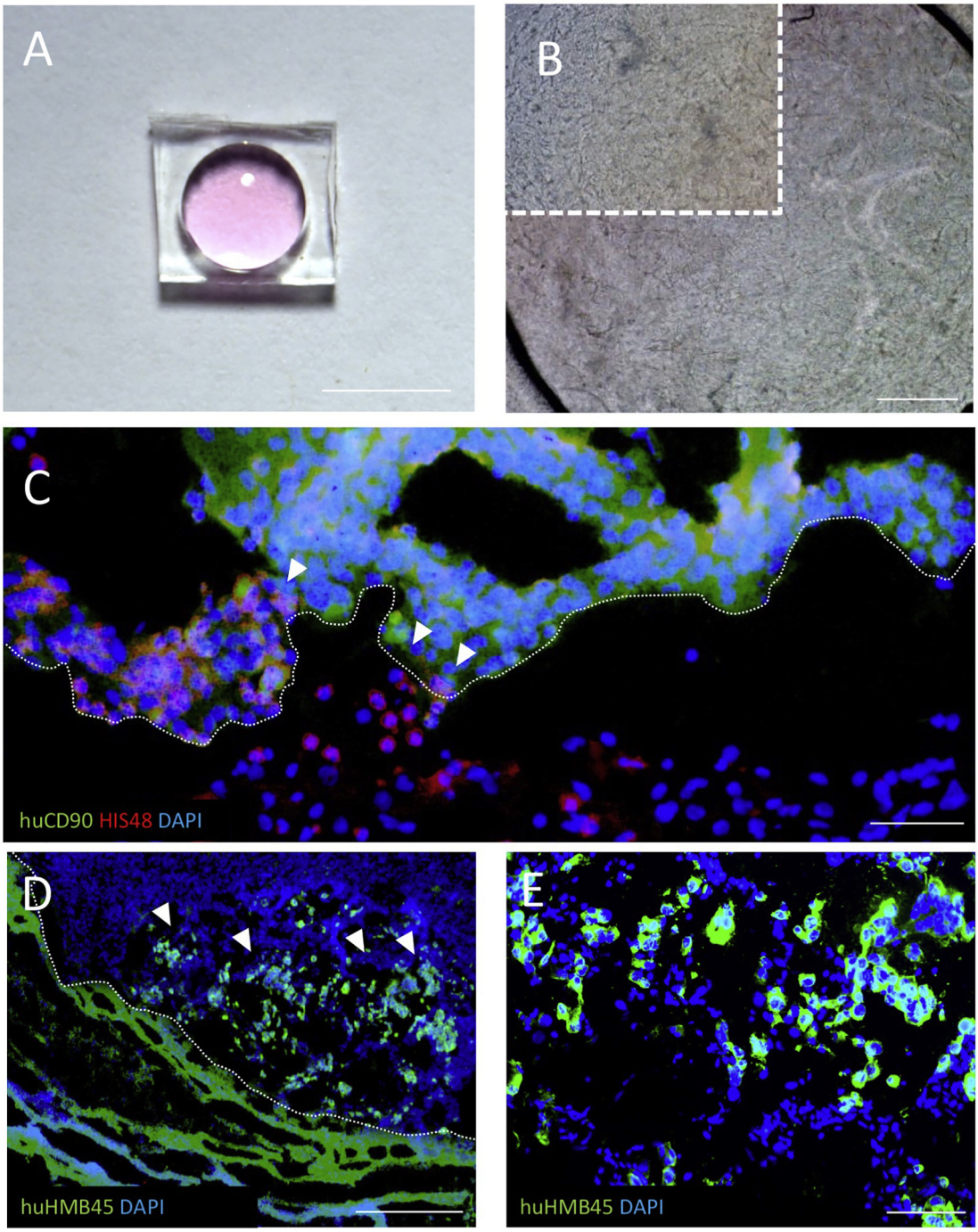

Fig. 5. *In vivo* transplantations of PIC polymer hydrogels. A. Mold used for subcutaneous transplantation filled with PIC hydrogel with cells. (Scale bar = 0.5 cm) B. Low and high magnification of human dermal fibroblasts growing in a well of the mold 4 days after seeding. (Scale bar = 500 μm) C. Cryo-section of rat tissue with implanted PIC hydrogel extracted 1 weeks after transplantation. Fibroblasts of human origin are stained with human specific anti-CD90 antibody and rat granulocytes are stained with HIS48 antibody. Dotted line marks the boarder of the hydrogel (scale bar = 20 μm). Rat granulocytes (marked with arrows) are observed only at the interface between rat tissue and the hydrogel. D. Cryo-section of tissue with transplanted PIC hydrogel with human melanoma cells, which were stained for HMB45. Cells that migrated out of the hydrogel were marked with arrows, whereas hydrogel is marked with dotted line. (Scale bar = 500 μm) E. Region with melanoma cells that migrated out in higher magnification. (Scale bar = 100 μm).

studies with PIC polymers may enrich understanding of niche sensing by the cells and further mechanisms governing stem cell fate.

5. Conclusions

We optimized novel synthetic, thermo-responsive and stress-stiffening polyisocyanopeptide (PIC) hydrogels for the use in organotypic 3D cell culture. We focused on the most suitable values of the two most crucial properties of PIC hydrogels: (1) the polymer concentration in a hydrogel and (2) the concentration of the GRGDS peptide conjugated to the polymer backbone. This fine-tuning of the PIC hydrogels enabled us to confirm that it supports complex processes including adipogenic differentiation and formation of blood capillaries. What is more we also showed that PIC hydrogels in molds can be subcutaneously transplanted.

Finally, we utilized the thermo-responsive properties of PIC hydrogels to show that they enable quick and easy retraction of viable cells from the gels, eliminating usage of enzymes or time-consuming procedures and digestion.

Taken together, we showed that PIC hydrogels, with their mimicking properties, versatility, tenability, and biocompatibility they are interesting candidates to be used for a broad spectrum of studies.

6. Author's contribution

JZ and JSP performed most of the experiments and analysis, JSP synthetized the PIC polymer, AK performed experiments with vasculogenesis, TB performed experiments with melanoma cells, QVM designed and produced PDMS molds, JZ, JSP, AK, TB, CJW, AR and ER wrote a manuscript, CJW, AR, ER designed and supervised the experiments.

7. Conflict of interests

Christopher J. Wilson and Alan E. Rowan hold shares in NovioTech BV, and own patent rights for the PIC gel and GRGDS conjugates (EP3021872B1).

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