A Temperature-Based Easy-Separable (TempEasy) 3D Hydrogel Coculture System

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1. Introduction

Tissues in our body are complex 3D structures and consist of multiple cell types within a dedicated extracellular matrix (ECM). Communications between different cells through soluble signaling molecules,[1,2] physical cell–cell and cell–ECM interactions[3–6] induce either positive or negative regulations, which influence cellular processes, such as proliferation and differentiation.[4,7–8] Cell coculture systems have been developed to better study the interactions between different cell populations.[9] In contrast to conventional monoculture systems, coculture systems may develop into superior culture platforms,[10–12] because they can mimic natural occurring interactions of cells with surrounding cells and with the matrix, which are both important in many fields, ranging from tissue engineering[13–15] to cancer research.[16–18]

Coculture approaches are classified into direct and indirect systems: in direct coculture systems, cells are mixed, without the ability to steer cell–cell interactions. Subsequent analysis of one specific cell type requires cell sorting. In indirect coculture systems, which are often performed in a Transwell setup, different cell types are physically separated and cultured without direct cell–cell interactions.[9,19] The latter models rely on cellular communication via soluble factors only, however, their main advantage is that different cell types can be easily separated from the coculture and quantitively assessed.[20–23]

In general, cocultures are often performed in 2D, however, tissues are inherently 3D and contain several different topographical cues that cells can sense and respond to.[24] To better recapitulate cellular interactions in an in vitro system, the effect of the ECM needs to be considered as it plays a pivotal role in providing cell support and maintenance of the cellular microenvironment, including growth factor release.[10,25–26] In recent years, great efforts have been made to develop 3D coculture materials based on electrospun fibers,[27–28] microfluidics,[29–31] and hydrogels.[32–34] Electrospun fibers combine controllable mechanical properties with a straightforward production method at low cost.[35–36] When used in cocultures, electrospun fibers provide a layered scaffold where each cell type grows on a layer,[27] giving rise to a sandwich structure. Microfluidics-based approaches give access to cost-effective, integrated, high-throughput cell culture systems with high controllability that are often studied to “organ-on-a-chip” applications.[37–38] Within the microchannels or compartments, different cells may be seeded in 2D or 3D cultures.[17,39] Because of their similarity on architecture and mechanical properties with ECM, hydrogels are widely used in
biomedical research, and they form a promising class of coculture matrix.\textsuperscript{[9,40]}

Despite significant progress in the field, it remains challenging to develop versatile and readily usable coculture systems. Electrospinning and microfluidic-based approaches require dedicated equipment and specific knowledge that many biology labs lack.\textsuperscript{[23,27,30,41]} Similarly, many labs lack the expertise to optimize the architecture and mechanical properties of hydrogels.\textsuperscript{[13–34]} which is necessary as different cell types require different microenvironments.\textsuperscript{[42–41]} A clear need remains for a user-friendly, well-controlled 3D coculture system that closely mimics the native ECM and allows tailoring to accommodate a wide variety of cell types.

In 2013, a novel hydrogel based on an oligo(ethylene glycol)-decorated polyisocyanide (PIC) was introduced,\textsuperscript{[44–45]} which has been developed into an artificial ECM that mimics the fibrous structure and the mechanical properties of biopolymer gels such as collagen and fibrin.\textsuperscript{[46]} PIC gels have been applied in a variety of biomedical applications in vivo\textsuperscript{[47–49] and in vitro,\textsuperscript{[50–54]}} A major advantage of PIC hydrogels is the versatility of the material: mechanical properties and decoration with bioactive components are readily customized. In addition, PIC gels are thermoresponsive,\textsuperscript{[55]} i.e., heating an aqueous PIC solution above the gelation temperature results in the formation of a gel; cooling returns the solution, which simplifies cell extraction enormously.

In this study, we developed PIC hydrogels into a Temperature-based Easy-separable (TempEasy) 3D cell coculture system. It is composed of two layers of PIC gel with a different gelation temperature ($T_{gel}$). In the layers, different cells are seeded, resulting in an indirect coculture approach. Uniquely, after the desired culture time, the entire coculture system is cooled below $T_{gel}$ of the top layer but above $T_{gel}$ of the bottom layer, allowing easy removal of the top PIC layer and subsequent cell analysis. Further cooling liquefies the bottom layer, which can be analyzed separately.

As a prove of concept and to demonstrate the feasibility and value of the TempEasy system, we studied the behavior of vaginal fibroblasts and human adipose stem cells (hADSCs); the latter is an abundant stem cell source with wide therapeutic applications, especially in repair and regeneration of acute and chronically damaged tissues.\textsuperscript{[56]} Our TempEasy coculture system showed that hADSCs promote cell–cell interactions of fibroblasts. At the same time, we found that fibroblasts enhance both cell proliferation and differentiation of hADSCs. We demonstrate that the TempEasy system has the ability to screen phenotypic and functional effects of cell–cell interactions in a quantitative way and we believe that it may serve as a promising platform for future cell–cell interaction studies.

2. Results

2.1. Setup and Characterization of TempEasy 3D Coculture System

As a synthetic material, the architecture and mechanical properties of PIC gels are readily tailored.\textsuperscript{[57]} Here, we prepared PIC polymers with a small fraction (3.3 mol\%) of azide-functionalized monomer that allow for postfunctionalization, analogous to earlier work.\textsuperscript{[58]} To vary the gelation temperature, two different polymers with different contour length $L_c$ were prepared through polymerizations with different monomercatalyst ratios. The reactions yielded a shorter polymer S-PIC-N$_3$ (contour length $L_c$ = 99 nm and viscosity average molecular weight $M_v = 251$ kg mol$^{-1}$) and a longer polymer L-PIC-N$_3$ ($L_c = 222$ nm, $M_v = 562$ kg mol$^{-1}$), as determined by viscometry measurements\textsuperscript{[58]} (see details in Supporting Information). To promote cell–gel interactions, the generic cell adhesive peptide GRGDS equipped with a DBCO-terminated PEG spacer was conjugated to azide-appended polymers, resulting polymer S-PIC and L-PIC with a coverage of $\approx 3.3\%$ of peptide on every monomer\textsuperscript{[45,54,59]} (Figure 1A).

To prepare hydrogels, both the short polymer S-PIC and long polymer L-PIC were overnight dissolved in Dulbecco’s modified eagle medium (DMEM) medium (3 mg mL$^{-1}$) at 4 °C. Oscillatory shear rheology was used to measure mechanical properties; S-PIC and L-PIC showed a different gelation temperature $T_{gel}$, characterized by a steep increase in the shear modulus (Figure 1B). Based on the heating ramp, $T_{gel}$ of S-PIC is approximately 30 °C, while $T_{gel}$ of L-PIC is 20 °C (defined as the onset of the increase in the modulus). From the cooling ramp, we observe some hysteresis and find that S-PIC and L-PIC exhibit gel-to-sol transitions at 17 and 7 °C, respectively. We note that the shear modulus at 37 °C of the L-PIC gels is higher than that of the S-PIC gel at the same concentrations. Tuning the concentration is an orthogonal strategy to tailor the PIC hydrogel stiffness that does not affect the gelation temperature.\textsuperscript{[57]} Based the difference in gelation temperatures, it is possible to separate S-PIC and L-PIC solutions by tuning the temperature (Figure 1C). Both S-PIC and L-PIC are elastic gels at 37 °C. By lowering the temperature from 37 to 15 °C, L-PIC remains in the gel state, while S-PIC returns to a low viscous solution, which can be removed selectively. Further cooling to 4 °C enabled harvesting of the L-PIC compartment. PIC gels show a fibrous hierarchical architecture with characteristic pore length scales in the micrometer\textsuperscript{[60]} and in the tens of nanometer\textsuperscript{[61]} regime, which ensures high diffusion rates for most biochemical cues. Earlier work has demonstrated that cytotoxicity of PIC gels is very low and independent of the molecular weight of the constituting polymers, as based on earlier Live-Dead assays.\textsuperscript{[51,58]} Additionally, we note that for the cell culture experiments the gel-solution transition is relatively fast; it only takes 1–2 min to remove the top layer from the culture and process it further. In summary, we use the difference in $T_{gel}$, induced by the difference in molecular weight of the polymers as the basis of our TempEasy 3D cell coculture system.

2.2. Fibroblasts Cell Morphology and Gene Expression in Monocultures

Human fibroblasts in standard 2D monolayer culture conditions revealed a well-known spread-out spindle-shaped morphology; locally, some cells aligned in parallel clusters. In the S-PIC 3D hydrogel culture, the majority of fibroblasts showed a different morphology, exhibiting long protrusions (Figure 2A; Figure S3, Supporting Information). In the absence of crowding, the cells in the 3D hydrogel freely stretched out without any clear orientation. Fibroblasts cultured in the L-PIC hydrogel showed a similar morphology with fibroblasts cultured in S-PIC hydrogel, however, the cell body appeared somewhat smaller and branches were
Figure 1. Structure and the mechanical properties of the PIC hydrogels. A) Molecular structure of the PIC polymer backbone in blue, the linking group in orange and the GRGDS peptide in green. B) Shear modulus $G'$ of L-PIC (gray) and S-PIC (pink) hydrogels as a function of the temperature (both at a concentration of 1.5 mg mL$^{-1}$). Heating and cooling rates 2 °C min$^{-1}$. C–F) Photos to illustrate thermoreversibility of the PIC gels with different gelation temperatures. S-PIC was dissolved in medium (pink) and L-PIC was dissolved in an iron oxide solution (black) to highlight the different layers, both in a concentration of 1.5 mg mL$^{-1}$. At room temperature L-PIC forms a hydrogel C). After placing S-PIC on top and heating to 37 °C, a double-layer gel is formed D). Cooling to 15 °C allows selective removal of the S-PIC layer E). With further cooling, the L-PIC layers is also removed F). Note that there is no visible contamination of L-PIC in the S-PIC vial.

Figure 2. Cell morphology and gene expressions of fibroblasts in 2D and 3D cultures. A) Representative bright field images of fibroblasts after 7 days in culture: spindle-like in 2D and branched morphologies in both S-PIC and L-PIC. Concentrations, cell densities and other conditions are given in the Experimental Section. B) Gene expression of FSP1 and CDKN1A was quantified using RT-qPCR. Data is normalized to the human acidic ribosomal protein (hARP) housekeeping gene. Differences in the relative expression were calculated by the $2^{\Delta\Delta Ct}$ method.[62] Sequences of qPCR primers were provided in Table 1. Data are shown as mean ± standard deviation (SD) of $n=5$ biological replicates. Statistics: n.s. = not significant ($p > 0.05$), by unpaired t-test.
Figure 3. Cell morphology and gene expressions of human adipose-derived stem cells in 2D and 3D cultures. A) Representative bright field images of human hADSCs after 7 days in culture: long shuttle-shaped cells in 2D and branched morphologies in both 3D cultures. Concentrations, cell densities and other conditions are given in the Experimental Section. B, C) Gene expression of CDKN1A and SOX2 B) and of several markers for intracellular junctions and attachment CD34, CDH5, VWF, and PECAM1 C) was quantified using RT-qPCR. Data is normalized to the human acidic ribosomal protein (hARP) housekeeping gene. Differences in relative expression were calculated by the 2ΔΔCt method. [62] Sequences of qPCR primers are provided in Table 1. Data are shown as mean ± SD, biological replicates n = 5. Statistics: n.s. = not significant (p > 0.05), *p < 0.05, by unpaired t-test.

shorter (Figure 2A). The difference in fibroblast morphology in S-PIC and L-PIC hydrogels may result from the difference in mechanical properties of the two matrices, analogous to what was observed in stem cell studies in PIC gels. [58]

Despite the clear difference in cell morphologies of the fibroblasts cultured in 2D and 3D S-PIC or L-PIC hydrogels, we studied the similarity of the cells by quantifying gene expression. We measured expression of FSP1, encoding for Fibroblast-Specific Protein 1, which is a reliable marker for detecting tissue-resident fibroblasts. [63] In addition, we tested expression of CDKN1A, encoding for p21, which is a member from the Cip/Kip family of cyclin-dependent kinase inhibitors and functions as a regulator of cell cycle progression at G1. [64] P21 is the key inhibitor of the cyclin E-Cdk2 complex, and, therefore, is widely used as a cell cycle marker. We found no significant differences between any of the cell culture conditions (Figure 2B). Similar expression levels of FSP1 indicate that both gels are appropriate cell culture media for the fibroblasts, despite the small difference in mechanical properties of the gels. The expression of CDKN1A is slightly lower in the L-PIC hydrogels, in comparison to 2D culture, indicating a higher cell proliferation and implying that the L-PIC hydrogel may provide better microenvironment than the S-PIC-based gel for human fibroblasts, although the differences are statistically not significant. Note that we also quantified expression of other genes that are introduced later in this work (Figure S1, Supporting Information); in line with FSP1 and CDKN1A, they do not show significant differences in expression levels between the different culture conditions. We highlight that in our matrices, cell behavior does depend on culture dimensionality as was previously found, [55-57] but that in terms of gene expression, the different cells show a largely similar response irrespective of their 2D or 3D environment.

2.3. Human Adipose-Derived Stem Cell Morphology and Gene Expression in Monocultures

Then, we explored if the difference in cell culture conditions affect cell behavior of the second cell line in our study, the human adipose stem cells (hADSCs). Earlier work on the stem cells in PIC matrices has shown that matrix properties will greatly affect cell morphologies and secretion of biological factors. [53,58] In 2D, hADSCs exhibited uniform long-shuttle shapes (Figure 3A; Figure S4, Supporting Information). In the 3D S-PIC hydrogel...
culture, the majority of hADSCs showed a highly branched morphology, while in L-PIC, most hADSCs are stretched and elongated. Notably, in both 3D cultures multiple cell–cell contacts are generated.

We assessed the effect of the different culture conditions at the molecular level through qPCR analyses (Figure 3B,C). Similar to the fibroblasts, expression of CDKN1A is lowest for the L-PIC hydrogels but the observed differences are statistically not significant (Figure 3B). In addition, we tested expression of the stem cell marker SOX2 (encoding for SRY-Box Transcription Factor 2). The similar SOX2 expression levels suggest that in all culture conditions stemness of the cells is maintained. The results suggest that, after 7 days in a 3D culture, hADSCs show a proliferation and stemness profile that largely agrees with the traditional 2D culture.

Prompted by the clear morphological changes, especially the increased cell interactions of hADSCs in the 3D culture, we analyzed genes that are related to intracellular junctions and attachment (Figure 3C). Expression of CD34, a surface glycoprotein that functions as a cell–cell adhesion factor, did not show any significant difference between different culture conditions. Expression of CDH5, encoding for VE-Cadherin, another glycoprotein that is involved in cell–cell adhesion and intracellular signal transduction as well as vascular remodeling showed small but significant differences between the S-PIC and L-PIC cultures. The reduced expression in L-PIC hydrogel suggests a lower cell adhesion capacity in the L-PIC hydrogel, which could be the result of a reduced cell migration due to the increased hydrogel stiffness, which is virtually the only difference between the two matrices. We also tested von Willebrand factor (VWF), a multimeric plasma glycoprotein that mediates platelet adhesion as well as leukocyte adhesion, but we did not observe any clear differences in its gene expression. Finally, we tested PECAM1, encoding for Platelet Endothelial Cell Adhesion Molecule 1 or CD31, which plays an important role in regulating intercellular junctions of endothelial cells and, therefore, endothelial barrier functions. PECAM1 is expressed in much higher levels (five-fold) in both 3D cultures, suggesting enhanced intercellular junction formation between hADSCs in 3D, which is in line with the brightfield morphology images (Figure 3A).

### 2.4. Cell Morphologies and Gene Expression of Fibroblasts in the TempEasy Fibroblast/hADSC 3D Coculture

After individual assessment of fibroblasts and hADSC behavior in S-PIC and L-PIC, we assembled the TempEasy 3D coculture system and study their behavior together. As both layers have different mechanical properties, we distinguish two cases, one with the fibroblast in the (softer) S-PIC top layer and one where they are seeded in the stiffer L-PIC bottom layer (Figure 4A). The stem cells are then seeded in the other, complementary layer. As controls, fibroblasts were exclusively cultured either in the upper S-PIC hydrogel or in the lower L-PIC hydrogel in the presence of empty (i.e., without cells) complementary layers. Note that these controls are expected to closely relate to the monoculture experiments discussed above.

With regard to their morphology, we observe that the fibroblasts displayed a more elongated cell morphology in 3D coculture with hADSCs, when compared to 3D monoculture (Figure 4B; Figure S5, Supporting Information). This more elongated and branchy cell morphology was observed in both cases where fibroblasts were seeded in the S-PIC and in the L-PIC hydrogel.

We then explored gene expression levels to study the influence of the presence of the stem cells, exploring the origins of the morphological changes (Figure 4C). Expression of FSP1, a fibroblast marker, was significantly higher for fibroblasts cultured in the L-PIC hydrogel coculture than in the corresponding monoculture. A similar difference was observed in the S-PIC layers, albeit statistically not significant, suggesting that the coculture with stem cells does not change the cell identity of the fibroblasts. Evaluation of CDKN1A expression did not show clear differences between the S-PIC and L-PIC cocultures and their corresponding monocultures, suggesting that the coculture with hADSCs did not affect fibroblast proliferation.

We also tested expression of the intracellular junction and attachment markers CD34, CDH5, VWF, and PECAM1 (Figure 4C). Expression of CDH5 was barely detectable and therefore excluded for further analyses. For both CD34 and VWF, we did not observe significant differences in gene expression, i.e., no effect of the presences of hADSCs in close vicinity. We did, however, observe a strong increase in the gene expression level of PECAM1 in the cocultures with fibroblasts either in the S-PIC or in the L-PIC layers and the stem cells in the other TempEasy compartment, as compared to the corresponding monoculture controls. This difference, as confirmed in five independent experiments, suggests that enhanced intercellular junctions are formed between fibroblasts when they are cocultured with hADSCs, which is in line with the observed morphological changes (Figure 4B). Taken together, coculturing with hADSCs seems to promote cell–cell interactions between fibroblasts, which may help to maintain the cell identity of fibroblasts as shown by higher FSP1 expression.

### 2.5. Cell Morphologies and Gene Expression of hADSCs in the TempEasy Fibroblast/hADSC 3D Coculture

Vice versa, we also observed clear differences in the hADSC morphology and gene expression when they are cocultured with fibroblasts (Figure 5). The experimental TempEasy setup is the same but now we study the stem cell behavior with the corresponding controls (Figure 5A).

In bright field microscopy experiments, we noticed only minor changes in hADSC cell morphology: the nucleus is less visible when cocultured with fibroblasts (Figure 5B; Figure S6, Supporting Information) in comparison to the 3D monoculture (Figure 3A). Overall, however, the hADSCs display a stretched morphology with branches in both S-PIC hydrogel and L-PIC hydrogel with or without fibroblasts present.

Despite the absence of obvious morphological variation, we nevertheless explored potential changes in gene expression of hADSCs induced by the fibroblast cocultured (Figure 5C). Analogous to the earlier experiment, we first tested the expression of CDKN1A and SOX2 after culturing the stem cells in either matrix for 7 days. A significantly lower gene expression levels of CDKN1A were observed in the TempEasy hADSCs/fibroblast cocultures, both for the stem cells in the soft S-PIC and the stiffer
Figure 4. Cell morphology and gene expression results of fibroblast in a TempEasy fibroblast/hADSC coculture experiment. A) Schematic illustration of the experimental setup of the TempEasy 3D coculture system: fibroblasts were embedded in either S-PIC or L-PIC, with (right, coculture) or without (left, monoculture) hADSCs in the other layer. Note that for the monocultures a layer of the complementary gel without cells was introduced in the wells. B) Representative bright field images of fibroblasts in the hADSC cocultures, 7 days after seeding. C) Gene expression of FSP1 and CDKN1A, and the markers for intracellular junction and attachment CD34, VWF, and PECAM1 as quantified using RT-qPCR after 7 days of culturing in 3D monoculture or coculture. Data is normalized to the human acidic ribosomal protein (hARP) housekeeping gene. Differences in the relative expression were calculated by the 2ΔΔCt method.[62] Sequences of qPCR primers are provided in Table 1. Data are shown as mean ± SD, biological replicates n = 5. Statistics: n.s. = not significant (p > 0.05), *p < 0.05, **p < 0.01, by unpaired t-test.

L-PIC layer. Because CDKN1A is a key inhibitor of cell cycle progression, this result suggests that a coculture with fibroblasts promotes cell proliferation of hADSCs. As for the stem cell marker SOX2, its expression was reduced in the presence of fibroblasts but only statistically significant with the stem cells in the top S-PIC layer (and the fibroblasts in the L-PIC gel). These results suggested that the coculture with fibroblasts not only promote proliferation but also differentiation of hADSCs.

For the intracellular junction and attachment markers CD34, CDH5, and VWF, we earlier observed very few differences in expression levels for different 2D or 3D cell culture conditions (Figure 3C). In the fibroblast cocultures, however, the expression changes (Figure 5C). The expression of CD34 significantly decreased in the coculture with hADSCs were cultured in the soft S-PIC hydrogel, compared to the monoculture. This decrease that is not observed with the stem cells in the stiffer bottom layer, is unlikely associated with endothelial differentiation, supported by gene expression levels of endothelial markers, CDH5 and VWF, both of which showed a reduced gene expression level in the cultures with fibroblasts. Taken together, we can consider that hADSC cocultures with fibroblast seem to promote both the proliferation and differentiation of hADSCs; however, hADSCs do not seem to differentiate towards an endothelial lineage according to the marker genes that we used so far.

3. Discussion and Conclusions

Cell coculture systems enable studies of cell–cell communication and interactions between different cell types, which are important to guide cell behavior, for instance in tissue engineering approaches and in tissue and organ development. Despite progress in the past several years, an urgent need remains for
Figure 5. Cell morphology and gene expression results of hADSCs in a TempEasy fibroblast/hADSC coculture experiment. A) Schematic illustration of the experimental setup of the TempEasy 3D coculture system: hADSCs were embedded in either S-PIC or L-PIC, with (right, coculture) or without (left, monoculture) fibroblasts in the other layer. Note that for the monocultures a layer of the complementary gel without cells was introduced in the wells. B) Representative bright field images of hADSCs in the fibroblasts cocultures, 7 days after seeding. C) Gene expression of FSP1 and CDKN1A, and the markers for intracellular junction and attachment CD34, CDH5, VWF, and PECAM1 as quantified using RT-qPCR after 7 days of culturing in 3D monoculture or coculture. Data is normalized to the human acidic ribosomal protein (hARP) housekeeping gene. Differences in the relative expression were calculated by the \( 2^{\Delta\Delta C_T} \) method. [62] Sequences of qPCR primers are provided in Table 1. Data are shown as mean ± SD, biological replicates \( n = 5 \). Statistics: n.s. = not significant \( (p > 0.05) \), * \( p < 0.05 \), ** \( p < 0.01 \), by unpaired t-test.
Beyond a welcome 3D alternative for the Transwell® coculture system, the PIC-based TempEasy system offers a variety of advantages over existing approaches.[32–34] First, from a practical perspective, no specialized technology or equipment is necessary,[23,27,30,41] which makes the technology available to any interested researcher. Secondly, the thermoreversibility of PIC gels facilitates a straightforward extraction route from each of the two TempEasy layers, which can subsequently be processed and submitted for downstream analysis. Lastly, one of the biggest advantages is the flexibility and versatility of the PIC gel itself. Recent work in PIC gel development has demonstrated the great tailorability of this material as a cell culture matrix, which is required when other cell types will be cultured.[52,54,59] Many different biomolecules can be introduced on the fibrous network, ranging to small cell-adhesive peptides to biomolecules as large as proteins and antibodies. To tune the physical properties of the gels, various (orthogonal) routes have been established that are able to (independently) manipulate the gelation temperature and the mechanical properties, including hydrogel stiffness and biomimetic stiffening behavior.[57–58,61] Moreover, through the formation of hybrids and composites, the mechanical properties of the PIC gels can be readily tailored or even adapted in situ by straightforward application of external cues, such as magnetic fields or small temperature steps.[82,83] When this technology is applied in both layers, a coculture system is obtained where the mechanical properties of both layers can be tuned independently and reversibly during a cell culture experiment.

To conclude, we believe that the TempEasy 3D cell coculture systems provide an attractive easily handleable alternative for indirect cocultures with in precisely defined cellular microenvironments with the option of easy cell or cell construct extraction.

4. Experimental Section

**Polymer Synthesis and Peptide Conjugation:** The synthesis of PIC polymers has been reported before.[81] Briefly, azide-functionalized PIC was obtained by copolymerization of a methacryl and an azide-appended monomer (ratio OMe to N₃ is 29:1) in toluene in the presence of Ni (ClO₄)₂ · 6H₂O solution as catalyst. To obtain polymer of different length, a monomer:catalyst ratio of 1:5000 was used for the long polymers L-PIC (low T₆⁰) and 1:500 was used for the short polymers S-PIC (high T₆⁰). The polymerization mixture was stirred overnight, and full monomer conversion was confirmed by the disappearance of the characteristic isocyanide peak at 2140 cm⁻¹ in the infrared spectrum. The polymers were isolated by precipitation in diisopropyl ether for three times and air drying, after which they were stored at −20 °C until use. Viscometry measurements[58] yielded molecular weights M₉ = 251 and 562 kg mol⁻¹, which corresponds to a degree of polymerization n = 784 and 1756 and a polymer contour length Lc = 99 and 222 nm.[57] For biofunctionalization with the cell adhesion peptide GRGDS, an earlier published protocol was used,[84] where the peptide is equipped with a dibenzocyclooctyne (DBCO)-terminated spacer and subsequently clicked to the PIC polymers using the highly efficient strain-promoted azide-alkyne cycloaddition reaction.

**Cell Culture and Encapsulation:** Human adipose stem cells (hASCs) were obtained from the Radboud Biobank and cultured in Gibco Minimum Essential Medium (α-MEM) (Invitrogen, Thermo Fisher, USA). Vaginal epithelial cells were cultured in Dubécco’s modified Eagle medium (DMEM). Cells were obtained after informed consent. Cell culture media was supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA) and 1% penicillin/streptomycin (final concentration of 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin, Gibco, Thermo Fisher, USA). All cultures were regularly tested for mycoplasma, and no contamination was observed. Cells were harvested by trypsin treatment once they reached 80–90% confluence, followed by centrifugation at 1300 rpm for 5 min. Then, fresh medium was added to re-suspend cells to a cell density of 2 × 10⁵ mL⁻¹. Cell densities were determined by a LUNA-FL dual fluorescence cell counter.

**Cell Coculture in TempEasy:** Dry PIC polymers were sterilized by UV for 20 min and then dissolved in ice-cold DMEM medium (3 mg mL⁻¹) for 24 h at 4 °C before cell encapsulation. Cells were mixed with the polymer solution once in a 1:1 ratio to achieve the desired cell density (1 × 10⁴ mL⁻¹) and polymer concentration (1.5 mg mL⁻¹). After thorough mixing of cells with the PIC gel, the S-PIC-cell mixture was put on ice, while the S-PIC-cell mixture was kept at 25 °C in a water bath to avoid a temperature drop below the gelation temperature. In a 24-well plate, 300 µL of the L-PIC-cell mixture was pipetted, which was placed in an incubator (37 °C, 5% CO₂) for 15 min to ensure full gel formation. Then, the 24-well plate was removed from the incubator and 300 µL S-PIC-cell mixture was pipetted on top of the L-PIC-cell mixture and the plate was returned to the incubator. After 15 min to allow for gel formation of the top layer, 300 µL culture medium (preheated at 37 °C) was carefully added (drop-wise) to cover the gel surface. Then all samples were subject to standard cell culture conditions (37 °C, 5% CO₂).

After 7 days of coculture of, the 24-well plate was removed from the incubator and 300 µL DMEM medium (precooled at 15 °C) was slowly added to each well to collect the S-PIC encapsulated cells. This step was repeated twice. Then 300 µL DMEM medium (precooled at 4 °C) was slowly added to each well to collect the L-PIC encapsulated cells.

**RNA Extraction and Reverse Transcription:** Collected cells were washed twice with ice-cold PBS buffer and centrifuged at 1300 rpm for 5 min. Then, 0.5 mL TRIzol™ Reagent (Invitrogen, Thermo Fisher, USA) was added to lyse cells. After incubation at room temperature for 5 min to permit the complete disassociation of nucleicprotein complexes, the samples centrifuged at 1300 rpm for 5 min to remove cell debris. The supernatant was transferred to a new tube and 0.2 mL of chloroform (Sigma-Aldrich, USA) per 1 mL of TRIzol™ Reagent was added, followed by vigorous vortexing for 15 s and incubation at room temperature for 2 min. After centrifugation at 12,000 × g for 15 min, the colorless upper aqueous phase containing the RNA was transferred into a fresh tube. An equal volume of isopropyl alcohol (Sigma-Aldrich, USA) was added for RNA precipitation. After incubation at −20 °C for 30 min, RNA was collected by centrifugation at 12,000 × g for 15 min. The supernatant was removed completely and the RNA pellet was washed twice with 70% ethanol. After removing the ethanol, the RNA was air-dried for 10 min and then dissolved in 20 µL DEPC-treated water. The concentration of RNA was measured with a Nanodrop 1000 (Thermo Fisher Scientific). The total RNA and cDNA were amplified using GoTaq qPCR (Promega) according to the manufacturer's protocol. The human acidic ribosomal protein (hARP) was used as the housekeeping gene for normalization purposes. Differences in the expression of each gene (relative expression) were calculated by 2ΔΔCt method.[62]

**Statistics:** Gene expression values were normalized to the housekeeping gene hARP following the standard 2ΔΔCt procedure (see above). The data shown in the box plots are mean ± SD of n = 5 biological replicates. For the cell morphology quantification (circularity), 6 representative isolated cells in the focal plane were selected, retraced and the cell shapes were analyzed using ImageJ. The data shown in the box plots (Supporting Information) are mean ± SD of all 6 cells (in one biological replicate). Significance evaluation after unpaired t-tests: n.s. = not significant (p > 0.05), p < 0.05, **p < 0.01 was carried out using Graphpad’s Prism software.
Table 1. RT-qPCR primers.

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<td>hARP-R</td>
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</tr>
<tr>
<td>CDH5-R</td>
<td>CAAATGTGTACTTGGTCTGGGTG</td>
</tr>
<tr>
<td>VWF-F</td>
<td>AGTGGGATCTGCCACATCTTCT</td>
</tr>
<tr>
<td>VWF-R</td>
<td>GATCCGGAGGTCTCACCCTTTCA</td>
</tr>
<tr>
<td>PECAM1-F</td>
<td>GCCGTCCACAGCAGCCAGGT</td>
</tr>
<tr>
<td>PECAM1-R</td>
<td>CGACCCCTTCCGTCTAGAT</td>
</tr>
</tbody>
</table>

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

cocultures, fibroblasts, human adipose-derived stem cells, polysaccharide hydrogels, synthetic extracellular matrix

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