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Dynamic polymer hydrogels as synthetic extracellular matrices for 3D cell culture

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CHAPTER 3

Co-assembly of integrin-targeting peptides on squaramide supramolecular materials facilitate 3D expansion of human induced pluripotent stem cells

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3.1 Abstract

Human induced pluripotent stem cells (hiPSCs) have recently advanced drug screening, disease modeling, and tissue engineering research because of their capacity to be differentiated into numerous cell types. To meet the large cell number required for these applications efficient and scalable cell expansion is a prerequisite. Herein, we develop a synthetically supramolecular hydrogels as a 3D bio-matrix for hiPSCs expansion, bearing two integrin-targeting peptides to provide cell-matrix interactions. The squaramide-based monomers co-assemble to form supramolecular polymers with a fibrous structure and eventually form a self-healable hydrogel with soft mechanical properties, as evidenced by spectroscopy and rheological measurements. A six-fold expansion was achieved over a four-day culture period in the absence of ROCK inhibitor. The hiPSCs embedded in this matrix displayed high cell viability, cell proliferation, maintenance of pluripotency and differentiation capacity after release. Starting from a single cell suspension, peptide-containing supramolecular hydrogel facilitated cells expansion by 4.8-fold of that without peptides in the condition of single cell seeding. Overall, this integrin-targeting supramolecular hydrogels was proven to be synthetically accessible, cytocompatible, and efficient as a bio-matrix for hiPSCs expansion, demonstrating its applicability for the expansion of hiPSCs.

3.2 Introduction

Human induced pluripotent stem cells (hiPSCs) are rapidly growing in use in the biomedical field because they can be differentiated into any cell type of the body. They can be used to develop various *in vitro* cell culture models for drug discovery research, and the lack the ethical controversies of human embryonic stem cells (hESCs) making them readily accessible.^[1-4] A key issue for their application in these areas is that large cell numbers are required, yet their expansion remains challenging.^[5-7] Current matrices to expand hiPSCs on two-dimensional (2D) surfaces involve animal-derived (*e.g.* Matrigel) and extracellular matrix (ECM) proteins under static conditions. Besides the limited scale up capacity, these biological matrices can be problematic for their immunogenicity and batch-to-batch variations due to the natural substrates used. An improved method involves the use of suspension culture in a matrix-free manner to overcome the limited scale up of capacity. The cells are cultured as aggregates and subject to shear forces and distribution of gas and nutrients through stirring, wave spinning, rotation or microfluidics throughout the culture vessel providing a new dimension to the culture, but also may compromise their viability and/or genetic stability.^[8] Hence, synthetic matrices have been applied in dynamic suspension culture, and are referred to as microcarriers that support cell adhesion and show the potential to protect cells from shear induced effects. Though they can positively impact dynamic culture, preparation of microcarrier can be labour-intensive based on the selected polymer and its subsequent removal from the cells can be time-consuming.^[9,10] Hence, developing defined, cytocompatible and accessible matrices that support easy processing, scalable and efficient 3D cell culture is appealing to produce hiPSCs for clinical and fundamental research.

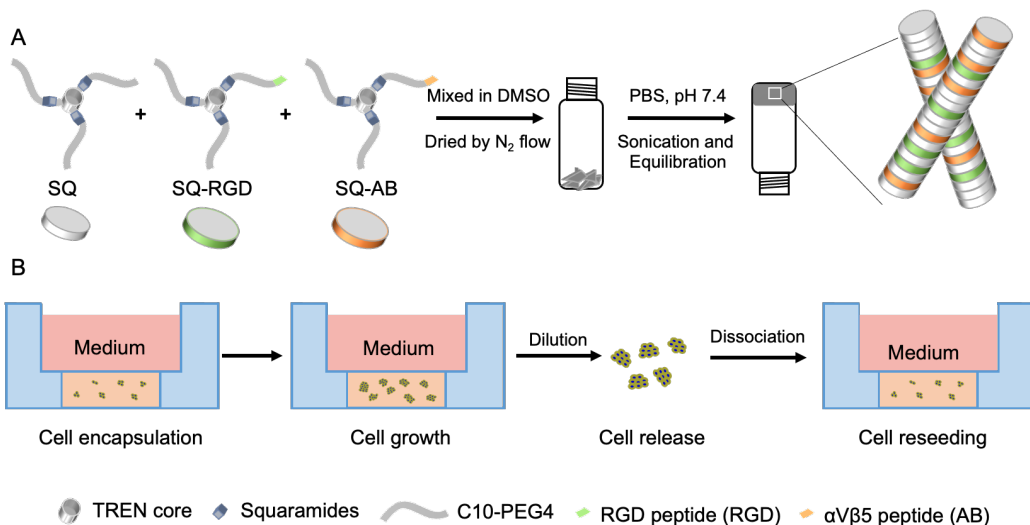
Synthetic hydrogels have been exploited in the field of 3D cell culture to provide a chemically-defined environment with structural support. Controlled mechanical

stiffness, incorporation of functional cargos (*e.g.* proteins, peptides, growth factors), degradability and variable topographies can be introduced into the materials to mimic the natural extracellular matrix.^[11–13] Synthetic polymer hydrogels based on thermoreversible and covalent polymers, such as poly(N-isopropylacrylamide)-copoly(ethylene glycol) (PNIPAAm-PEG), and hyaluronic acid-PNIPAAm were previously used by Schaffer and co-workers highlighting their applicability for induced pluripotent stem cell culture.^[14–16] However, these hydrogels are based on high molecular weight polymers that can be challenging for the introduction of bioactive cues that are necessary for applications such as cell differentiation, highlighting the need for alternative synthetic polymer hydrogel strategies. Filamentous supramolecular polymers, in which well-defined small molecular monomers self-assemble through non-covalent interactions, can provide a viable alternative to overcome this challenge through their straightforward combination with short peptides to form bioactive 3D cell culture materials that are dynamic and responsive.^[17–20]

In native ECM, the filamentous ECM proteins, *i.e.* collagens, elastins, fibronectins and laminins support and interact with cell surface receptors, *e.g.* integrins, engaging in bidirectional signalling to drive numerous cellular processes.^[21,22] Therefore, there is an interest in using short peptides in biomaterials to provide a cell relevant environment in a facile manner, both chemically and synthetically. A short peptide routinely used to guide cell adhesion and proliferation in biomaterials is RGD. This peptide originates from the 9th-10th domain of fibronectin and targets various integrin receptors.^[23,24] Another short peptide that can target integrin receptors, namely $\alpha v\beta 5$ that binds vitronectin, a common substrate used in iPSC culture, is KKQRFHRNRKG (AB). This peptide has been employed to support stem cell adhesion, proliferation, maintenance of pluripotency and differentiation.^[25–31] Moreover, 2D substrates covalently functionalized with these two short peptides supported stem cell culture without the use of ROCK inhibitor.^[27] However, cells interact with their microenvironment in 3D

and in a dynamic manner, and how these two short peptides can affect stem cell behaviour in 3D in a dynamic material has not been explored.^[32,33] We therefore examine the combination of these peptides within a supramolecular material and investigate their potential for 3D iPSC expansion.

We previously reported the formation of a filamentous supramolecular hydrogel that can be used for hiPSCs culture using amphiphilic monomers that rely on directional hydrogen-bonding between squaramide units and hydrophobic interactions.^[34–37] However, these materials lacked possibilities to introduce bioactivity or change their mechanical properties. In order to introduce bioactive cues into these materials, we designed an azide-terminated squaramide-based monomer for further click reaction with peptides (Chapter 2). Using a supramolecular co-assembly approach, RGD-functionalized squaramide-based supramolecular hydrogels that support HepG2 spheroids assembly, proliferation and differentiation were obtained. To examine and compare the effect of integrin targeting peptides, RGD and KKQRFHRNRKG (AB) on hiPSCs culture in 3D, we synthesized two peptide-functionalized monomers, **SQ-RGD** and **SQ-AB**. Supramolecular co-assembly of these two peptide-functionalized monomers with native monomer **SQ** result in hydrogels with tunable bioactive character for stem cell culture. We examine their material properties and potential to effect hiPSC viability, proliferation and expansion to better understand the materials features needed to facilitate hiPSCs growth (Scheme 3.1).



Scheme 3.1. Integrin-targeting squaramide-based supramolecular hydrogels for hiPSC expansion. A) Preparation of multicomponent squaramide-based hydrogels through supramolecular co-assembly of monomers **SQ**, **SQ-RGD** and **SQ-AB**. B) hiPSCs cultured within multicomponent squaramide-based hydrogels: cells were encapsulated by gently mixing cells and hydrogels using a pipet; cells aggregate and grow during the culture; expanded hiPSCs were released from hydrogel by dilution and dissociated for the next round of seeding and culture.

3.3 Results and Discussion

Design and synthesis of integrin-targeting monomers and their co-assembly into squaramide-based supramolecular hydrogels. The native tripodal squaramide-based monomer (**SQ**) was synthesized as previously published and purified by High Performance Liquid Chromatography (HPLC) prior to hydrogel preparation.^[37] An azide-terminated tripodal squaramide-based monomer (**SQ-N₃**) was synthesized and purified by silica column chromatography using the protocol described in Chapter 2. RGD and AB (KKQRFRRHRNRKG) peptides were prepared by solid-phase peptide synthesis and

were made alkyne-terminated through the use of 4-pentynoic acid in a final coupling step at the N-terminus. Similarly to Chapter 2, peptide coupling on **SQ-N₃** monomers by copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) was performed and purified by HPLC to obtain the peptide-labelled squaramide monomers **SQ-RGD** and **SQ-AB**. The synthetic details are included in the supporting information.

Co-assembled integrin-targeting squaramide-based hydrogels were prepared similarly as described in Chapter 2. Briefly, DMSO stock solutions of **SQ**, **SQ-RGD** and **SQ-AB** monomers were prepared at concentrations of 25 mM, 5 mM and 5 mM, respectively. The monomer solutions, **SQ** with **SQ-RGD** and/or **SQ-AB**, were mixed at various ratios, vortexed and then dried overnight under nitrogen flow to obtain a white film. The dried solid was rehydrated in PBS (pH 7.4), vortexed, and subsequently sonicated in an ice bath (4°C). The obtained transparent solution was incubated at 37°C for 30 min and equilibrated overnight at room temperature prior to further study. UV-Vis spectroscopy measurements were then performed to understand the effect of the mixing protocol on the squaramide-based supramolecular polymer at the molecular level. In DMSO, **SQ-AB** monomers showed a single band at 293 nm that is consistent with depolymerization of the squaramide monomers (Figure S3.2). The sample with 10 mol% of SQ-RGD (SQ-10RGD) displayed two absorption bands at 262 nm and 322 nm upon aggregation that correspond to the HOMO–LUMO and HOMO–LUMO+1 transitions, respectively. These bands are identical to self-assembly of the native **SQ** monomers on its own suggesting that the mixing of the **SQ-RGD** does not alter their assembly at the molecular level (Figure 3.1D). In the samples with 10 mol% of SQ-AB (SQ-10AB) and 10 mol% of both **SQ-RGD** and **SQ-AB** (SQ-10COM), the two HOMO–LUMO and HOMO–LUMO+1 transitions were less red and blue-shifted at 267 nm and 319 nm, and are thus indicative of a lower degree of aggregation compared to that of native **SQ** monomers. Collectively, these results suggest a depolymerization of all monomers in DMSO and their likely co-assembly in buffered solutions.

Physicochemical properties of squaramide-based supramolecular hydrogels. Squaramide-based hydrogels co-assembled with **SQ-RGD** and **SQ-AB** at various monomer ratios were first evaluated for their capacity to gelate by the gel-inversion method. The total squaramide-based monomer concentration was maintained at 3.1 mM, in which increasing peptide-functionalized monomers were added from 5-20 mol% (Figure S3.1). Gel phase materials were observed on increasing the **SQ-RGD** monomer concentration within this range. Similarly, when **SQ-AB** monomers were added, non-flowing hydrogels were obtained up to 10 mol%, whereas samples with higher than 15 mol% of **SQ-AB** lacked the capacity to form immobile gels. This weakening of the supramolecular hydrogels may be due to the increased charge and length of the AB peptide relative to RGD peptide resulting in its higher aqueous solubility and decreased aggregation observed in UV-Vis spectroscopy. Hence, the **SQ-AB** monomer was kept less than 15 mol% in the preparation of the integrin-targeting peptide hydrogels. The optimal ratio between RGD and AB peptides for 2D pluripotent stem cell culture was previously demonstrated by Kiessling et al. to be 3:7 resulting in long term maintenance of pluripotency even in the absence of ROCK inhibitor. [27] On this basis, **SQ-RGD** and **SQ-AB** were mixed in a molar ratio of 3:7 for integrin-targeting squaramide hydrogel (SQ-xCOM). The combination of **SQ-RGD** and **SQ-AB** peptides at a 15 mol% concentration (SQ-15COM) resulted in non-flowing hydrogels.

Oscillatory rheology measurements further confirmed the trends observed in gel inversion tests and provided a means to quantify the effect of the integrin-targeting peptides on hydrogel properties. Time sweep measurements showed comparable profiles and storage moduli at plateau for the native squaramide hydrogel (SQ, $G' = 60.8 \pm 4.5$ Pa) and 10 mol% RGD-containing squaramide hydrogel (SQ-10RGD, $G' = 46.7 \pm 5.9$ Pa). On the other hand, lower storage moduli were recorded in comparison to the SQ for 10 mol% AB-containing squaramide hydrogel (SQ-10AB, $G' = 24.7 \pm 1.1$ Pa), and 10 mol% integrin-targeting squaramide hydrogel (SQ-10COM, $G' = 28.3 \pm 0.8$ Pa) (Figure

3.1A). During an amplitude sweep, the storage modulus (G') and loss modulus (G'') of SQ-10RGD remained constant until a strain of 5% was applied. In the case of SQ-10AB and SQ-10COM, both moduli (G' and G'') remained constant before the application of 15% strain. For all three hydrogels, G' was an order of magnitude higher than G'' from 0.01 Hz to 2 Hz in frequency sweep measurement confirming the formation of viscoelastic hydrogels (Figure S3.4, S3.5 and S3.6). Lastly, step-strain measurements were performed to evaluate the effect of the added peptides on the self-recovery of these hydrogels (Figure 3.1B). The peptide-containing squaramide hydrogels showed similar behavior, compared with SQ, namely the decrease and inversion of both moduli (G' and G'') in response to large amplitude strain, and recovery of the material to its initial state after its removal over two cycles. To explore the limit of co-assembly, hydrogels with higher ratio of AB peptide were prepared and their rheological properties were tested. Similar to 40mol% of **SQ-RGD** in Chapter 2, G' was slightly higher than G'' for the sample that contains 20mol% of **SQ-AB** ($G' = 2.53$ Pa, $G'' = 0.47$ Pa), indicative of the formation of a weak gel (Figure S3.7).

Cryogenic transmission electron microscopy (cryo-TEM) was performed to provide insight into the self-assembly of the SQ-10COM (Figure 3.1C). Cryo-TEM images of the SQ-10COM (3.1 mM, PBS) showed a nanofibrous structure indistinguishable from the SQ confirming that hydrogel formation is driven by fiber entanglement. The fibers from SQ-10COM displayed a width of 6.2 ± 0.7 nm, which is slightly larger than that of the SQ. This larger fiber width likely results from weaker hydrophilic-hydrophobic interactions after introducing the larger AB peptide, which is on par with gel-inversion and rheological measurements.

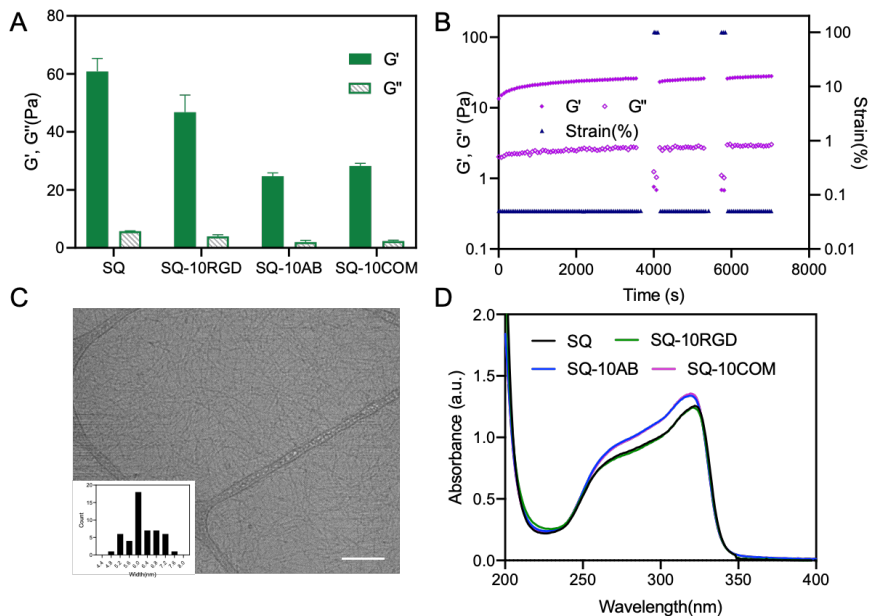


Figure 3.1. A) Averaged storage (G') and loss (G'') moduli of hydrogels (3.1 mM, PBS) collected at 37°C by time-sweep measurement with a fixed frequency of 1 Hz and strain of 0.05% until a plateau in the storage moduli was obtained. (N = 3) B) Step-strain measurements of SQ-10COM (3.1 mM) at 37°C with a frequency of 1 Hz; Frequency sweep (from 0.01 to 2 Hz with strain at 0.05%) was performed prior to application of high strain (100%). C) Cryo-TEM image of SQ-10COM (3.1 mM, pH 7.4 PBS) showing entangled nanoscale fibres. Insert: Histograms of the fiber width distribution for a sample size of n = 50, Scale bar: 200 nm. D) UV-Vis spectra of **SQ** and co-assembled 10mol% of **SQ-RGD** and/or **SQ-10AB** (c = 30 μ M) in PBS (pH7.4).

hiPSCs 3D encapsulation and release from squaramide-based supramolecular hydrogels. To apply the integrin-targeting squaramide hydrogel for 3D hiPSCs culture, we first evaluated the effect of encapsulation and release from the materials on cell viability due to the sensitivity of the hiPSCs. As illustrated in Figure S8, hiPSCs were first dissociated into small clumps by EDTA (0.5 mM). By gentle pipetting, dissociated hiPSCs were mixed and seeded within SQ-10RGD (3.1 mM, PBS). The cell-gel mixture was left at 37°C for 15 min to gelate using the self-recovery property of the hydrogel. The cell-

gel construct was cultured at 37°C for 2 h with medium added on top. After that, the cell-gel construct was disrupted gently by pipetting and dilution with excess PBS (~100 times) to release the cells as aggregates. The released cell aggregates were stained with LIVE/DEAD and showed high cell viability (Figure S3.8), indicating that these sensitive cells can be maintained viable during the encapsulation, release and centrifugation process.

Impact of the integrin-targeting peptides RGD and AB on the growth of encapsulated hiPSCs. The growth of hiPSCs in synthetic materials in 3D can be affected by multiple factors, such as use of RhoA GTPas signaling effector Rho kinase (ROCK) inhibitors in the culture, whether the cells are dissociated into clumps or singlets prior to seeding, as well as starting cell seeding density in the materials. In this study, a control hiPSC line L44 was first seeded as small clumps and cultured in SQ. The two common reagents to reduce dissociation-induced cell death are ROCK inhibitor Y-27632 (R) and RevitaCell™ (C), we thus compared the effect of the R and C on the 3D culture of hiPSCs in the SQ. As shown in Figure S9, hiPSCs treated with R or C in a 5-day culture period did not show a significant difference in morphology and/or size distribution; consequently, the more specific inhibitor C was used for subsequent experiments. Notably, cells treated with R or C for 4 days displayed a more narrow size distribution to those treated for 1 day, therefore further experiments were performed with 4-day treatment. To screen the optimal concentration of peptides, cell metabolic activity test were performed for cells encapsulated in hydrogel with various mol% of **SQ-RGD** and **SQ-AB**. It turned out that cell metabolic activity peaked at 10 mol% of **SQ-RGD** and 5 mol% of **SQ-AB** in the tested range (Figure S3.10). Compared with SQ, cells in SQ-10RGD and SQ-5AB showed 1.3 and 3.3 times enhancement in cell metabolic activity, respectively, which strongly suggests that peptide modification, especially the use of the AB peptide, supports increased metabolic activity of cells. Moreover, cells expanded in SQ, SQ-10RGD and SQ-5AB showed high cell viability as evidenced by

LIVE/DEAD staining of spheroids in 3D. The released hiPSCs, when stained with 5-ethynyl-2'-deoxyuridine (EdU), showed a significant number of proliferative cells (Figure S3.11 and S3.12). Thereafter, the integrin-targeting squaramide hydrogels were prepared in a molar ratio **SQ-RGD:SQ-AB** of 3:7. At the end of the culture period, large numbers of cells were observed to be viable and proliferating from LIVE/DEAD and EdU staining (Figure S3.13). Importantly, increased cell expansion can be reached within the integrin-targeting squaramide hydrogels after a 3-day culture, with an increase of 5.0, 7.9 and 3.7-fold relative to the initial cell number in SQ-5COM, SQ-10COM and SQ-15COM, respectively. After a 5-day culture, an increase in expansion was obtained in these hydrogels that was 6.9, 6.3 and 6.3-fold relative to the cell number, respectively (Figure S3.13). Expansion of cells in SQ-10COM decreased slightly from 3-day culture to 5-day-culture, hinting that the cells may reach a maximum in their growth a couple days before passage when cultured in SQ-10COM for 5 days. In addition, hiPSCs released from the integrin-targeting squaramide hydrogel were further analyzed using fluorescence activated cell sorting (FACS) to assess the pluripotency marker expression. As shown in Figure S3.13D, over 75% and 96% cells were positive in expression of pluripotency markers TRA-1-60 and SSEA4 pointing to a retention of pluripotency after release.

Overall, hiPSCs seeded as clumps in SQ-10COM using 4-day RevitaCell™ supplement treatment resulted in 6.3-fold expansion after 5 days of culture. High cell viability of hiPSCs, active proliferation throughout the spheroids and expression of pluripotency markers were confirmed in this supramolecular hydrogel through the use of various fluorescent techniques after the 3D culture.

To explore the application potential of this matrix, the hiPSC line L20 that is fully characterized for its cardiac differentiation potential was seeded as both small clumps (clumps seeding, CS) and single cells (single cell seeding, SS) in SQ-10COM. First, the effect of using the RevitaCell™ treatment on the culture was evaluated after

incubation on 0(C0), 1(C1) and 4(C4)-days. Brightfield microscopy images over 4 days of culture showed the formation of compact hiPSCs spheroids and their increase in size over time in SQ-10COM. The average diameter of the hiPSC spheroids measured on D1 and D4 were $111 \pm 36 \mu\text{m}$ and $158 \pm 48 \mu\text{m}$, respectively, based on a population of 250 spheroids (Figure 3.2B). Among them, 55% of counted spheroids showed diameters over $100 \mu\text{m}$ on D1, which turned into 90% on D4. Notably, treatment of the hiPSCs with the RevitaCell™ supplement for 0, 1 and 4 days resulted in comparable expansion folds, which were 5.7 (C0), 5.1 (C1) and 5.8 (C4)-fold, respectively, indicating that the integrin-targeting squaramide hydrogel can support hiPSC growth with the use of less ROCK inhibitor. After optimization of RevitaCell™ treatment, cell expansion within hydrogels presenting different peptide were assessed. In case of cell expansion, presentation of either the RGD and AB peptide resulted in an improved expansion fold of 2.2 and 3.8-fold, respectively. Notably, by introducing RGD and AB peptides, SQ-10COM supported hiPSCs expansion to a greater extent with a higher fold of 5.8 indicating a combinational effect between these two peptides with respect to supporting hiPSCs growth (Figure 3.2D). One major goal for using rock inhibitor for hiPSCs culture is to reduce cell death during the dissociation. Therefore, hiPSCs were dissociated by Accutase to obtain singlets and encapsulated within SQ-10COM. As seen in the brightfield microscopy images in Figure S3.14A, compact hiPSCs spheroids were formed and increased in size over the 4-day culture. The average diameter of hiPSC spheroids measured on D1, D2 and D4 were $57 \pm 13 \mu\text{m}$, $75 \pm 19 \mu\text{m}$ and $91 \pm 25 \mu\text{m}$, respectively, based on a population of 300 spheroids (Figure S3.14B). Among them, 15% of counted spheroids showed diameters over $70 \mu\text{m}$ on D1, which turned into 55% on D2 and 84% on D4, respectively. In the case of SQ, there were far fewer spheroids formed after 4 days of culture. Importantly, expansion of the hiPSCs in SQ-10COM significantly increased in comparison to the SQ, with values of 5.8 and 1.2-fold, respectively (Figure S3.14C). In addition, hiPSCs after culture in the SQ-10COM showed over 77% and 97% expression of pluripotency markers TRA-1-60 and SSEA4,

respectively (Figure S3.14D). In all, the combination of the RGD and AB peptides in the supramolecular hydrogels enable rock inhibitor-independent growth of L20 hiPSCs from their seeding as clumps, proving as an effective matrix for the 3D culture of hiPSCs.

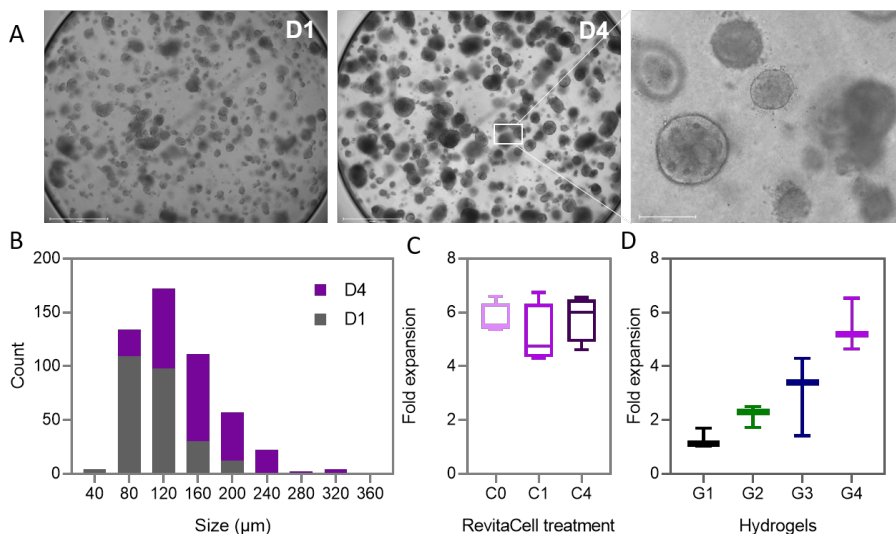


Figure 3.2. A) Bright field microscopy images of spheroids cultured in SQ-10COM with 1(C1)-day treatment of RevitaCell™ supplement, scale bar: 750 μm (left), 750 μm (middle) and 200 μm (right). B) Size distribution of hiPSCs during 4-day culture in SQ-10COM (C1), 250 spheroids were counted. C) Expansion fold of hiPSCs during 4-day culture with 0(C0), 1(C1) and 4(C4)-days incubation of RevitaCell™ Supplement. D) Expansion fold of hiPSCs during 4-day culture in SQ (G1), SQ-10RGD (G2), SQ-10AB (G3), and SQ-10COM (G4).

Viability, proliferation, pluripotency marker expression and differentiation of hiPSCs after expansion in integrin-targeting squaramide hydrogel. Similar to L44 hiPSCs, cell viability, proliferation and pluripotency of L20 hiPSCs after culture in the SQ-10COM was assessed on D4 (Figure 3.3). LIVE/DEAD staining was performed in 3D, generally showing a large population of live cells in tightly compacted colonies. To assess hiPSC proliferation, the colonies were first gently released from the hydrogel by dilution with

PBS prior to use of the EdU staining kit (Figure 3.3B). Notably, the cells were confirmed to be proliferating through the spheroid on staining with EdU, consistent with the observed increase in cell number. Subsequently, pluripotency of the hiPSCs was further assessed with fluorescently-labelled antibodies, TRA-1-60 and SSEA-4, by FACS. HiPSCs were released as single cells after a 4-day culture in SQ-10COM. As plotted in Figure 4C, hiPSCs expanded in SQ-10COM showed a SSEA4 positive population (94%) that was similar to 2D cultured cells. On the other hand, hiPSCs cultured in 2D and 3D showed different percentages in TRA-1-60 expression. In comparison to 2D (~74%), cells expanded in SQ-10COM showed a slight increase in expression of TRA-1-60, with percentages of $83.9 \pm 2.0\%$, $79.1 \pm 2.0\%$ and $76.7 \pm 3.0\%$ in C0, C1 and C4 culture. Importantly, removal of RevitaCell™ supplement did not alter pluripotency marker expression. Overall, LIVE/DEAD and EdU staining showed high cell viability and average proliferation through the hiPSC spheroids and a high maintenance of pluripotency markers after 3D expansion in the integrin-targeting squaramide hydrogel.

To assess the differentiation potential of hiPSCs after expansion in SQ-10COM, we released hiPSCs as clumps and used the STEMdiff™ Cardiomyocyte Differentiation Kit to prepare cardiomyocytes. The released hiPSCs grew as a monolayer and started to polarize and elongate on day 4. Clear network formation and gaps between cells can be identified on day 6. The elongated cells formed thicker networks that were distinguished clearly from the background from day 10 to day 16. In the meantime, increased and stronger spontaneous beating was observed on day 8 and day 16 (Supplementary Video S3.1, S3.2 and S3.3). The cardiomyocytes differentiation of hiPSCs released from the integrin-targeting squaramide hydrogel support the maintenance of pluripotency in culture and their further downstream application.

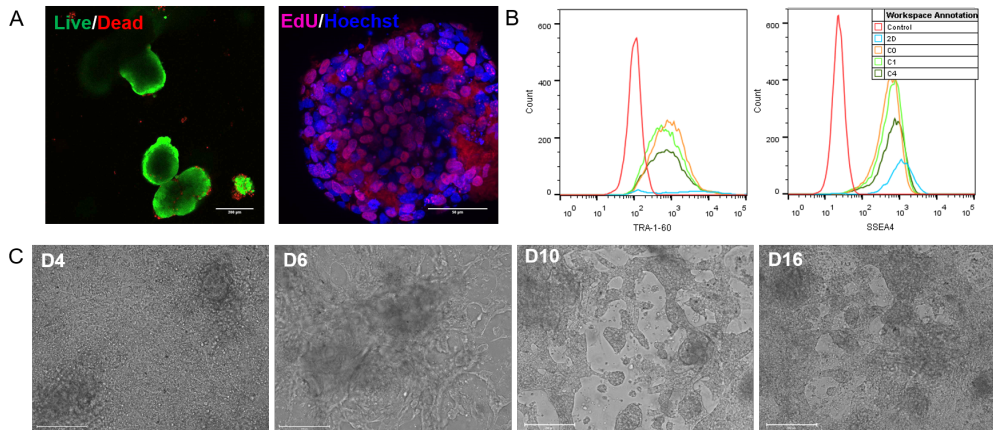


Figure 3.3. Characterization of L20 hiPSCs after 4-day culture in SQ-10COM. A) LIVE/DEAD staining of hiPSCs with calcein AM (viable cells, *green*) and propidium iodide (PI) (dead cells, *red*). Scale bar: 200 μ m. Representative images of EdU staining in hiPSCs. Proliferating cells were labelled with EdU-Alexa fluor 594 (*red*) and cell nuclei were stained with Hoechst 33342 (*blue*). Scale bar: 50 μ m. B) FACS analysis of pluripotency markers in hiPSCs expanded in SQ-10COM and that maintained on 2D. C) Bright field images of hiPSCs that were expanded and released from SQ-10COM and further processed for cardiomyocyte differentiation. Scale bar: 200 μ m.

hiPSC release, re-seeding and expansion in integrin-targeting squaramide hydrogels.

Several strategies have been used to release cells embedded in hydrogels including introducing enzymatic degradation, thermo or photo-responsiveness of the materials. These strategies usually require an initial investment into their synthetic design and end with limited modularity. Because squaramide-based supramolecular hydrogels can be easily broken down by dilution using excess PBS or medium based on their soft and non-covalent nature, cell release can be achieved by easily using gentle dilution. As illustrated in Figure 3.4A, expanded hiPSC spheroids were first gently released by dilution, dissociated and re-seeded into integrin-targeting squaramide hydrogel. From bright field microscopy images, hiPSCs were re-seeded as small clumps (D0) and grew into spheroids with larger size (D4). Even though dead cells can be identified both

around and within the aggregates, these passaged and re-grown hiPSCs aggregates displayed high cell viability observed from the fluorescent images by LIVE/DEAD staining (Figure 3.4B and 3.4C).

One concern in the long-term culture of hiPSCs is the retention of their genetic status. To further assess whether these hiPSCs retain their genetic stability, i.e. that no abnormalities have arisen during culture, we performed Global Screening Array of genome DNA samples that were collected from hiPSCs cultured in integrin-targeting hydrogel in 3D and on 2D substrates. We quantified the relative signal intensities of each single nucleotide polymorphism (SNP) and detected no copy number abnormalities (resolution: ~50kb) (Figure 3.4D). Comparing 700 k SNPs of hiPSC lines under different culture conditions, we found no significant difference between cells cultured within 3D hydrogels and those maintained on 2D. This result confirms that hiPSCs maintained their genetic characteristics after culture in the integrin-targeting squaramide hydrogels.

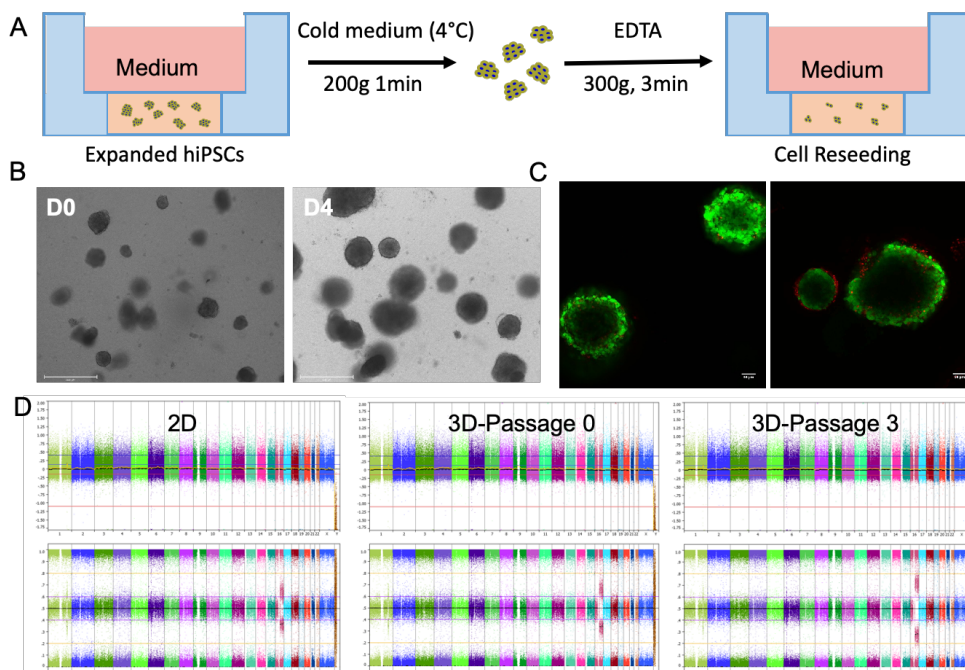


Figure 3.4. A) Cell release and re-seeding protocol in SQ-10COM. B) Bright field microscopy images of hiPSCs after re-seeding in SQ-10COM. Scale bar: 500 μm. C) LIVE/DEAD staining confirms viability of hiPSCs after re-seeding. Scale bar: 100 μm. D) Global Screening Array analysis of 700k single nucleotide polymorphisms (SNP). Genomic DNA were collected from the 2D culture condition on vitronectin and in SQ-10COM in 3D after one round and three rounds, respectively.

3.4 Conclusions

A multicomponent supramolecular self-assembly system with integrin-targeting peptides was successfully designed, synthesized and characterized. The native **SQ** and integrin-targeting monomers **SQ-RGD** and **SQ-AB** co-assemble into filamentous supramolecular polymers and entangle together to form a hydrogel which maintains the optical clarity, soft texture and self-healable properties. The integrin-targeting squaramide hydrogel boosts hiPSC growth with cell seeding either as single cells or clumps showing high cell viability, active proliferation and expansion fold during a 4-

day culture. Moreover, comparable cell expansion was achieved in the absence of ROCK inhibitor by introducing these two integrin-targeting peptides. In addition, the easy cell release by dilution and pipetting, together with retention of pluripotent markers, confirm this hydrogel to be a potent bio matrix for hiPSCs culture. Furthermore, the hiPSCs are genetically stable after three rounds of passage within this integrin-targeting squaramide hydrogel, demonstrating their great potential in hiPSCs expansion.

3.5 References

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3.6 Supporting Information

3.6.1 Materials

All chemicals for the synthesis **SQ** and **SQ-N₃** were obtained from commercial suppliers and used without further purification. Vitronectin XFTM, CryoStor CS10 and ROCK inhibitor Y-27632 were obtained from Stem Cell Technologies. EssentialTM 8 medium was received from Thermo Fisher Scientific. Ethylenediaminetetraacetic acid disodium salt (EDTA), Dulbecco's Phosphate Buffered Saline (DPBS), calcein AM (AM = acetoxymethyl) and propidium iodide (PI) were obtained from Sigma-Aldrich. The Click-iT EdU Imaging kit and RevitaCellTM supplement were obtained from Invitrogen. Anti-TRA-1-60-PE and anti-SSEA4-FITC monoclonal antibodies were from Miltenyi Biotec. ReliaPrepTM gDNA Tissue Miniprep System was purchased from Promega.

3.6.2 Instruments

HPLC purification of the monomers was executed on setup equipped with C18 column. A gradient from 10%-90% CH₃CN (0.1% TFA) in H₂O (0.1% TFA) over 15 min at a flow rate of 12 mL/min was used. LC-MS analysis was performed on a TSQ Quantum Access MAX system equipped with a Gemini 3 μm C18 110 Å 50×4.60 mm column (UV detection at 214 nm and 254 nm). The mobile phase was a gradient of 10-90% of H₂O-CH₃CN with 0.1% trifluoroacetic acid over 13.5 minutes. High resolution mass spectra (HR-MS) were collected on a Thermo Finnigan LTQ Orbitrap mass spectrometer that equipped with an electrospray ion source in positive mode (resolution R=60000). The spectra were collected by direct injection of samples (2 μM in H₂O-CH₃CN 50/50 v/v) and recorded with a mass range of 150-2000 using dioctylphthalate (m/z = 391.28428) as a "lock mass". Oscillatory rheology experiments were executed on a Discovery HR-2 hybrid rheometer using a cone-plate geometry (40 mm, 1.995°) at 37 ± 0.2 °C with a Peltier-based temperature controller and solvent trap. Cryo-TEM images were acquired

on a Tecnai F12 (FEI) equipped with a field emission gun operating at 120 keV using a Gatan UltraScan charge-couple device (CCD) camera (Gatan) with a defocus between -6 and -9 μm . UV absorption spectra were recorded on a Cary 300 Bio UV-vis spectrometer, scanning from 200 nm to 400 nm with 1 nm intervals with a scan rate of 120 nm/min. Bright field images were taken on an EVOS FL AUTO2 equipped with temperature and a CO_2 gas controller. Confocal fluorescent images were acquired on Leica SP8 confocal laser scanning microscope equipped with 10 \times air objective, 40 \times oil objective and 63 \times oil objective. Images were processed in the Fiji Image J software.

3.6.3 Synthetic procedures

Synthesis of SQ and SQ-N₃. Native (**SQ**) and azide -functionalized (**SQ-N₃**) squaramide-based monomer synthesis are described in Chapter 2.

Peptide synthesis. Detailed synthetic procedures for RGD peptides, and the conjugation between RGD peptides and **SQ-N₃** are described in Chapter 2. $\alpha\beta 5$ -binding peptides (KKQRFRRHRNRKG) (**AB**) was synthesized on an automatic CEM peptide synthesizer on a 100 μmol scale. Fmoc-Rink amide AM resin with a loading of 0.39 mmol/g was used for AB peptides. Amino acid coupling was performed with 4 eq. of the amino acid, 4 eq. of the activator HCTU and 8 eq. of DIPEA. Fmoc deprotection was executed with pyridine: DMF (2:8 v/v). 4-Pentynoic acid was coupled to the N-terminus of the peptide using 5 eq. of HCTU and 10 eq. of DIPEA in DMF at room temperature for 1 h. The alkyne functionalized peptides were cleaved from the resin by a TFA solution (2.5% H_2O and 2.5% TIPS) for 4h, precipitated in cold diethyl ether, dissolved in water, and lyophilized to obtain the white solid. The alkyne functionalized AB peptides are labeled as **AB_A**. The sequence was confirmed by mass collected from LC-MS. **AB**: LC-MS: t =1.47 min, m/z: 1609.68; **AB_A**: LC-MS: t =0.81 min, m/z: 1689.51.

Synthesis of SQ-AB. Sodium L-ascorbate (36.5 mg, 184.3 μmol) and Copper(II) sulfate pentahydrate (6.1 mg, 24.4 μmol) were first dissolved in water (100 μL) separately and

mixed by vortex for 30 s to obtain bright yellow solution. Afterwards, tris(3-hydroxypropyltriazolylmethyl) amine (THPTA, 5.3 mg, 12.2 μmol) dissolved in ethanol (200 μL) and SQ-C10-Azide (47 mg, 29.2 μmol) dissolved in DMF (1.2 mL) were added to the mixture. Lastly, **AB_A** (125.4 mg, 74.2 μmol) dissolved in DMF: H₂O (400 μL , v/v 1:1) was added and the reaction mixture was stirred at room temperature for 2h. The crude was first dialysed against water (Mw 500-1000 Da) for 48 h and purified by HPLC. The product was concentrated by evaporation and lyophilized overnight to obtain a white solid. Yield: 17%, 16.5 mg. LC-MS: t = 5.51 min, m/z: 1099.51, 1650.42. HR-MS: [M+4H]⁴⁺: calcd: 826.2516, found: 826.2514; [M+5H]⁵⁺: calcd: 661.2027, found: 661.2022.

3.6.4 Hydrogel preparation protocol.

SQ was prepared as previously published.^[1] The multicomponent hydrogels with short peptides were prepared as described in Chapter 2. Briefly, DMSO stock solutions of **SQ** (25 mM), **SQ-RGD** (5 mM) and **SQ-AB** (5 mM) were made separately, and then pipetted in the appropriate ratios into a new vial and vortexed to obtain a transparent mixture. The mixed solutions were left under nitrogen flow overnight to remove DMSO. The obtained films were suspended in PBS (pH 7.4) under sterilized flow cabinet resulting in a final monomer concentration of 3.1 mM, followed by 30 s vortex and sonication in ice-water bath until transparent solutions were obtained (~30 min). Lastly, the transparent solutions were incubated at 37°C for 30 min and left on the bench overnight at room temperature prior to further testing.

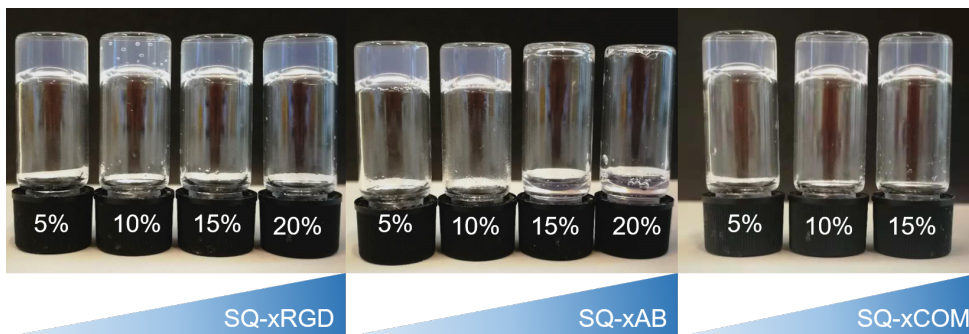


Figure S3.1. Gel inversion test of hydrogels based on **monomer SQ** and increasing mol% of monomer **SQ-RGD** (left), **SQ-AB** (middle) or both monomers with molar ratio of 7:3 (right). All monomers concentration were kept at 3.1 mM in PBS (pH7.4).

3.6.6 UV-Vis Spectroscopy

UV-Vis spectra were recorded on a Cary 300 UV-Vis spectrophotometer using a quartz cuvette with a path length of 1 cm. Stock solutions of monomers **SQ**, **SQ-RGD** and **SQ-AB** in DMSO were first made separately, and mixed in various ratios in a separate vial and vortexed. The mixed monomers were left overnight under nitrogen flow to remove the DMSO. Then, the obtained films were suspended in PBS (pH7.4) with a final monomer concentration at 1.0 mM, followed by vortexing for 30 s and sonication in ice-water bath to obtain transparent solutions. The solutions were left on the bench overnight and diluted prior to the measurement.

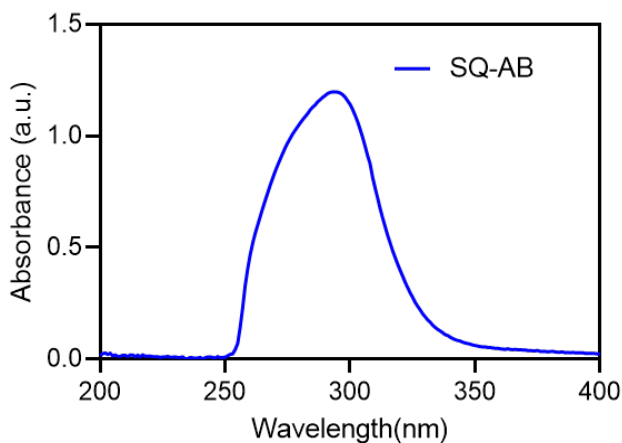


Figure S3.2. UV-Vis spectra of **SQ-AB** in DMSO with monomer concentration of 15 μ M.

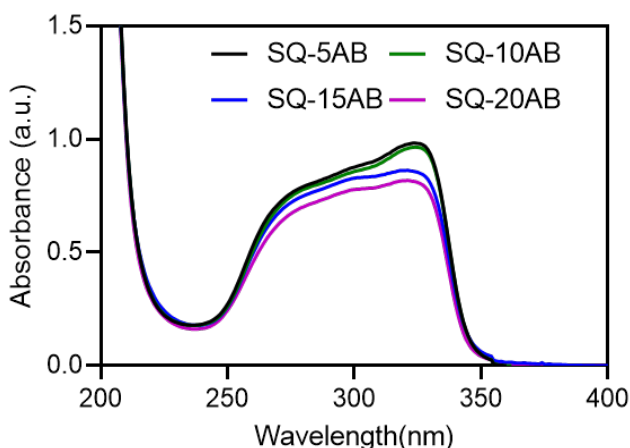


Figure S3.3. UV-Vis of **SQ** with increasing mol% of **SQ-AB**. All monomers concentration were kept at 30 μ M in PBS (pH7.4).

3.6.5 Oscillatory Rheology

Oscillatory rheology experiments were carried out on a Discovery HR-2 hybrid rheometer using cone-plate geometry (40 mm, 1.995 $^\circ$) at 37 \pm 0.2 $^\circ$ C with a Peltier-based temperature controller and a solvent trap. The SQ, SQ-10RGD, SQ-10AB and SQ-10COM were prepared according to the protocol above in a final monomer

concentration of 3.1 mM. The hydrogels (600 μL), after being left to equilibrate overnight, were pipetted onto the bottom plate and the geometry was lowered to a gap distance of 54 μm . Time sweep measurements were executed at a frequency of 1.0 Hz and strain of 0.05%, and frequency sweeps were conducted from 0.01–2 Hz with 0.05% strain. Subsequently, a step-strain measurement was performed after a plateau was reached in the storage modulus in the time sweep. Then, 100% strain was applied on the hydrogels for 120 s. The hydrogels were left to recover for 20 min while measuring at 0.05% strain ($f = 1.0$ Hz) and continued with a frequency sweep (from 0.01 to 2 Hz, $\gamma = 0.05\%$), during which the storage modulus returned to the original plateau. This measurement was repeated for two cycles.

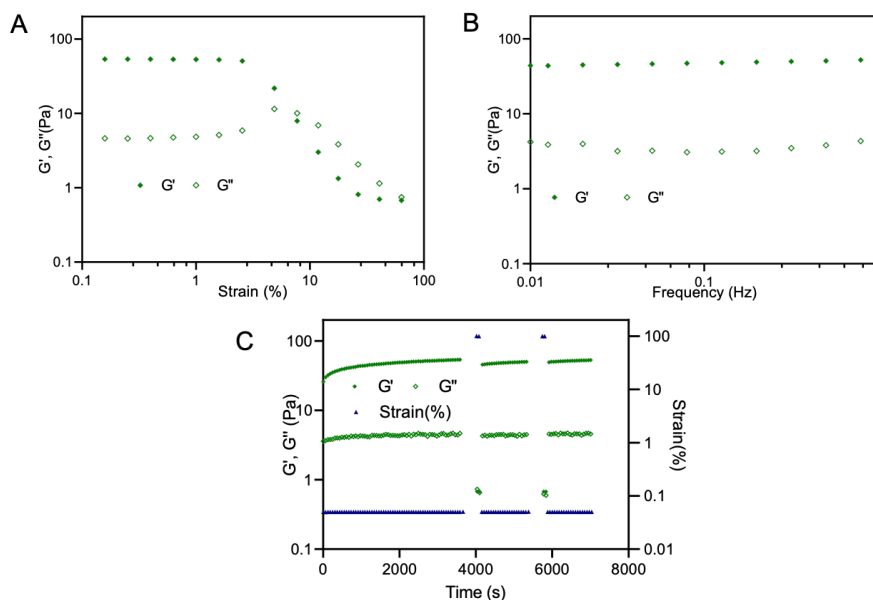


Figure S3.4. Oscillatory rheology measurements of SQ-10RGD (3.1 mM) in PBS at 37 °C: A) Amplitude sweep with a frequency of 1 Hz and strain from 0.1% to 100% (1 Hz). B) Frequency sweep in a range of 0.01 Hz to 2 Hz with strain of 0.05% (N = 3). C) Step-strain measurements with a frequency of 1 Hz.

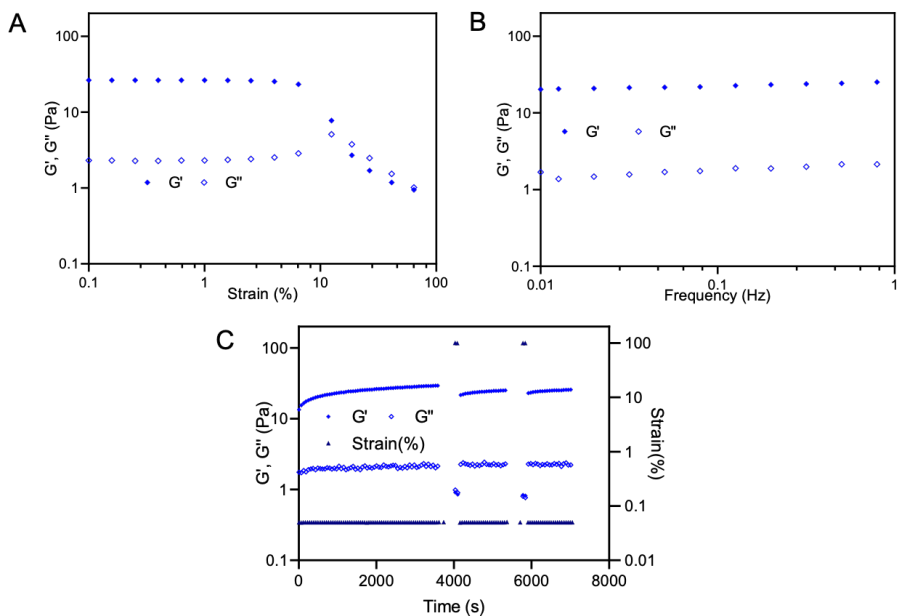


Figure S3.5. Oscillatory rheology measurements of SQ-10AB (3.1 mM) in PBS at 37 °C: A) Amplitude sweep with a frequency of 1 Hz and strain from 0.1% to 100% (1 Hz). B) Frequency sweep in a range of 0.01 Hz to 2 Hz with strain of 0.05% (N = 3). C) Step-strain measurements with a frequency of 1 Hz.

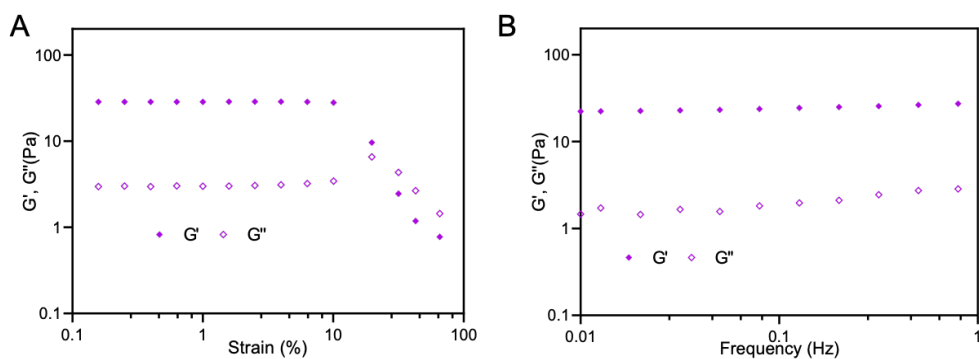


Figure S3.6. Oscillatory rheology measurements of SQ-10COM (3.1 mM) in PBS at 37 °C: A) Amplitude sweep with a frequency of 1 Hz and strain from 0.1% to 100% (1 Hz). B) Frequency sweep in a range of 0.01 Hz to 2 Hz with strain of 0.05% (N = 3).

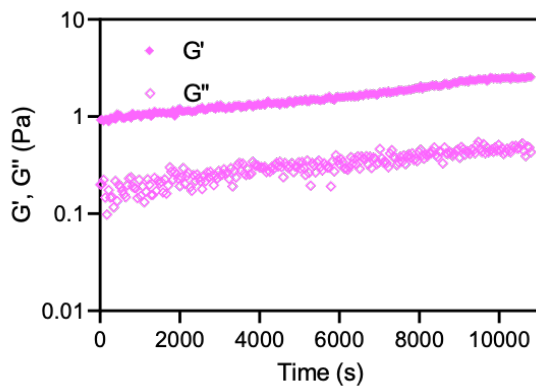


Figure S3.7. Time sweep measurement of SQ-20AB at 37 °C with strain at 0.05% and frequency of 1 Hz.

3.6.5 Cryo-TEM

Cryo-TEM images of vitrified samples were acquired on a Tecnai F20 (FEI Company, The Netherlands) equipped with a field emission gun operating at 200 keV using a Gatan UltraScan charge-couple device (CCD) camera (Gatan company, Germany) with a defocus between -6 and -9 μm . Cryo-TEM samples were prepared by applying 3 μL of SQ-10COM (3.1 mM) without further dilution to a freshly glow-discharged 300 mesh copper grid with a lacey-carbon support film (Supplier-Electron Microscopy Sciences, Pennsylvania, USA). Excess liquid was blotted away for 2 s (95% humidity, 21 °C, Whatman filter paper) and plunge-frozen in liquid ethane at -183 °C using a Leica EM GP (Leica Microsystems, Wetzlar, Germany) before imaging.

3.6.7 Cell Culture on 2D

Human induced pluripotent stem cell line (hiPSCs) LUMC0044iCTRL44.9 (L44) was obtained from Leiden University Medical Center (LUMC). hiPSCs were cultured on six-well plates coated with vitronectin in E8 medium (Stem Cell Technologies). Cells were

passed every 6 days with 0.5 mM EDTA (Sigma-Aldrich) with split ratio of 1:20. Specifically, on the passage day, cells were first washed with PBS and incubated with EDTA (0.5 mM) for 5 min at 37°C followed by 1 min at room temperature. After aspirating the EDTA solution, E8 medium (2 mL) was added to each well. A 2 mL pipet was used to vigorously wash cells with the E8 medium to dissociate them (6-8 times). The replated cells were first treated with E8 medium supplied with Revita- Cell™ (1:200) for 24 h and then refreshed with E8 medium free of RevitaCell™ every day prior to passage. Another hiPSCs LUMC0020iCTRL-6.9 (L20) were maintained by passaging twice per week with the same protocol with a split ratio of 1:10.

3.6.8 hiPSCs encapsulation, release and passage in 3D

For encapsulation, hiPSCs were first dissociated from the vitronectin-coated plates by EDTA (0.5 mM) described above. A small amount (100 µL) of dissociated cells suspension were collected and treated with Trypsin/EDTA (0.05%) at 37°C for 5 min, followed by counting with a hemocytometer to determine the cell number. hiPSCs were pelleted and re-suspended in E8 medium containing RevitaCell™ (1:200) to reach 2.5×10^7 cells/mL, respectively. The prepared cell suspension (20 µL) was mixed with SQ (180 µL) by gently pipetting before being transferred to a µ-Slide well plate (12 µL/well). After gelation at 37 °C for 15 min, the hydrogels were mounted with an additional amount of E8 medium (48 µL) containing RevitaCell™ (1:200) or ROCK inhibitor (10 µM) for culture. The medium was refreshed every 8h for all cultures. For single cell seeding, hiPSCs were dissociated by incubation with Accutase™ (1 mL/well) for 5 min at 37°C, following with counting, pelleting by centrifuge and re-suspending in E8 medium containing RevitaCell™ (1:200) and seeding into hydrogel as described above.

To release the cells as spheroids, the cell-gel mixtures were gently pipetted, diluted by PBS (pH 7.4) (5 mL) and collected by centrifuge at 100 g for 3 min. The

released cell aggregates were further stained by EdU. To release hiPSCs as single cells, the cell-gel mixtures were gently pipetted and diluted by trypsin/EDTA (0.05%) (125 μ L), following with incubation at 37°C for 5 min. The dissociation was stopped by adding E8 medium containing RevitaCell™ (1:200) (500 μ L) and pelleted by centrifuge at 300 g for 5 min. The cell pellet was ready to use for following analysis.

To passage the hiPSCs in squaramide hydrogels, the cell-gel mixtures were gently transferred from 15-well slide into a 15-mL tube that contains 5 mL cold E8 medium. After gently pipetting with a 2-mL serological pipette three times, cells were released and collected by centrifuge (200 g, 1 min). The obtained cell pellets were incubated with EDTA solution (0.5 mM) for 4 min at room temperature. The dissociation was stopped by removing EDTA solution and adding E8 medium containing RevitaCell™ (1:200) (500 μ L) and pelleted by centrifuge at 300 g for 5 min. Finally, the cells were encapsulated in the SQ-10COM gel for further culture as described above.

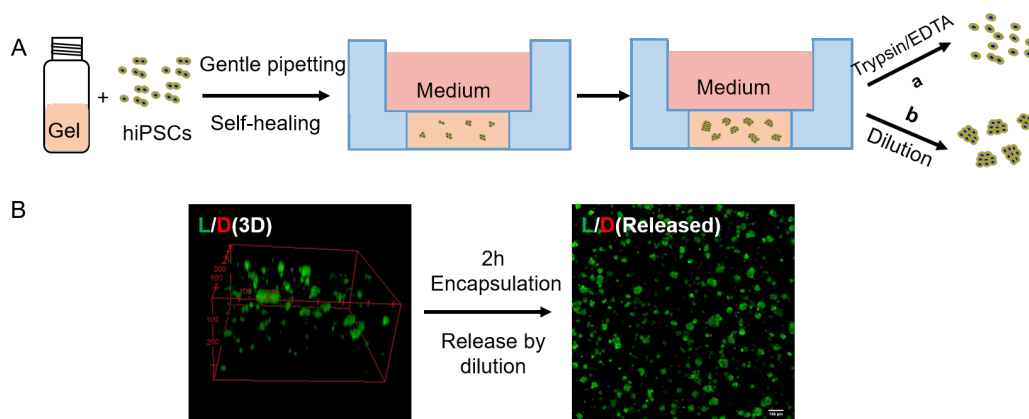


Figure S3.8. A) Encapsulation and release of hiPSCs from SQ-10RGD: Encapsulation was achieved using the self-healing property of the supramolecular materials on gently pipetting with hiPSCs, and gel dissociation was performed by dilution and gentle pipetting with PBS (pH 7.4) which resulted in the release of hiPSC as clumps, or with Trypsin/EDTA (0.05%) which released hiPSCs as single cells for counting and FACS

measurement. B) Clumps released from hydrogels showed high cell viability with LIVE/DEAD staining. Scale bar 100: μm .

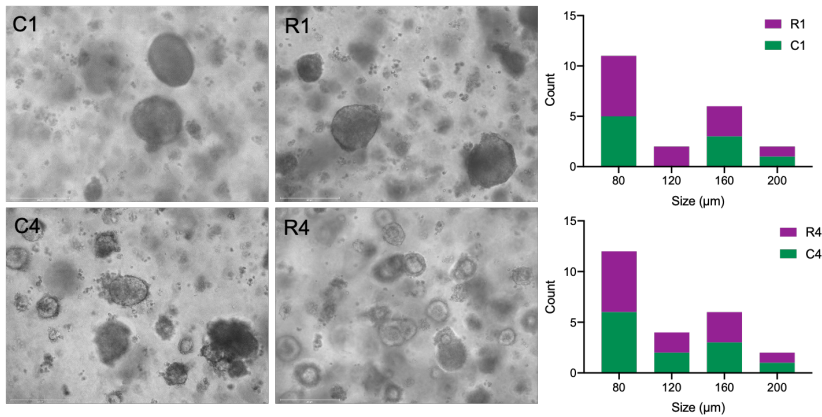


Figure S3.9. Morphology and size distribution of hiPSCs spheroids after 5-day culture in SQ hydrogels. hiPSCs were treated with Rock Inhibitor Y-27632 for 1(R1) and 4(R4) days or RevitaCell™ Supplement for 1 (C1) and 4 (C4) days. Scale bar: 200 μm .

3.6.9 Determination of spheroids diameter and expansion fold.

At pre-determined time points, hiPSCs spheroids were imaged using the EVOS FL AUTO2 equipped with a temperature and CO_2 gas controller. Z-stack images were acquired under a 10 \times objective. The spheroid diameter was measured in Fiji by drawing a straight line horizontally (angle < 3 $^\circ$) with the corresponding scale. During the imaging, the EVOS incubator was turned on to maintain an environment of 37 $^\circ\text{C}$ and 5% CO_2 for the hiPSCs. To calculate the expansion fold, hiPSCs were released from hydrogel as single cells by the method described above. The released cells were counted using a hemocytometer and divided by the number of cells initially seeded within the gels to determine the expansion fold.

3.6.10 Metabolic activity of hiPSCs

The Resazurin metabolism assay was performed to examine metabolic activity of hiPSCs in the supramolecular hydrogels on day 1 and 3. hiPSCs were encapsulated in hydrogels as described above and cultured at 37°C. At pre-determined time points, medium (40 μ L) was removed from each well. The cell-gel mixture (20 μ L) was pipetted into a black 96-well plate, and fresh medium (70 μ L) and resazurin stock solution (10 μ L, 5.4 mM) were added to each well. The plate was incubated at 37°C for 2h and protected from light. Medium without cells was used as a negative control. The fluorescence intensity was recorded on the TECAN plate reader with an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

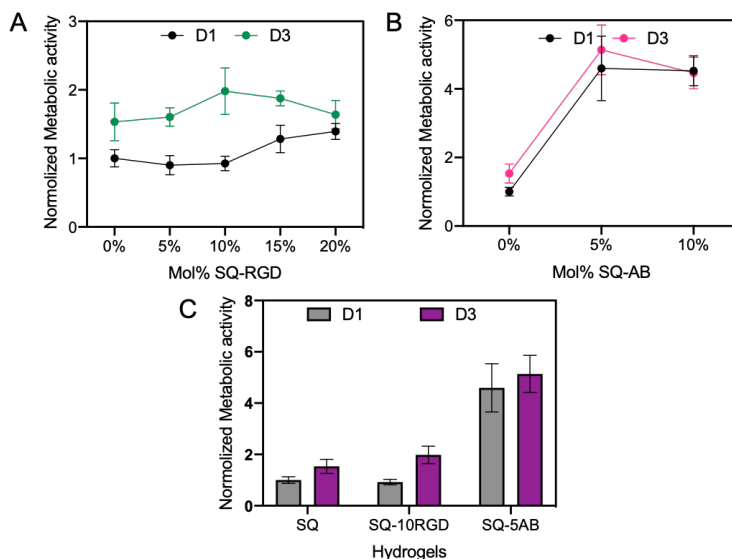


Figure S3.10. A) hiPSCs cultured in the RGD-containing squaramide hydrogel show concentration-dependent metabolic activity in the range of 5 – 20 mol%, with the optimal amount of **SQ-RGD** being 10mol %; B) hiPSCs cultured in AB-containing squaramide hydrogels showed a maximum in metabolic activity using 5 mol% of **SQ-AB**. C) Metabolic activity of hiPSCs cultured in SQ-10RGD and SQ-5AB hydrogels normalized against the SQ hydrogel.

3.6.10 LIVE/DEAD staining

LIVE/DEAD staining of released hiPSCs. The viability of released hiPSCs from hydrogels was assessed using a LIVE/DEAD staining test with calcein AM and propidium iodide. Briefly, released hiPSCs aggregates were first pelleted by centrifuging at 100 g for 3 min and then treated with PBS (200 μ L) containing calcein AM (2 μ M) and propidium iodide (1.5 μ M) at room temperature for 10 min. After staining, the cells were washed with PBS (200 μ L) and resuspended in PBS (50 μ L) before imaging. The cell suspension was transferred to a chamber slide and imaged on a Leica SP8 confocal laser scanning microscope equipped using a 10 \times objective. Fluorescent images were acquired at a resolution of 1024 \times 1024 pixels using an excitation wavelength of 488 nm and an emission filter of 500–545 nm for calcein AM and an excitation wavelength of 532 nm and an emission filter of 594–686 nm for propidium iodide.

LIVE/DEAD staining of hiPSCs in 3D. The viability of expanded hiPSCs in the hydrogels in 3D were assessed using the same LIVE/DEAD staining reagents above. At pre-determined time points, the medium was removed from top of the hydrogel by pipetting, rinsed with PBS two times (pH 7.4, 45 μ L) and incubated with the staining solution (calcein AM (2 μ M) and propidium iodide (1.5 μ M)) at 37 $^{\circ}$ C for 30 min. Afterwards, the staining solution was removed and the hydrogel was rinsed again with PBS two times before imaging. The stained cell-laden hydrogel was imaged with Leica SP8 confocal laser scanning microscope equipped under 10 \times and 40 \times objectives.

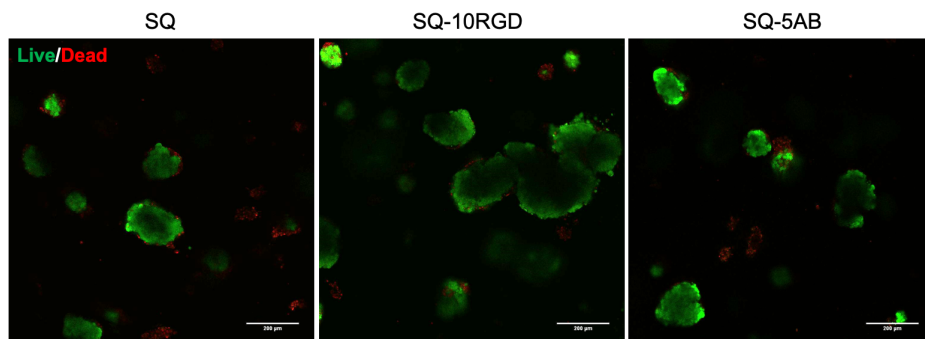


Figure S3.11. Cell viability assay of hiPSCs after 5 days of culture in SQ, SQ-10RGD and SQ-5AB, respectively. LIVE/DEAD staining with calcein AM (viable cells, *green*) and propidium iodide (PI) (dead cells, *red*). Scale bar: 200 μm .

3.6.12 EdU staining

Before EdU staining, hiPSCs spheroids were released from hydrogel by the method described above. The labelling of proliferating cells was executed according to Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen). Briefly, the released hiPSC spheroids were first incubated with EdU solution (10 μM) at 37 $^{\circ}\text{C}$ for 4h. After labelling, hiPSCs spheroids were fixed with 5% (w/v) paraformaldehyde solution at room temperature for 15 min and then incubated with PBS containing 0.5% Triton X-100 at room temperature for 20 min. After washing, the Click-iT[®] reaction cocktail was first added and incubated for 30 min, followed by a second wash and Hoechst 33342 (5 $\mu\text{g}/\text{mL}$) incubation for another 30 min to stain nuclei. The stained hiPSCs spheroids were transferred into a chamber slide after washing with PBS and imaged with Leica SP8 confocal laser scanning microscope equipped with a 63 \times oil objective. Fluorescent Z-stack images were acquired at a resolution of 1024 \times 1024 pixels with a step size at 1 μm . The Z-stack images from 3D hiPSCs spheroids were projected into 2D by the Fiji software.

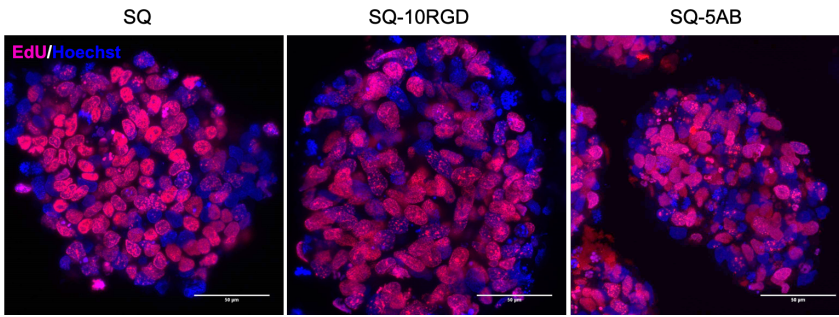


Figure S3.12. Representative images of EdU staining in hiPSCs spheroids released from SQ, SQ-10RGD and SQ-5AB hydrogels, respectively. Proliferating cells were labelled with EdU-Alexa fluor 594 (*red*) and cell nuclei were stained with Hoechst 33342 (*blue*). Scale bar: 50 μm .

3.6.13 Flow cytometry measurement

HiPSCs were released from the supramolecular hydrogels as single cells with trypsin/EDTA (0.05%) to assess the pluripotency marker expression of cells expanded in 3D hydrogels. The single cell suspension was incubated with TRA-1-60-PE and SSEA-4-FITC fluorescently labeled antibodies (1: 10 dilution in FACS buffer) at 4 °C for 30 min. After washing with PBS, hiPSCs were suspended in FACS buffer and recorded on a Guava® easyCyte Flow Cytometer.

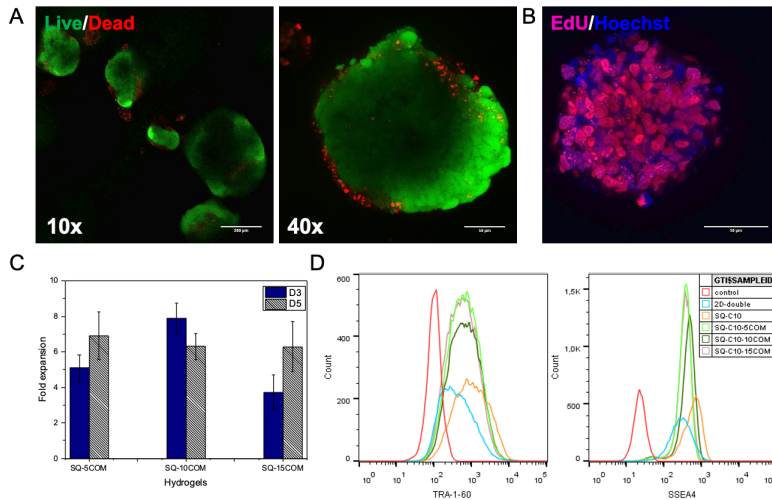


Figure S3.13. A 5-day culture of hiPSCs seeded as clumps in integrin-targeting squaramide hydrogels. A) LIVE/DEAD cell staining of hiPSCs after 5 days of culture in SQ-10COM. Scale bar: 200 μm (10x) and 50 μm (40x). B) Representative images of EdU staining from hiPSCs spheroids that were released from SQ-10COM. Scale bar: 50 μm . C) Expansion fold of hiPSCs in integrin-targeting squaramide hydrogels that were quantified on day 3 and 5. D) FACS measurement of pluripotency marker expression of TRA-1-60 and SSEA4 from hiPSCs after 5 days of culture.

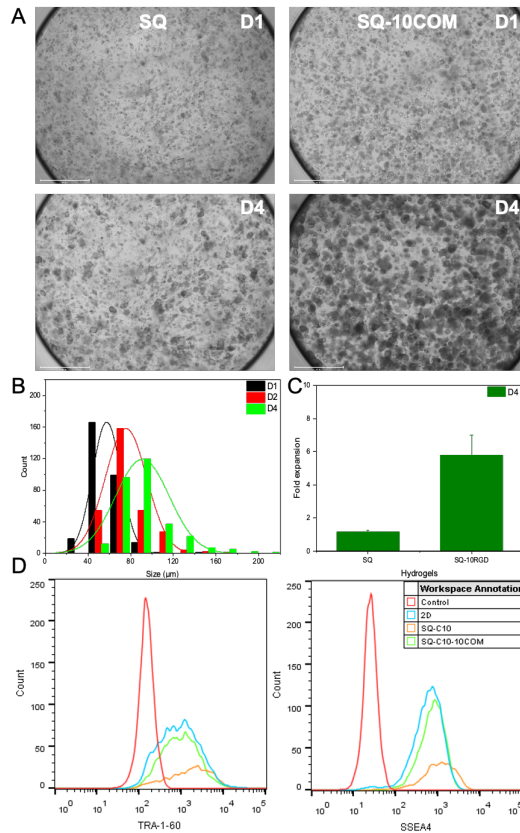


Figure S3.14. A 4-day culture of hiPSCs seeded as single cell in SQ-10COM. A) Brightfield microscopy images of hiPSC spheroids. Scale bar: 750 μm . B) Size distribution of hiPSC spheroids expanded in SQ-10COM, 300 spheroids were counted. C) Expansion fold of hiPSCs in SQ and SQ-10COM hydrogels quantified after 4 days of culture. D) FACS measurement of pluripotency marker expression of TRA-1-60 and SSEA4 from hiPSCs after 4 days of culture.

3.6.14 Cardiomyocytes differentiation

HiPSCs were released as clumps from a Matrigel coated plate according to the protocol described in Figure S3.8 (path b). The differentiation of hiPSCs were performed using the STEMdiff™ Cardiomyocyte Differentiation Kit from StemCell Technologies. At pre-determined time points, hiPSCs were imaged using the EVOS FL AUTO2 equipped with

a temperature controller and CO₂ gas. The video of beating hiPSCs-derived cardiomyocytes were recorded on the EVOS FL AUTO2.

Video S3.1. Spontaneously beating cardiomyocytes cells differentiated from hiPSCs (Day 8) (<https://surfdrive.surf.nl/files/index.php/s/HiVGGR1yG2MneDg>)

Video S3.2. Spontaneously beating cardiomyocytes cells differentiated from hiPSCs (Day 10) (<https://surfdrive.surf.nl/files/index.php/s/g9jFZlHaMHfPBxg>)

Video S3.3. Spontaneously beating cardiomyocytes cells differentiated from hiPSCs (Day 16) (<https://surfdrive.surf.nl/files/index.php/s/IJWA8LmopH5Xvd8>)

3.6.15 Global screening array

The Global Screening Array (GSA) (Illumina) was used according to standard procedures, followed by an analysis in GenomeStudio software (Illumina) using the GSA manifest files. GenomeStudio final reports were used to analyze and visualize in Nexus Discovery (BioDiscovery El Segundo). A report resolution of ~50 kb was used to analyze the data for chromosomal aberrations.

3.6.16 Reference

- [1] C. Tong, T. Liu, V. Saez Talens, W. E. M. Noteborn, T. H. Sharp, M. M. R. M. Hendrix, I. K. Voets, C. L. Mummery, V. V. Orlova, R. E. Kieltyka, *Biomacromolecules* **2018**, *19*, 1091.