Disulfide-Based Diblock Copolymer Worm Gels: A Wholly-Synthetic Thermo-reversible 3D Matrix for Sheet-Based Cultures

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ABSTRACT: It is well known that 3D *in vitro* cell cultures provide a much better model than 2D cell cultures for understanding the *in vivo* microenvironment of cells. However, significant technical challenges in handling and analyzing 3D cell cultures remain, which currently limits their widespread application. Herein we demonstrate the application of *wholly synthetic* thermo-responsive block copolymer worms in sheet-based 3D cell culture. These worms form a soft, free-standing gel reversibly at 20-37 °C, which can be rapidly converted into a free-flowing dispersion of spheres on cooling to 5 °C. Functionalization of the worms with disulfide groups was found to be essential for ensuring sufficient mechanical stability of these hydrogels to enable long-term cell culture. These disulfide groups are conveniently introduced via statistical copolymerization of a disulfide-based dimethacrylate under conditions that favour intramolecular cyclization and subsequent thiol/disulfide exchange leads to the formation of reversible covalent bonds between adjacent worms within the gel. This new approach enables cells to be embedded within micrometer-thick slabs of gel with good viability, permits cell culture for at least 12 days, and facilitates recovery of viable cells from the gel simply by incubating the culture in buffer at 37 °C (thus avoiding the enzymatic degradation required for cell harvesting when using commercial protein-based gels such as *Matrigel*).

Introduction

Three-dimensional (3D) cell culture systems are attracting increasing attention because they provide a microenvironment that more closely resembles that of tissue *in vivo*, than conventional two-dimensional (2D) cell culture systems.[1-4] The 3D microenvironment includes cell-cell and cell-extracellular matrix (ECM) interactions, which are both known to regulate signalling and differentiation of cells. The 3D structure also influences local gradients that govern mass transport of oxygen, glucose, metabolites, and signalling molecules.[4-6] However, measuring cellular viability and phenotype in hydrogel-based 3D cell cultures[7] often requires specialized histology[8] and/or optical techniques that restrict their widespread use in cell-based assays.

Herein, we describe the application of a thermo-responsive diblock copolymer for embedding cells in mesh sheets for 3D cell culture. This poly(glycerol monomethacrylate)-block-poly(2-hydroxypropyl methacrylate) (PGMA-PHPMA) diblock copolymer is synthesized via RAFT aqueous dispersion polymerization.[9-11] By targeting an appropriate copolymer composition, highly anisotropic worm-like particles are formed *in situ* by polymerization-induced self-assembly (PISA). These worms can form relatively soft, free-standing hydrogels at either ambient (20°C) or physiological temperatures (37°C). However, on cooling to 4°C, the PGMA-PHPMA worms become plasticized and are converted into spheres, which leads to *in situ* degelation and produces a free-flowing dispersion.[11, 12] This transition is fully reversible: on warming the solution, one-dimensional fusion of multiple spheres leads to reformation of the original worms at 20 °C, with concomitant rapid regelation.

Thus this diblock copolymer provides a highly convenient thermo-responsive hydrogel from which cells can be embedded and recovered without recourse to proteolytic enzymes such as

trypsin. Moreover, commercially available protein-based gels for cell culture are relatively expensive and must be stored at low temperature ($\leq$ 4 °C) to prevent their irreversible chemical degradation. In contrast, this new wholly-synthetic hydrogel can be stored indefinitely at room temperature, and still gel reversibly on demand.

Over the past two decades, various hydrogels have emerged as a useful matrix to create 3D structures for either supporting or encapsulating cells *in vitro*.[13] For example, Bissell *et al.* demonstrated that malignant and non-malignant breast cancer cells can be distinguished on the basis of differences in cell morphology and gene expression when cultured in 3D using *Matrigel*[5,14-17] (a protein-based hydrogel extracted from Engelbreth-Holm-Swarm mouse sarcoma cells), but not when cultured in 2D.[17] In principle, hydrogels suitable for 3D cell culture can either be derived from natural biopolymers,[7, 18] or from synthetic polymers.[19] Protein-based hydrogels (e.g., *Matrigel*, collagen, silk), polysaccharides (e.g., hyaluronate, chitin) and polynucleotides (e.g., DNA, RNA) typically contain various bio-active species such as laminin, collagen, and entactin that promote cellular growth and signalling.[13, 20] Moreover, these components often vary in composition and concentration between batches, which can introduce artifacts in cell biology studies. [7, 21] Furthermore, such biopolymers have a limited shelf-life and are relatively expensive.[7]

In contrast, synthetic hydrogels based on poly(ethylene glycol), polyacrylamide, poly(*N*-isopropylacrylamide), poly(vinyl alcohol) or poly(acrylic acid)[19] have a user-defined composition and provide a cost-effective, reproducible and tuneable environment for 3D cell culture studies. Nevertheless, these synthetic hydrogels lack the chemical functionality required to promote biologically-relevant cell-matrix contacts. Furthermore, efficient harvesting of the embedded cells requires enzymatic, thermal, chemical, or optical disruption

of the hydrogel cross-links. These degradation strategies can compromise cellular viability[18] and this problem, in addition to other challenges associated with 3D cultures, has hitherto limited the applicability of such hydrogels in 3D cell culture.[7]

In this context, our PGMA-PHPMA worm gels offer a number of potentially decisive advantages. Their wholly synthetic nature is important because this ensures better batch-to-batch reproducibility, which is a general problem for animal-derived products. Moreover, the low-viscosity fluid obtained on cooling these worm gels is amenable to cold ultrafiltration, which provides a facile route to sterilization compared to other synthetic hydrogels.[11] Small molecule impurities such as unreacted HPMA monomer can be readily removed by dialysis, and subsequent freeze-drying enables the worms to be conveniently redispersed in a wide range of cell culture media.[22] Such reconstituted worm gels appear to be a promising matrix for 3D cell culture since embedded mammalian cells exhibit good viability.[11] Very recently, we reported that the gel modulus can be fine-tuned by placing disulfide groups on the surface of the worms, since this leads to covalent bonds being formed between adjacent worms via thiol/disulfide exchange.[23] As we demonstrate in this work, such 'second generation' worms are essential if more resilient hydrogels are required for long-term cell studies.

Previously, we and others have demonstrated that multi-zone sheets of paper or polymer-based mesh (Figure 1) can be used to support cell-embedded gels (so-called "Cells-in-Gels-in-Paper", CiGiP and "Cells-in-Gels-in-Mesh", CiGiM).[24-29] Upon spotting with a micropipette, a cell suspension in *Matrigel* at 4 °C readily wicks through the sheets of paper or mesh. Upon incubation in media at 37 °C, the suspension forms a gel within the voids of the sheets. This approach provides a powerful method for manipulating and analysing 3D cell cultures.[24-29]

Here we report the use of disulfide-functionalized PGMA-PHPMA worm gels as a cost-effective alternative to *Matrigel* for embedding cells in sheets of polymer mesh or paper-based sheets. This combination of the synthetic worm gel and the mesh offers several important advantages over the analogous *Matrigel*-mesh constructs: (i) the thickness of the cell culture can be controlled by choosing mesh sheets of appropriate size, (ii) the progress of cell growth and proliferation can be monitored *in situ*, since the mesh sheets allow transmission of light with minimal scattering, (iii) the cells can be harvested by simply immersing the sheets in cold buffer or media (instead of requiring enzymatic degradation), (iv) the gel can be heated and cooled reversibly without significantly changing its physical properties, and thus does not require stringent long-term storage conditions; (iv) the synthetic worm gel avoids the use of biologically-derived materials.

Experimental

Synthesis and Preparation of Worm Gels

Materials

Glycerol monomethacrylate (GMA; 99.8 %) was donated by GEO Specialty Chemicals (Hythe, UK) and used without further purification. 2-Hydroxypropyl methacrylate (HPMA) and 4,4'-azobis(4-cyanopentanoic acid) (ACVA; V-501; 99 %) were purchased from Alfa Aesar (Heysham, UK). 2-Cyano-2-propyl dithiobenzoate (CPDB, 80 % as judged by ^{1}H NMR spectroscopy) was purchased from Strem Chemicals (Newton, UK). Bis(2-(methacryloyloxy)ethyl disulfide monomer (DSDMA) was synthesized according to a protocol reported by Rosselgong et al.[31] CD_3OD (99.8 %) and CD_2Cl_2 (99.8 %) were purchased from Goss Scientific (Nantwich, UK) and used as received. All solvents were of

HPLC quality; they were purchased from Fisher Scientific (Loughborough, UK) and used as received.

Synthesis of $PGMA_{54}$ macro-CTA

CPDB RAFT agent (0.864 g, 3.9 mmol) and GMA monomer (25.0 g, 156.1 mmol) were weighed into a 100 mL round-bottomed flask and purged under N_2 for 30 min. Into the same flask, ACVA initiator was added (218.6 mg, 0.78 mmol; CTA/ACVA molar ratio = 5.0) followed by anhydrous ethanol (49.6 mL; previously purged with N_2 for 30 min). The flask was subsequently sealed and immersed in an oil bath set at 70 °C. After 100 min, the polymerization was quenched by exposing to air, immersing in liquid nitrogen for 30 seconds followed by diluting the solution with methanol (100 mL). A final GMA conversion of 78 % was determined by ^{1}H NMR analysis. The methanolic solution was precipitated into a ten-fold excess of dichloromethane. After filtering and washing with dichloromethane, the crude polymer was dissolved in water and the residual dichloromethane was evaporated under vacuum. The resulting aqueous solution was freeze-dried overnight to yield a pink powder. ^{1}H NMR analysis indicated a mean degree of polymerization of 54 for this PGMA macro-CTA. Using a refractive index detector and a series of near-monodisperse poly(methyl methacrylate) calibration standards, DMF GPC analysis indicated an M_n of 14,700 g mol^{-1} and an M_w/M_n of 1.11 (Figure S1).

Copolymerization of DSDMA with GMA via RAFT to afford P(GMA_{55}-stat-$DSDMA_{0.50}$)

CPDB RAFT agent (80 % purity; 0.192 g, 0.69 mmol), GMA monomer (5.00 g, 31.3 mmol) and DSDMA monomer (0.101 g, 0.347 mmol) were weighed into a 100 mL round-bottomed flask and purged under N_2 for 30 min. ACVA initiator (38.9 mg, 0.139 mmol; CTA/ACVA

molar ratio = 5.0) and anhydrous ethanol (47.6 mL; previously purged with N_2 for 30 min) were then added to the same flask and the resulting red solution was degassed for an additional 10 min. The flask was subsequently sealed and immersed in an oil bath set at 70 °C. After 18 h, the polymerization was quenched by immersing the flask in liquid nitrogen. A final GMA conversion of 90 % was determined by ^{1}H NMR analysis. Overnight storage of this ethanolic reaction solution at -25 °C caused precipitation of the $PGMA_{55}$-$DS_{0.50,}$ and thus enabled collection of the precipitate by decanting the supernatant solution containing the residual comonomers. This precipitate was dissolved in methanol (100 mL) and then reprecipitated into a ten-fold excess of dichloromethane. After filtering and washing with dichloromethane, the copolymer was dissolved in water and the residual dichloromethane was evaporated under vacuum. The resulting solution was freeze-dried overnight to yield a pink powder. ^{1}H NMR analysis of this PGMA macro-CTA indicated a mean degree of polymerization of 55. DMF GPC analysis indicated an M_n of 16,100 g mol^{-1} and an M_w/M_n of 1.27 (see Figure S1 in Supporting information).

Synthesis of disulfide-functionalised poly(glycerol monomethacrylate)$_{55}$-poly(2-hydroxypropyl methacrylate)$_{130}$ [0.30P(GMA$_{55}$ -stat-DSDMA$_{0.50}$) + 0.70PGMA$_{54}$]-H$_{130}$ worm gel via RAFT aqueous dispersion polymerization of HPMA

$PGMA_{54}$ macro-CTA (2.519 g, 0.279 mmol), $PGMA_{55}$ -$DSDMA_{0.50}$ macro-CTA (1.080 g, 0.120 mmol), HPMA monomer (7.49 g, 51.94 mmol; target DP = 130), ACVA (22.3 mg, 0.080 mmol; CTA/ACVA molar ratio = 5.0) and, 0.15 M PBS (44.0 g, to produce a 20% w/w aqueous solution) into a 100 mL round-bottomed flask. The flask was placed on ice and purged with N_2 for 30 min. Following this degassing protocol, the flask was immersed in an oil bath set to 70 °C. The reaction solution was stirred for 3 h before the RAFT

polymerization was quenched by exposure to air. Full monomer conversion was confirmed by ^{1}H NMR spectroscopy (complete disappearance of vinyl signals) and DMF GPC analysis indicated an M_n of 40,300 g mol^{-1} and an M_w/M_n of 1.17 (see Figure S1 in the Supporting information).

Transmission Electron Microscopy (TEM)

Copolymer dispersions, micropipet tips, water, staining agent and TEM grids were incubated at the desired temperature (37°C or 4°C) before the copolymer dispersion was diluted to 0.20% w/w, which is well below the critical gelation concentration for such worm gels. Copper/palladium TEM grids (Agar Scientific, U.K.) were coated in-house to produce a thin film of amorphous carbon. These grids were then treated with a plasma glow discharge for 30 s to create a hydrophilic surface. Each aqueous diblock copolymer dispersion (12 μL; 0.20% w/w) was placed on a freshly treated grid for 1 min and then blotted with filter paper to remove excess solution. To stain the deposited nanoparticles, an aqueous solution of uranyl formate (9 μL; 0.75% w/w) was placed on the sample-loaded grid via micropipet for 20 s and then carefully blotted to remove excess stain. Each grid was then carefully dried using a vacuum hose. Imaging was performed using a FEI Tecnai Spirit TEM instrument equipped with a Gatan 1kMS600CW CCD camera operating at 120 kV.

Evaluation of the Mechanical Properties of the Worm Gels via Oscillatory Rheology Studies

Experiments were conducted using an AR-G2 rheometer (TA Instruments) equipped with a variable temperature Peltier plate, a 40 mm 2° aluminium cone and a solvent trap to prevent

evaporation of water over the time scale of the experiment. The loss moduli (G") and storage moduli (G') were recorded as a function of temperature to determine the gel strength and critical gelation temperature (CGT). Temperature sweeps were conducted at a constant angular frequency of 1.0 rad s^{-1} and a constant strain of 1.0 %. The temperature was increased by 1.0 °C between each measurement, allowing an equilibration time of 2 min in each case.

Fabrication of PVC-Polyester Mesh Composite Sheets

The scaffolds were prepared using a protocol modified from that described by Simon et al.[24] A craft cutter (Graphtec Craft ROBO) was used to cut the patterns and perforations (~ 3mm in diameter) in a 130 μm-thick sheet of poly(vinyl chloride) (PVC; Warp Bros); the pattern was designed in Adobe Illustrator C4 and can be provided upon request. A heated press was used to melt the sheet of PVC between two ~90 μm-thick sheets of polyester mesh (McMaster-Carr); the sheets were pressed at 211 °C in three to four cycles of 10-15 seconds to form a single composite sheet (~200 μm thickness). The composite sheets were subsequently placed in a glass petri dish and sterilised by autoclaving. The autoclaved composite sheets were stored in a dry Parafilm-sealed container prior to use.

Cell Culture of A549-GFP Cells

A549-GFP cells were cultured in 1X Dulbecco's Modified Eagle Medium (Gibco) with 10% (v/v) fetal bovine serum (HyClone), 1% (v/v) Penicillin-Streptavidin (Gibco). The cells were maintained as adherent cultures in a vented tissue culture flask (Corning) at 37°C and 5%CO_2, and were passaged every 4 to 5 days until use. Green fluorescence protein was expressed in A549 cells (American Type Culture Collection) by transduction with Cignal

Lenti GFP (Quiagen) and 5 mg/mL polybrene (Santa Cruz Biotech), as described by Mammoto et al.[31]

Preparation of 3D Cell Culture

Micropipette tips, worm gels, and composite sheets were chilled in a 4°C refrigerator at least 24 h before seeding the cells. The cells were detached from the tissue culture flask by treatment with TrypLE™ Express (Gibco) for 5 min at 37°C, suspended in media and pelletised by centrifugation at 1,500 rpm. In a typical experiment, the suspension of cells was prepared in either cold 10 % w/w worm gel or cold *Matrigel* at a concentration of 3×10^4 cells/μL suspension. The suspensions, micropipette tips, and unused composite sheets were stored at 0°C to avoid premature gelation of the cell suspensions. 1 μL of the cell suspension was spotted into the zones of the composite sheets, followed by their immersion in warm media (37°C) in 6-well plates.

Imaging of Cells in the Scaffolds

The A549-GFP cells cultured in the composite sheets or in the 96-well plates were visualised using a fluorescence microscope with a CCD camera. A Typhoon FLA 9000 gel scanner (General Electric) with a resolution of 50 μm and a photomultiplier tube setting of 300 V were used to analyse the intensity of GFP expressed by A549 cells in the composite sheets. The fluorescence intensity for each zone was calculated from the corresponding image using Image J software (NIH).

Recovery Protocol

Cells were recovered by incubating the composite sheets in a 6-well plate containing 1 mL per well of the recovery solution. Cold PBS (4°C, pH 7.4) was used as the recovery solution

for cells embedded in worm gels, and warm *Accumax* (37°C) was used for cells embedded in *Matrigel*. After recovery, cells were washed with cold PBS, and the recovery solution was removed by centrifugation at 1,500 rpm.

Culture and Viability Assay of Recovered Cells

Suspensions of the recovered cells were prepared in media at a concentration of 3×10^4 cells/mL media. 100 μL of the cell suspension was dispensed into 96-well plates and cultured at 37 °C and 5 %CO_2 prior to cell viability measurements using CellTiter-Glo® (CTG) assay. The CTG reagent was prepared as recommended by the manufacturer (Promega). Into each well, 100 μL CTG reagent was added, and the samples were incubated for 20 min. The luminescence was measured using a PHERA star FS microplate reader (BMG Labtech).

Results and Discussion

In preliminary experiments, *non-functionalised* PGMA-PHPMA[11, 12] worm gels were supported on either paper or a composite sheet of polyester mesh and poly(vinyl chloride). However, these 'first-generation' worm gels proved to be insufficiently robust and became partially detached from the sheets after 9 days (see Figure S2 in the Supporting information). Therefore, we designed a 'second-generation' worm gel that adhered more strongly to the mesh sheet; this new gel contained disulfide bonds within some of the PGMA stabiliser chains (Scheme 1), which were introduced using a strategy previously reported for disulfide-functionalised nano-objects.[23, 32] Briefly, a disulfide dimethacrylate (DSDMA) comonomer was statistically copolymerised with GMA via RAFT solution polymerization to produce a well-defined poly(glycerol monomethacrylate)-*stat*-disulfide dimethacrylate ($PGMA_{55}$-$DSDMA_{0.50}$) macromolecular chain transfer agent (macro-CTA) (M_n = 16,100 g mol^{-1},

M_w/M_n = 1.27). This copolymerization was conducted in relatively dilute solution (10 % w/w) in order to suppress intermolecular branching, and hence favour intramolecular cyclization.[33] A 7:3 binary mixture of $PGMA_{54}$ and $PGMA_{55}$-$DSDMA_{0.50}$ macro-CTAs was used for the subsequent RAFT dispersion polymerization of HPMA (Scheme 1) to produce well-defined diblock copolymer chains (M_n = 40,300 g mol^{-1}, M_w/M_n = 1.17; see GPC curves shown in Figure S1).

Polymerization of HPMA using the binary mixture of $PGMA_{54}$ and P(GMA_{55}-stat-$DSDMA_{0.50}$) macro-CTAs at 20 % w/w solids produced a free-standing worm gel at 20 °C.

For rheological measurements, the worm dispersion was diluted to 8 % w/w solids; this dispersion remained a free-standing gel at 37 °C and was transformed into a free-flowing fluid at 4 °C (Figure 2). TEM images confirmed the presence of worms at the former temperature, and a mixture of spheres and rather short worms at the latter temperature. Rheology measurements indicated that the 8.0 % w/w worm gel exhibited an initial storage modulus (G') of around 20 Pa in pH 7.4 PBS at 37 °C (Figure 3a).

To evaluate the effect of long-term storage, we incubated five identical worm gels at 37 °C (pH 7.4), and then performed rheological measurements after 1, 2, 3, 4 or 5 days. After initial, physical gelation, the G' value increased from 20 Pa to 50 Pa over this time period (see Figure 3b), which suggests the formation of inter-worm covalent bonds via thiol-disulfide exchange as the gel is aged at 37 °C.[24] We also monitored changes in the physical properties of these dispersions, which were stored as gels at 37 °C for 5 days, then cooled to 2 °C to induce degelation. The data shown in Figure 3b (green data set) indicate (i) a slight shift in the critical gelation temperature (CGT) to 13 °C and (ii) an increase in G' from 0.03 Pa to 0.40 Pa for the cold, de-gelled samples. This suggests the formation of additional inter-

particle disulfide bonds. Nevertheless, the G' of the aged worm gel remained relatively low and, in particular, its highly desirable thermo-reversible behaviour was retained.

Because thiol-disulfide exchange led to inter-worm cross-linking, we hypothesised that this 'binary mixture of macro-CTAs' approach should produce worm gels that were strong enough to prevent premature detachment from the supporting mesh sheet. To examine whether this was the case, [26, 27] we spotted 1 μL suspensions of fluorescent 10 μm polystyrene microspheres into cold (2 °C) worm gels within zones of sheets of mesh (or paper),[24, 25, 34] and then immersed the sheets in warm PBS (37 °C, pH 7.4) for nine days. Fluorescence images revealed that the microspheres, and consequently the disulfide-functionalised worm gel, remained within the original zones (Figure 4). This confirmed that functionalization with disulfide groups produces more robust worm gels with better long-term stability with respect to degelation.

To determine whether cells embedded in these disulfide-based worm gels were still capable of proliferation, we spotted 1 μL suspensions of A549-GFP cells (3×10^4 cells per zone) into worm gels within zones of the mesh sheets, and observed changes in cell density over the course of 12 days. Optical microscopy studies indicated that the density of A549-GFP cells increased progressively from the first to the fifth day of culture (Figure 5a). Cellular densities of A549-GFP cells became indistinguishable after 5 days of culture, so we imaged the cell-impregnated sheets with a fluorescence gel scanner to assess whether proliferation continued over longer time periods. Fluorescence intensities increased linearly over time (Figure 5b), which confirms that these cells remain viable and proliferative for at least 12 days while embedded within the worm gel. Unlike protein-based hydrogels such as *Matrigel*, synthetic worm gels lack the chemical functionality (e.g., peptides such as RGD, growth

factors, focal adhesion proteins, etc.) required to promote cell growth.[7, 21] In view of this, it may be somewhat surprising that proliferation is observed at all, but it should be noted that it is well-known that such A549 cancer cells require little or no stimulus to proliferate.

Conventional cell culture methods require proteases (e.g., trypsin, *Accumax*, *Accutase* etc.) to release cells from 2D surfaces or to recover cells from protein-based gels.[35] In contrast, cells cultured within thermo-responsive gels (e.g., poly(*N*-isopropylacrylamide)-based gels)[36] can be isolated by simply cooling to liquefy the gel, thus releasing the cells from the gel matrix. To evaluate the latter strategy for worm gels, we incubated the mesh sheets containing A549-GFP cells embedded in worm gels in cold (4 °C) Dulbeccos's phosphate buffer saline (DPBS), and imaged the mesh sheets containing the cells immersed in worm gels using an optical microscope and a fluorescence gel scanner. The cell-embedded worm gels gradually detached from the mesh after incubation in cold DPBS (Figure 6a). We estimated the percentage of cells retained in the mesh sheets by measuring the fluorescence intensities from the zones as a function of time, and then calculating the reduction in fluorescence at each time point relative to the fluorescence of the zones before cooling. Figure 6b shows the reduction in GFP intensity over time: approximately 91 ± 6 % of the cells detached from the mesh sheets after 60 min in cold DPBS. GFP intensities determined for each time point indicated that the efficiency in the recovery of A549 cells in worm gels on cooling is comparable to that achieved when cells were recovered enzymatically from *Matrigel*.

Cell recovery from the worm gels requires non-physiological conditions (i.e. incubation in cold media), which could potentially compromise cellular viability. To determine whether the recovered cells remained viable, we suspended A549-GFP cells (isolated either from worm gels or *Matrigel*) in culture media, dispensed the suspensions (~2,000 cells per zone) in 96-

well plates, cultured for seven days, and measured the luminescence from the reaction of ATP (indicating metabolically-active cells) at various time points using CellTiter-Glo® (CTG) assay. We estimated the viability of the recovered cells over time by calculating the ratio of the luminescence intensity at each time point relative to that determined at the start of the culture (24 h after dispensing the recovered cells in the well plates). The cellular density and normalized ATP levels of A549-GFP cells recovered from the worm gels increased monotonically up to a week after recovery (Figures 7a and 7b), which indicates good viability. However, cells recovered from worm gels proliferated less readily than those recovered from *Matrigel* (Figure 7b). This difference most likely reflects the fact that, unlike *Matrigel*, the worm gels lack the various proteins and growth factors that are known to promote cellular growth and proliferation.[7 21, 37]

Conclusions

In summary, sheet-supported 3D cell culture provides a convenient means of handling and analysing 3D cell cultures, while thermo-responsive hydrogels provide a convenient vehicle to deliver and embed cells into the sheets. The disulfide-functionalized PGMA-PHPMA diblock copolymer hydrogel described herein is expected to be preferable to commercial protein-based gels, particularly for applications where the biological effects of such animal-derived gels are not acceptable, or are simply too variable.[38-40] This new synthetic hydrogel permits 3D culture of cells supported in mesh sheets and can be used to evaluate the effects of cell-ECM proteins for at least 12 days. The efficiency of cell recovery is comparable to that achieved via enzymatic degradation. A549-GFP cells released from such worm gels remain viable and can be further cultured or analysed directly. In principle, thiol-disulfide chemistry can be used for convenient chemical functionalization of these hydrogels with

RGD, DNA or adhesion proteins to evaluate how such biomolecules influence cellular growth and proliferation. Thus these third-generation hydrogels should enable the effects of the bio-active species to be decoupled from the effect of growth factors that are typically present in protein-based gels.

ASSOCIATED CONTENT

Supporting Information. Microscopy images of PVC-lens paper composite sheet impregnated with first-generation worm gels and GPC chromatograms obtained for the various polymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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List of Schemes

Scheme 1. Synthesis of disulfide-functionalized PGMA-PHPMA worms via RAFT aqueous dispersion polymerization of HPMA at 70°C

List of Figures

Figure 1. (a) Fabrication of PVC-mesh composite sheets. (b) Digital photograph of a multi-zone mesh sheet.

Figure 2. Digital photographs and TEM images obtained at 37 °C and 2 °C illustrating the temperature-dependent (de)gelling behaviour of the disulfide-functionalized copolymer worms used in this work.

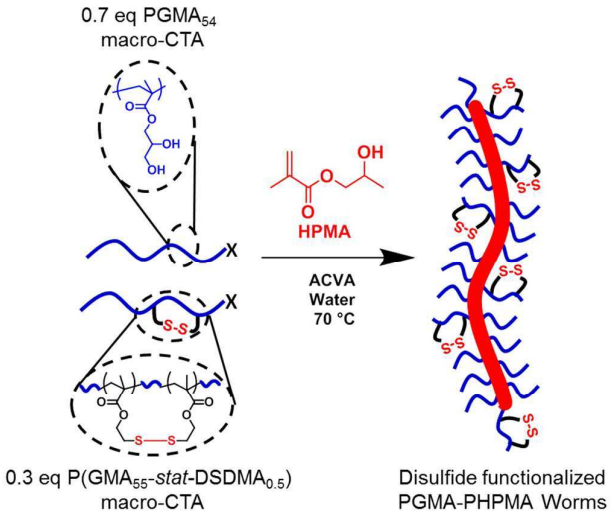
Figure 3. (a) Temperature-dependent oscillatory rheology measurements conducted on two identical 8.0 % w/w disulfide-functionalized copolymer worm gels before and after incubation in pH 7.4 PBS at 37°C for 120 h. (b) Variation of the storage modulus (G') with ageing time for a series of five identical 8.0 % w/w disulfide-functionalized copolymer worm gels incubated for up to five days at 37°C.

Figure 4: Optical and fluorescence microscopy images showing a portion of a zone of sheets of (a) Single lens paper and (b) PVC-mesh composite containing 10-μm fluorescently-labelled polystyrene particles embedded in a 10 % w/w disulfide-functionalized copolymer worm gel. Images were obtained nine days after immersion in 0.15 M PBS solution at 37°C.

Figure 5. Worm gels enable A549 cells to remain viable within mesh sheets for up to 12 days. (A) Bright field images of A549 embedded worm gels supported on the PVC-mesh composite sheets. (B) Growth curve of A549-GFP-embedded worm gels supported on PVC-mesh composite sheets. Standard deviations were calculated based on seven replicates (N =7 zones).

Figure 6. Recovery of A549-GFP cells from mesh sheets. Mesh sheets containing A549-GFP cells embedded in worm gel were incubated in cold PBS (4°C), while samples embedded in *Matrigel* were incubated in warm *Accumax* (37°C). (a) Fluorescence images of mesh sheets containing worm gel-embedded A549-GFP cells during recovery in cold PBS. (b) Extent of removal (as judged by normalized % GFP intensity) of A549-GFP cells embedded in either 10 % w/w disulfide-functionalized copolymer worm gel or *Matrigel*. Standard deviations were calculated based on thirty replicates (N = 30 zones).

Figure 7. (a) Fluorescence images of 2D cultures of recovered A549-GFP cells. (b) Comparison of cellular viability of A549-GFP cells after recovery from worm gel or *Matrigel*, respectively. Recovered cells were suspended in media, seeded in a 96-well plate (2,000 cells/zone), and ATP levels were measured using CellTiter-Glo® (CTG) Assay. Luminescence was normalized to the first day of recovery. Standard deviations were calculated based on five replicates (N = 5 wells).


0.7 eq PGMA54
macro-CTA
OH
HPMA
ACVA
Water
70 °C
0.3 eq P(GMA55-stat-DSDMA0.5)
macro-CTA
Disulfide functionalized
PGMA-PHPMA Worms

Scheme 1.

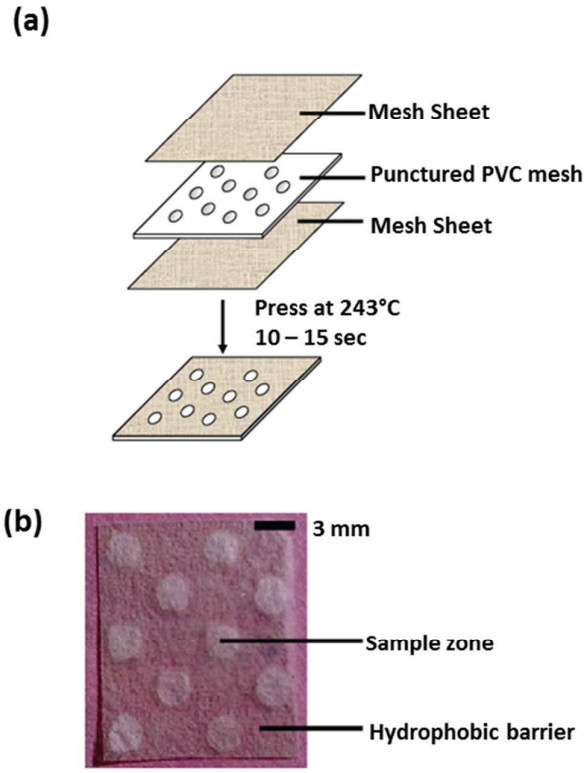

(a)
Mesh Sheet
Punctured PVC mesh
Mesh Sheet
Press at 243°C
10 – 15 sec
(b)
3 mm
Sample zone
Hydrophobic barrier

Figure 1.

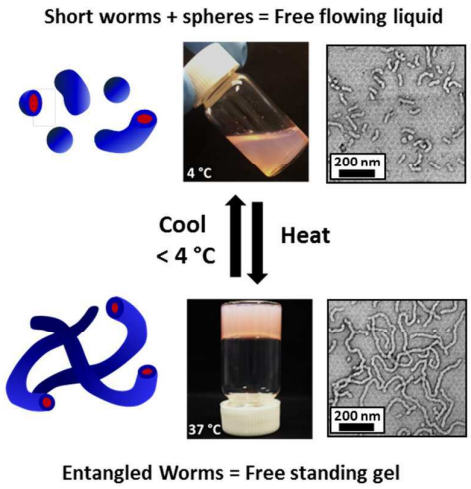

Short worms + spheres = Free flowing liquid
4 °C
200 nm
Cool
< 4 °C
Heat
37 °C
200 nm
Entangled Worms = Free standing gel

Figure 2.

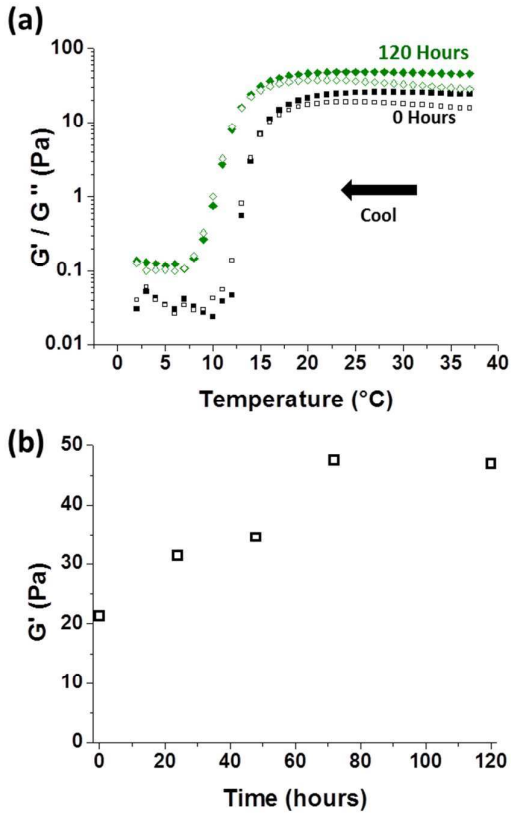


Figure 3.

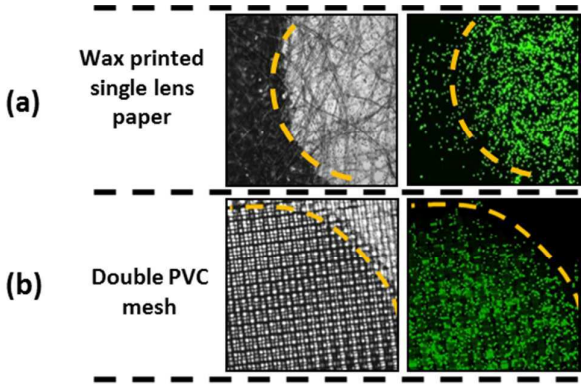


Figure 4.

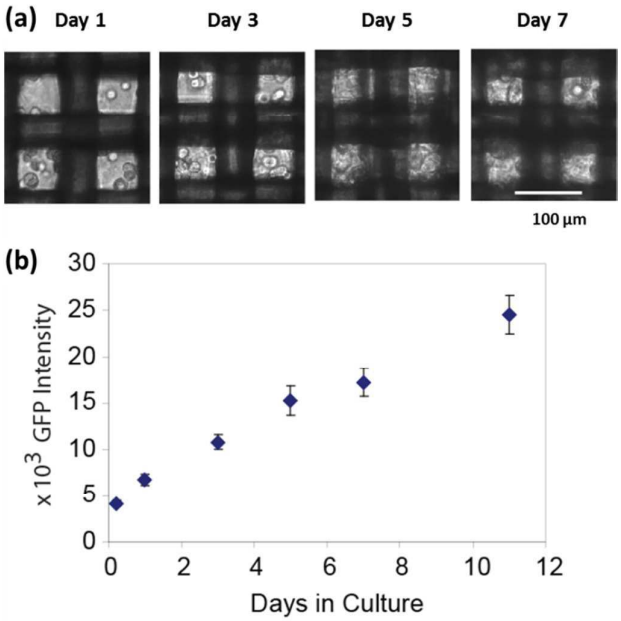

(a)
Day 1
Day 3
Day 5
Day 7
100 µm
(b)
x 10^3 GFP Intensity
0
5
10
15
20
25
30
0
2
4
6
8
10
12
Days in Culture

Figure 5.

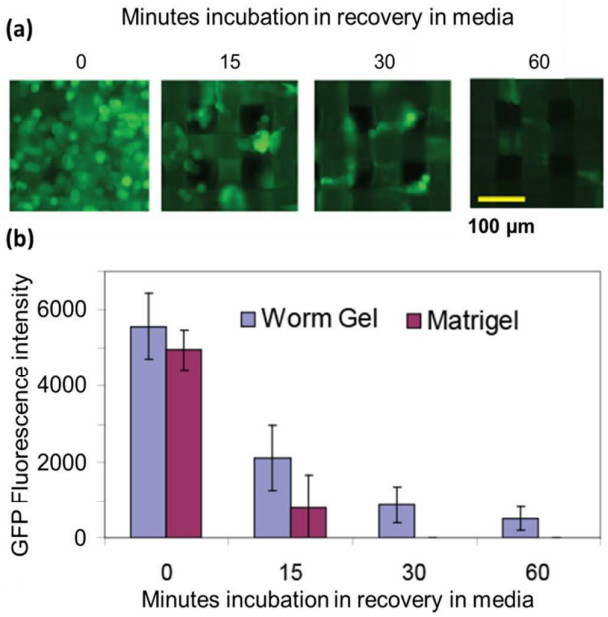

(a)
Minutes incubation in recovery in media
0
15
30
60
100 µm
(b)
GFP Fluorescence intensity
6000
4000
2000
0
Worm Gel
Matrigel
0
15
30
60
Minutes incubation in recovery in media

Figure 6.

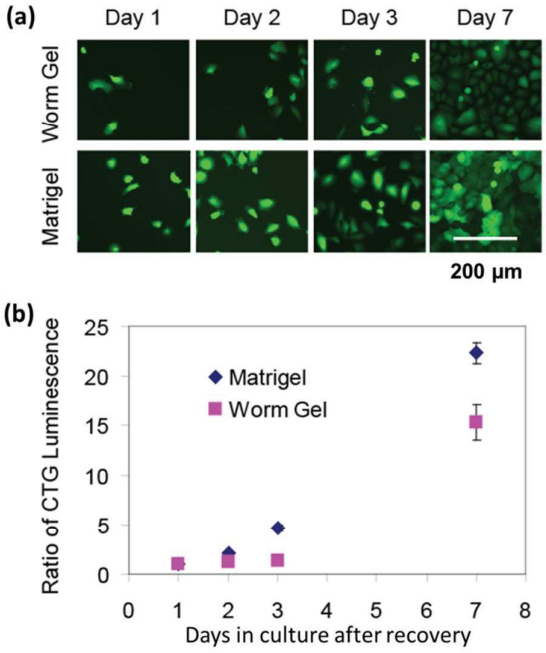
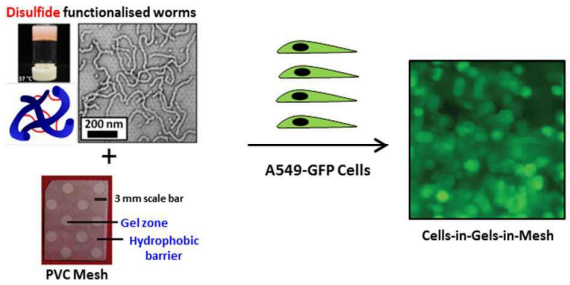

(a)
Day 1
Day 2
Day 3
Day 7
Worm Gel
Matrigel
200 µm
(b)
Ratio of CTG Luminescence
25
20
15
10
5
0
Matrigel
Worm Gel
0 1 2 3 4 5 6 7 8
Days in culture after recovery

Figure 7.

TOC Graphic


Disulfide functionalised worms
200 nm
3 mm scale bar
Gel zone
Hydrophobic barrier
PVC Mesh
A549-GFP Cells
Cells-in-Gels-in-Mesh