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Pseudo-Double Network Hydrogels with Unique Properties as Supports for Cell Manipulation

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Pseudo-double network hydrogels based on vinyl pyrrolidone and anionic methacrylic units were prepared, for the first-time, via a simple one step radical polymerization procedure using thermal or photo initiation. These networks showed improved mechanical properties, in the hydrated state, compared with their single network cousins and were capable of hosting cells to confluence. Rapid cell detachment can be induced through simple mechanical agitation and the cell sheets can be transplanted easily without the need for a cell superstrate. The results reported in this work suggest that these hydrogels could be used as support systems for cell manipulation and are candidates to compete with the conventionally used thermoresponsive cell platforms based on poly-N-isopropylacrylamide (pNIPAm).

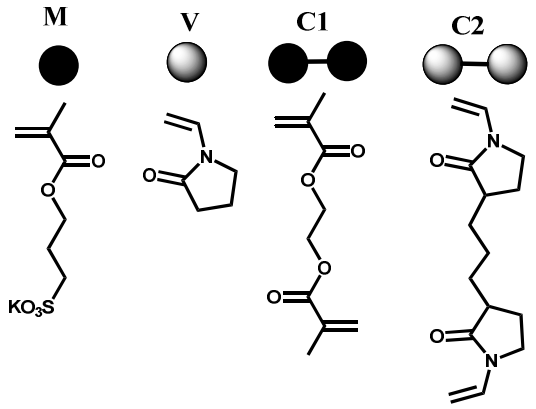
Introduction

Traditional methodologies used for the detachment of cells involve the almost ubiquitous use of aggressive proteolytic enzymes (most commonly trypsin) or cell scraping. Both approaches can be injurious to the harvested cells with studies showing that the damage caused is associated with the disruption of the cellular membrane.[1] For example, mechanical cell-disaggregation has been shown to lead to the loss of intracellular DNA, low numbers of cells, and cells with a crystalline type matrix surrounding the recovered cells which is indicative of intracellular leakage due to membrane rupture.[2] Such cell disruption constitutes a significant deficiency in these cell disaggregation approaches and alternative and gentler cell detachment methods are therefore desirable. In the early 1990's, thermoresponsive, polymeric based platforms were developed that were capable of hosting cells to confluence and subsequent cell detachment was induced by the modulation of the ambient temperature. [3-4] This cell detachment approach represents a more gentle and non-destructive mode of cell harvesting and overcomes the presented drawbacks of the established techniques.

The high numbers of publications over the past couple of decades dedicated to the development and refinement of these types of thermoresponsive platforms underpins the importance of this field in biomedicine. The vast majority of these publications report on poly-*N*-isopropylacrylamide (pNIPAm) based substrates, which possess inherent thermoresponsive behavior in a physiologically relevant range. At temperatures above their lower critical solution temperature (LCST), pNIPAm substrates assume a hydrophobic collapsed state in aqueous based media, that under certain and sometimes stringent conditions (such as substrate thickness), is conducive to cell adhesion and subsequent proliferation. [5-7] Once cell growth has reached the desired level of confluence, a cell sheet can be detached by simply lowering the ambient temperature to below the LCST. This serves to render the pNIPAM substrate hydrophilic and the polymer layer imbibes the aqueous media which in-turn repels the cellular layer and a cell sheet is harvested. A cell sheet detached in this way maintains its cell to cell and cell to ECM junctions thus mimicking the native architecture of tissues so that these cell sheets may be used for 2D or 3D biomedical constructs or in tissue damage repair. [7] Successful manipulation of the cell sheet requires the development of a cell superstrate (a flat membrane overlayer),which facilitates the harvesting of a cell sheet that does not fold in on itself and deters cell sheet shrinkage upon detachment.[1]

These types of cell regenerative substrates are now available on the world retail market (UpCell™) but their high cost renders them outside of the economic remit for most laboratories, when warranted for routine cell culture use.[8]

Many techniques used to produce the pNIPAm based coatings report thickness-cell adhesion dependencies. Therefore, the vast majority of approaches used to produce surfaces for successful pNIPAm-mediated cell detachment, employ technologically expansive and economically expensive methods for ultra-thin film formation. Therefore, it remains attractive to develop further alternatives for the gentle detachment of cells and cell sheets. An example of such an alternative was reported by Zhang *et al*. They report on the cost-effective production of thermoresponsive hydrogels based on 2-(diethylamino)ethyl acrylate for the regenerative culture of human embryonic stem cells.[9] Haraguchi *et al.* report on cell detachment via thermoresponsive polymer/clay nanocomposite hydrogels, Chen *et al.* on thermoresponsive hydrogels with interpenetrating multi-walled



Scheme 1.Structures of the monomers and crosslinkers used in this work.

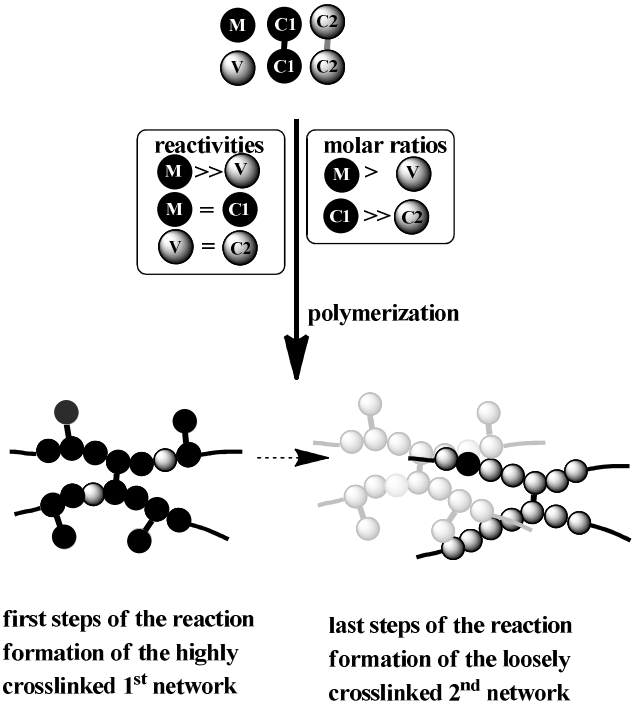
carbon nanotubes for cell detachment and Hong *et al*. on cell detachment modulated by light sensitive polymers.[10-12] Inaba *et al*. report on multi-layer cell sheets that were constructed from cell sheets detached from electro-chemical sensitive substrates.

Ito *et al*. exploited the magnosensitivity of peptide-conjugated magnetite cationic liposomes to yield contiguous cell sheets and Chen *et al*. employed pH sensitive modulation for cell regeneration.[13-15] The above strategies, along with countless others, highlight the increasing popularity of this area in tissue engineering strategies.

Here, we report on the development of new pseudo–double network (pseudo-DN) hydrogels for cell sheet detachment. Unlike all of the examples given above, the systems described here are not based on smart or stimuli responsive polymers. Cell detachment is instead initiated by simple mechanical agitation, via purposeful cell medium disruption, rather than through a change in an ambient parameter such as temperature or pH *etc*. The hydrogels, based on sulfopropyl methacrylate and vinylpyrrolidone, were prepared, for the very first time, by a one-step procedure, by carefully designing the relative ratios of monomers and crosslinkers in the polymerization process. This one step procedure is practically very simple; a bulk conventional free radical polymerization, initiated thermally or by UV exposure. Post synthesis, the hydrogels were subjected to physical and chemical characterization before being tested for possible biomedical relevant applications. As will be outlined in detail later, the term pseudo-DN refers to the structural tendency of these materials to form DNs, which can be described as interpenetrating polymer networks (IPNs) comprised of two highly asymmetric crosslinked networks. [16]

Experimental

Potassium sulfopropylmethacrylate (*M*), vinylpyrrolidone (*V*), ethylene glycol dimethacrylate (*C1*), 1-hydroxyl cyclohexyl phenyl ketone (HCPK) and azobisisobutyronitrile (AIBN) were purchased from Sigma. *V* was distilled prior to use and stored at 4 ºC. AIBN was recrystallized from ethanol and stored at 4 °C. The divinylic compound *C2* shown in **Scheme 1**, which is a *V* derivative, was synthesized in our laboratory as previously described.[17] C2C12-GFP (Green Fluorescent Protein) was sourced from ATTC, Fetal Bovine Serum was purchased from GIBCO, UK and Penicillin-Streptomycin was sourced from



Scheme 2. Scheme of the formation of the pseudo-DN.

Lonza. 24 well (both treated and non-treated) and 6 well plates were purchased from Costar, all other cell culture plastic-wear was sourced from Deltalab.

Networks synthesis

In this work, materials with different *M/V* and *M/C1* molar ratios have been prepared. The *V/C2* molar ratio was always 1000 (0.1 mol%). See **Scheme 2** for a visualization of the pseudo DN formation, which is further expanded on in the results section. Hydrogels were synthesized in a one-step conventional radical polymerization using Milli-Q water as the solvent. Briefly, an aqueous solution of *M* (1 mol/L) and *V* (concentrations ranging between 1 and 8 mol/L, which corresponds to the M/V molar ratios of 1/1 to 1/8) was prepared and the crosslinker *C1* was added at the selected molar percentage with respect to the *M* content. Two *M/C1* molar ratios were studied, 25 and 8.3, which correspond to 4 and 12 mol% of *C1* with respect to *M*. To this solution the crosslinker *C2* at 0.1 mol% with respect to the *V* content was added.

Two types of networks with different means of initiation were prepared and evaluated: samples obtained from 1) thermally initiated reactions which resulted in hydrogels which were several mm thick and 2) UV initiated reactions which resulted in hydrogels with thicknesses of less than 1mm.

For thermally initiated reactions, AIBN was added to the reaction mixtures (1.5×10^{-2} M) as the initiator. After bubbling with N_2 the solutions were poured into a Teflon mold and the system was closed. The reactions were then carried out at 60 °C over 24 H.

For photo-initiated reactions, 0.5 wt % of HCPK was added to the reaction mixtures and after bubbling with N_2 the solutions

were transferred to polypropylene molds via syringe. The polypropylene molds were separated by silicone spacers to attain samples of 0.4 mm in thickness. The polymerization was carried out for 40 minutes under UV radiation (λ=365 nm) in a UVP ultraviolet lamp (model CL-1000L, 230V).

For both types of reactions, after the selected reaction time, the networks were recovered from the molds and were allowed to swell in Milli-Q water until equilibrium was reached. Subsequently, they were exhaustively washed with water to remove any soluble material and finally they were cut into cylinders. Samples were stored in Milli-Q water at room temperature until needed for experimentation.

Analysis of the networks

A gravimetric study was carried out to determine the gel content. The initial weights of the monomers before polymerization were compared to the weight of the dry polymerized network after exhaustive extraction of any soluble materials with water, over 48 H. These washed materials were then freeze dried, after which they were analyzed by FTIR-ATR. Furthermore, another set of samples were subjected to soluble component extraction in deuterated water and the extractable content, (residual monomer and polymeric sol content), was directly analyzed using ^{1}H NMR.

Swelling at equilibrium

The swollen cylinders were accurately weighed and then allowed to dry until a constant weight was reached. The swelling (S) was defined as the amount of water per gram of dry network in the equilibrium state (equation 1).

$$S = \frac{(W_h - W_d)}{W_d} \qquad [1]$$

Where W_h is the swollen weight and W_d is the dried weight. This test was performed on each sample type in triplicate.

Methods

^{1}H NMR analysis was performed in D_2O using a Bruker Avance-300 spectrometer (300 MHz). Chemical shifts are given in the δ scale relative to TMS. Spectra were analyzed using MestReNova software. ATR-FTIR measurements were carried out using an FTIR spectrometer Spectrum One from Perkin Elmer.

The different thicknesses of the two types of samples required the use of different mechanical testing methods due to the technical limitations associated with the instrumentation used. Therefore, thicker samples, obtained by thermal initiation, could only be tested by compression while thinner samples obtained by photopolymerization could only be tested using the tensile test. The mechanical properties of samples obtained by thermal initiation were measured using the universal tester (Instron 3366, UK). The compressive fracture stress (σ_{max}) and the fracture strain (ε_{max}) were measured via a compressive test on the cylindrical gels (5 mm in thickness and 4.3 mm in radius). The elastic modulus (E) was calculated from the slope of the compresive stress-strain curves. The mechanical properties of samples obtained by photopolymerization were measured by a tensile test (MTS Synergie 200), where sample strains with lengths of 10 mm and 5mm width, were subjected to a constant strain of 5mm/min until they broke. The tensile modulus was calculated from the slope of the tensile stress-strain curves. Each test was performed on each sample type 5 times.

Cell culture

C2C12 mouse muscle myoblastic cells were used as the model cell line in this work. This cell line was treated with a lentivirus to integrate a GFP gene into the C2C12 genome. Cells were cultured in high glucose DMEM, supplemented with 10 % Fetal Bovine Serum plus 1 % antibiotics. Culture conditions were 37 °C in a humidified 5 % CO_2 atmosphere. Cell passaging was performed when cell growth reached ≈80 % confluence.

General set up for all cellular studies

The hydrogel samples were cut to form cylinders (16.26 mm diameter) to fit exactly into the wells of a standard 24 well plate. Sterilization was achieved by firstly immersing the gels in a solution of ethanol overnight followed by exhaustive washing in Milli-Q water to remove residual alcohol. This was followed by 3 cycles of washes with phosphate buffer solution after which the hydrogels were exposed to UV irradiation overnight. Each sterile hydrogel was placed into a well of a non-pretreated 24-well plate. The hydrogels were finally washed 3 times with culture medium (DMEM) prior to cell seeding. Cells were seeded at a density of 10,000 cells/cm^2 and a minimum of 3 hydrogels of each sample type was used for each experiment. Afterwards, 800 µl of pre-warmed complete culture medium was added to each well and all the samples were housed in a humidified 37 °C, 5 % CO_2 atmosphere incubator. Samples were observed and fluorescent images were captured at specific time points using an Olympus BX51 microscope.

To initiate cell detachment the culture media was removed and new media was added followed by media agitation by repetitive pipetting or simply by purposeful pipetting of the media in the well. The cell sheet was observed and images were captured using the fluorescent microscope after the hydrogel had been removed.

To investigate confluent cell sheet transplantation the hydrogels were removed using a tweezers and placed upside down on a 6 well plate (34.6 mm diameter) followed by re-incubation to allow the cells to attach to the new TCP substrate (the hydrogels now playing the role of transplantation superstrate). After incubation the hydrogels were removed and discarded. The transplanted cell sheets were observed and digital images were taken using the fluorescent microscope. These transplants were then reincubated and images were digitally captured incrementally to assess the transplanted cell morphology and behaviour in terms of further proliferation post hydrogel removal.

Cell sheets detached from other samples were split into single cells by gentle repetitive pipetting and these cells were replated on TCP to observe if the cells were viable and would attach and grow in the normal way expected for this type of cell adherent dependent cell line and again images were captured via fluorescent microscopy. Finally, the cells sheets which were transplanted and allowed to grow over 48 H were trypsinized and replated to further investigate if cells detached in this way from the hydrogels were compromised due to having being in contact with the hydrogels over a one week period.

Flow assay test

A syringe pump (Kd Scientific, Holliston, MA, USA) was connected to a homemade sterile methacrylate chamber equipped with inflow and outflow ports. A square (1.5 x 1.5cm) of hydrogel (type 4), upon which a confluent layer of C2C12 cells had been cultured over a six day period, was placed inside the chamber with cell culture medium. Using a controlled rate syringe inflow, supplemented high glucose DMEM was injected into the chamber. The apparatus was equipped with an outflow tube, so that the volume of media in the chamber was approximately constant throughout the experiment. The outflow volume, along with any collected detached cells, was collected in a vial after each experimental step. The aliquots collected were centrifuged after each increment to assess if a pellet of cells had been collected. Once detachment was achieved the cells were centrifuged, resuspended, counted and 2,500 cells/cm^2 were reseeded in a 25 cm^2 culture flask to assess post detachment behavior. The experiment was performed with different flux settings (from 1 to 20 ml/min over 20 seconds) and several samples were collected.

AlamarBlue cell proliferation assay

The alamarBlue metabolic activity assay was performed as per the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). In order to avoid any interference coming from cells adhered onto the plastic surface of the well, every hydrogel was transferred to a new well and fresh complete culture medium was added before prior to taking measurement. Briefly, 80 µl of the alamarBlue dye (10 % of the culture volume) was added to each well and incubated for 90 minutes. The fluorescence emitted at 590 nm - using λex = 530 nm - was recorded (using a Synergy HT, Biotech plate-reader) and blank readouts were subtracted from the data collected and the data was normalized against an alamarBlue assay performed on day zero (4 H after seeding). This assay was performed on each sample type in triplicate.

Trypan Blue Assay

The Trypan Blue assay was performed as per the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA), and counted in a Neubauer haemocytometer under the microscope.

Results and Discussion

For clarity's sake the results and discussion are split into 2 distinct sections: 1st the development of this new class of pseudo-DN hydrogel is outlined along with the associated characterization followed by a 2nd section detailing the application of the materials for cell and cell sheet delivery.

Pseudo- double network development and characterization

The novelty of these hydrogels, which are prepared in a single-step scenario, centers on their tendency to mimic, to some extent, the structure and properties of DNs that are prepared in a 2 step procedure, such as favorable mechanical properties (compared to single networks), even when highly swollen. DNs can be described as interpenetrating polymer networks (IPNs) comprised of two highly asymmetric crosslinked networks. The first DN was reported by Gong *et al*. and consisted of a first highly crosslinked network (4 mol% of crosslinker) of ionic poly(2-acrylamide-2-methyl-propane sulfonic acid (PAMPS) and a second slightly crosslinked network (0.1 mol% of crosslinker) of non-ionic and water soluble polyacrylamide (PAAm). The global AMPS/AA molar ratio approximately ranged between 1 and 0.05.[16] Many more DNs have been reported since, including DN cryogels, DNs of thermoresponsive components or DNs with the opposite characteristics to Gong *et al.'s* example (a first highly crosslinked neutral network and a second loosely crosslinked ionisable network).[18-20]

These DNs must be prepared in a two-step procedure to obtain the first and the second network, respectively. We propose here that vinylpyrrolidone (*V*)-based pseudo-DNs (structures that tend towards true DNs), which exhibit favorable mechanical properties even when highly swollen, can be obtained by a very simplistic, single-step methodology. This approach is based on the well-known high differential reactivity of vinylpyrrolidone, (a very low activated monomer in radical polymerization) compared to other commercial monomers such as (meth)acrylics. Put simply, this strategy is based on the fact that vinylpyrrolidone is far less reactive than other comonomers in radical copolymerization.

We have previously reported that the topology and the properties of a two-component network comprised of two monomers with high differential reactivity, such as *M* and *V,* may be controlled by the nature of the crosslinker used.[21-23] The high differential reactivity of *M* and *V* in copolymerization gives rise to two main types of chains. The 1st type is rich in the more reactive *M* (and formed in the first steps of the copolymerization). The 2nd contrastingly, is very rich in *V* and formed after the consumption of *M* (see **Scheme 2**). Upon crosslinking, these two types of chains may become the components of the 1st and 2nd network of the pseudo-DN.

By copolymerizing *M* and *V* in the presence of the commercially available crosslinker, ethylene glycol dimethacrylate (*C1)*, which is homologous to monomer *M,* and a non-commercial crosslinker *C2*, which is homologous to *V* (shown in Scheme 1), a network with a structural tendency towards a double network is formed. That is; a 1st network rich in *M* and highly crosslinked by *C1* and a 2nd one very rich in *V* and loosely crosslinked by *C2*. Notably, both are formed in the single polymerization procedure.

In addition to the simplicity of this proposed procedure, it was hoped that the targeted *V*-based hydrogels would combine the favorable mechanical properties with the desirable biocompatibility of poly-vinylpyrrolidone, which is a polymer that is used for many biomedical applications and has been approved for use in food by the FDA.[24]

The reaction and the properties of the networks formed depend mainly on the feed ratio of the four components *M/C1/V/C2*. In an effort to mimic the characteristics and the crosslinking asymmetry of the true DNs, a series of different materials were prepared according to the following constraints: a *M* concentration of 1 mol/L; a *M/V* molar ratio ≤ 1; a low *M/C1* ratio (two *M/C1* ratios were evaluated, 25 and 8.3) and a high *V/C2* ratio (1000). Focusing on the ratio of the main components *M* and *V*, **Fig. 1** describes the theoretical compositional drift of reactions with *M/V* molar ratios ranging between 1/1 and 1/8, which was the interval used in this study. This simulation was

built using Copol software which was designed by one of the

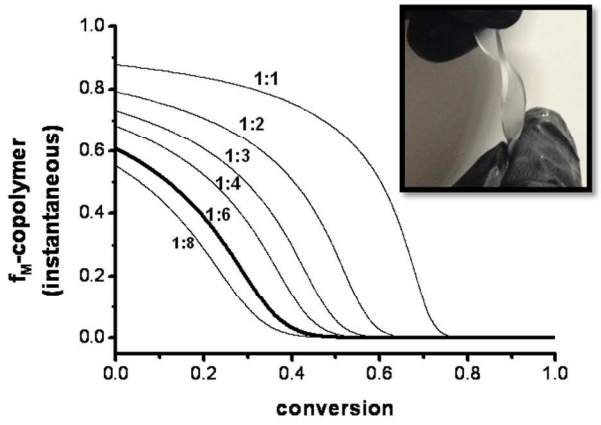


Fig. 1 Theoretical instantaneous molar fraction of *M* in the copolymer as a function of the conversion, for reactions with feed *M/V* ratios indicated by the labels. The thickest line represents the ratio selected for the cellular studies. **Insert.** Image showing a hydrated network with the selected *M/V* ratio of 1/6. The hydrogels were robust and easy to 'handle' and maneuver.

authors.[25] It was performed assuming that the reaction can be described compositionally using the terminal model and using the reactivity ratios r_M=6.6 and r_V= 0.06, as described in the bibliography. [26-27] The copolymerization terminal model can be used for descriptive purposes, although it is well documented that it is only an approximate model. [28-29]

Fig. 1 depicts the instantaneous *M* molar fraction in the copolymer (f_M) versus the conversion for the six reactions initially tested. There is a 'dramatic' compositional heterogeneity as previously described, with 2 different types of chains being formed as the reaction proceeds. The analysis of this graph highlights an important feature: while the chains rich in *V* are almost pure poly-*V*, the chains rich in *M* always incorporate a significant amount of *V* functionalities including the vinyl *C2* groups. The double functionality of *C2* may lead to poly-*V* grafting onto the chains rich in M, if one of the vinyl groups is incorporated into the initial chains containing mainly *M* and the second to the poly-*V* chains formed during the last steps of the reaction. This information is highly relevant, as it has been described that the enhanced mechanical properties of true DNs are related to residual heterolinks between the 1st and 2nd networks. [30]

Briefly, pseudo-DNs with the six *M/V* ratios, shown in Fig. 1, and two *M/C1* ratios of 25 and 8.3 (which corresponds to 4 and 12 mol% of *C1* with respect to *M)*, were prepared by conventional radical polymerization, either by thermal initiation (T samples) or UV irradiation (P samples). All of the samples were allowed to swell until equilibrium was reached and then the samples were exhaustively washed with water, and studied in this hydrated state. The pseudo-DN samples with low *M/V* molar ratio (from 1/1 to 1/3) turned out to be weak materials which broke during the initial swelling and they were discarded. The materials with *M/V* ratios higher than 1/6 were susceptible to autoacceleration during synthesis which affectively resulted in ruined samples. Samples with *M/V* ratio of 1/4 and 1/6 were robust and easy to handle, although the 1/4 materials exhibited poor cell adhesive properties in preliminary studies compared to 1/6, which is in agreement with the lower amount of *V* (less biocompatibility). Furthermore, these 1/4 samples showed worse mechanical performance than the hydrogels with 1/6 *M/V* ratio (the fracture stress was almost one order of magnitude inferior, see **Fig. S3** in Supplementary Information (SI)). Therefore, in view of the results and restrictions outlined above, 4 sample types were chosen to be evaluated for possible biomedical applications. These samples correspond to the M/V molar ratio of 1/6, i.e. the thickest trace-line in Fig. 1, with M/C1 ratios of 25 and 8.3, which were prepared through thermal initiation or photoinitiation. For ease of understanding, the sample types are labeled from 1 to 4 and details on their composition and mode of initiation are summarized in **Table 1**. The insert in Fig. 1 shows a twisted hydrated sample with a *M/V* ratio of 1/6 which demonstrates its easy 'handling'. A video further underlining this is included in the SI.

Table 1. Summary of results taken from network compositional characterization and water uptake experiments.

ID	*M/V* (Feed ratio)	*M/V* (Experimental Value)	*M/C1* (Feed ratio)	Swelling (g H_2O/g polymer)
1^T	6	5.1	25	10.2±0.2
2^T	6	5.1	8.3	4.1±0.1
3^P	6	5.4	25	9.6±0.2
4^P	6	4.9	8.3	4.1±0.1
$SN_{V2}{}^P$	-	-	-	9.8±0.1

P: photoinitiation , **T**: thermal initiation. Standard deviation is representative of 3 tests performed on each type of hydrogel in the case of the swelling. *M/V* molar ratios were experimentally deduced as shown in the SI.

Network formation and incorporation of *M* and *V* into the final materials (type 1 to 4) were analyzed gravimetrically and by NMR and FTIR-ATR techniques as outlined in SI. Briefly, around 90 mol% of the initial monomers are incorporated into to the network. The residual water soluble material is mainly poly-*V* chains formed during the last steps of the reaction, which means that the final networks, (post water rinsing), are enriched in *M* compared to the nominal feed ratio of 1/6. The corrected *M/V* molar ratio values, calculated and shown in SI, are close to 1/5, and have been included in Table 1.

For comparative purposes, single networks composed solely of sulfopropylmethacrylate (*M*) or vinylpirrolidone (*V*) were also prepared. Networks composed exclusively of *M,* referred to as SN_M and highly crosslinked with the structurally homologous *C1* (*M/C1* ratio of 25), burst during the initial swelling process (similar to samples with low *M/V* ratios, which are structurally the closest to SN_M), and hence were unusable. This behaviour is in agreement with the reported properties of highly crosslinked polyelectrolyte gels such as the PAMPS single networks reported by Gong *et al.*, which were reported as rigid and brittle materials. [16] Two types of single networks, composed solely of *V* were also prepared: The first, referred to as SN_{V1}, was weakly crosslinked with the structurally homologous crosslinker, *C2* (*V/C2* ratio of

1000, as with all of the samples outlined here). The 2nd, referred to as SN_{V2}, and structurally more related to the pseudo-DNs, was not only crosslinked with *C2*, but was additionally crosslinked with a high amount of *C1* (i.e. *V/C2* ratio of 1000, and *V/C1* molar ratio of 150, similar to the pseudo-DNs types 1 and 3). These two *V*-based single networks, SN_{V1} and SN_{V2} exhibited low Young's Moduli and behaved like ductile materials with a compression or elongation strain at rupture of around 80-90 and 160 % respectively (see **Figs. S4 and S5** in the SI). This is in agreement with the reported behavior of slightly crosslinked neutral hydrogels such as the PAAm single networks reported by in Gong *et al*.. [16]

Table 2. Summary of results taken from mechanical assays

ID	E KPa	ε_{max}%	$\sigma_{max.}$ KPa
1^T	380 ±2	57±3	660±15
2^T	501±5	63±3	1980±120
3^P	2.6±0.3	25±4	92±18
4^P	6.2±0.1	112±10	475±55
$SN_{V2}{}^P$	0.09±0.01	155±20	95±0.22

P: photoinitiation , **T**: thermal initiation. **E:** Young´s modulus, ε_{max}: maximum compression or elongation; σ_{max} maximum stress before breaking. Standard deviation is representative of 5 tests performed on each type of hydrogel (T samples measured by compression assay, P samples measured by tensile assay).

The relative strength of SN_{V2} was clearly superior to SN_{V1} (the stress at rupture was one order of magnitude higher, see Fig. S5 in SI), which can be attributed to the much higher amount of relative crosslinking. In addition, SN_{V1} type materials have been described previously as biocompatible but lacking in cell adhesive properties.[17] Considering the above, photopolymerized SN_{V2} samples were chosen to be used as the single network controls in the cell studies.

Tables 1 and 2 summarize the composition and properties of the samples used in the biocompatibility studies. The pseudo-DNs and the control samples imbibed a significant amount of water, in some cases up to 10 times their dry weight. The degree of swelling is obviously governed by the relative degree of crosslinking (*M/C1* ratio) i.e. the higher the degree of crosslinking the lower the swelling was, Table 1. Even in such a highly swollen state, the pseudo-DNs samples (samples 1 to 4) exhibited very favourable mechanical properties, compared to the single networks, Table 2, which allowed for easy handling (See photo insert in Fig. 1). Figs. S4 and S5 in SI show the stress-strain curves of the photo and thermo initiated samples, respectively. The pseudo-DN materials exhibited Young's moduli one or two orders of magnitude higher than the V-based SNs. Besides, most of the pseudo-DNs displayed a compression or elongation strain at rupture of 60 (types 1 and 2) and 110 % (type 4) respectively, approaching the values of those V-based SNs. The pseudo-DNs also exhibited relatively high stress at rupture. The comparatively high elastic modulus of the pseudo-DNs (compared to V-based SNs) can be attributed to the presence of the ionic M component and the structural tendency of the network to the DN formation, which implies the presence of charged and highly crosslinked chains rich in M. The high strain at rupture of samples 1, 2 and 4, (compared to SN_M,), and their relatively high stress at rupture, may be attributed to the poly-*V* chains and to the aforementioned heterolinks that anchor some of the poly-*V* chains in the initial network rich in *M*. On the other hand, the mechanical properties depend on the degree of crosslinking degree: the higher the crosslinking degree (the lower the M/*C1* molar ratio), the higher deformation at rupture is.

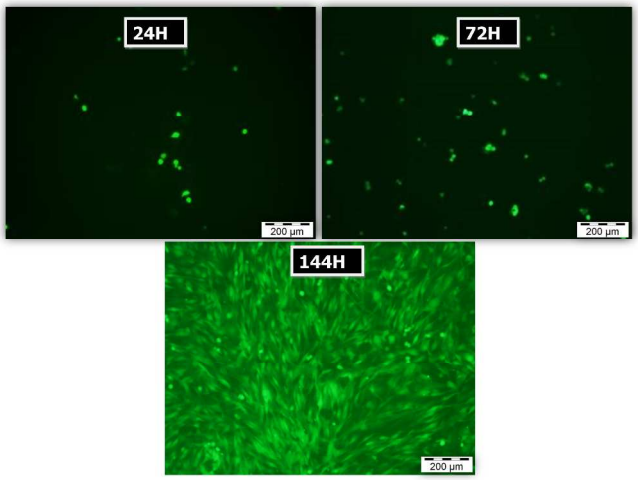


Fig. 2 Initial cell adhesion and proliferation was retarded but after a few days incubation cell numbers started to increase on all hydrogel types, and after 144 H confluent cell growth was achieved in sample types 3 and 4. These micrographs represent cell growth on hydrogel type 4 after 24 H, 48 H and 144 H respectively. The scale bar denotes a distance of 200 µms.

Pseudo-double networks as cell culture delivery systems

The biocompatibility of the hydrogels was assessed by seeding and incubating cells (10,000 C2C12-GFP cells per cm^2) on all of the hydrogels described in Table 1. Cell growth was observed microscopically over the following days. An initial lag in cell attachment and proliferation was observed for the first 2 days in all cases (compared to cells seeded on tissue culture plastic (TCP) controls). By day 3 increased cell proliferation was evident on all of the samples, with the exception of the vinylpyrrolidone SN_{V2} controls, where almost no proliferation was observed. The most improved growth was observed on sample types 3 and 4 (the photopolymerized pseudo-DNs). After 6 days incubation confluent sheets had grown on types 3 and 4 (**Fig. 2**), with significantly less cell proliferation observed on types 1 and 2. More details on cell growth, on all of the hydrogel supports, are given in **Fig. S6** in the SI. Confluent cell coverage on types 1 and 2 was achieved upon a further 2 to 3 days re-incubation.

Additionally, cells were seeded on other sample sets and alamarBlue assays were performed on cells seeded on each hydrogel type (in triplicate), to assess the daily relative cell metabolic activity. The results, represented graphically in **Fig. 3**, show that the day by day growth on each hydrogel followed the same growth trend as was seen microscopically i.e. the highest metabolic activities were observed for cells grown on hydrogel types 3 and 4, with less activity detected on hydrogels types 1 and 2, Fig. 3. The poorest hydrogel type, in terms of cell metabolic activity, was the type 5, the SN_{V2} control.

Upon replenishing the cell culture medium (on day 6) a surprising event occurred, when the cells attached to the hydrogel- pseudo-DNs spontaneously detached from the hydrogels' surface. In the case of hydrogels types 1, and 2 this

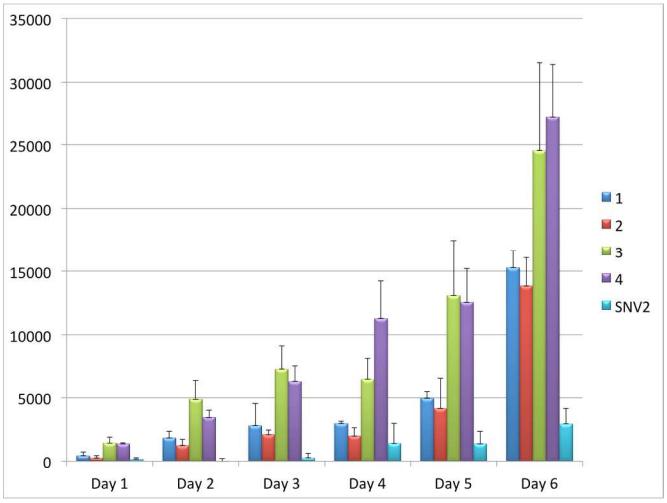


Fig. 3 AlamarBlue metabolic assay results performed on days 1 to 6 of cell culture on the prepared hydrogels. The result were normalized against an alamarBlue assay preformed on Day 0, 4 H after cell seeding.

took the form of small clumps or small cell sheets but in the cases of sample types 3 and 4, large confluent cell sheets were observed floating in the cell media, **Fig. 4**. The reproducibility of this cell harvesting outcome was confirmed by repeating the cell detachment experiments using further sets of samples. The pseudo-DN samples were therefore, not only capable of hosting cells to confluence, but also rapid cell sheet detachment, (detachment occurred upon mechanical stimulation in under 1 minute), could be induced by simple hydrogel mechanical agitation.

We additionally sought to develop a method to quantify the level of agitation which is needed to detach a cell sheet. To this end, we built a home-made system through which we could assess the level of agitation required to initiate the detachment of a confluent cell sheet. The system allowed for the housing of the hydrogel, (type 4, the type which exhibited the most desirable results in terms of their relative ability to host cells to confluence in the least amount of time), in a methacrylate chamber where a reservoir of media supplemented the growing cells. The chamber was equipped with inflow and outflow ports to facilitate the pumping of media over the cell sheet. The inflow line was connected to a syringe which was mounted on a motor driven syringe pump which served to control the rate at which media was administered over the hydrogel, **Fig. S7 A and B**. The outflow line ensured that the volume of media in the chamber was kept constant. In this way it was possible to administer media at a predetermined rate after which the hydrogel and cell sheet was assessed visually for cell detachment. The initial flow rate was kept low, 1 ml/min for 20 seconds, followed by increasing the flow in increments over the same time frame (2 ml/min for 20 seconds, and so on up to 20 ml/min for 20 seconds). Outgoing media was collected in vials for each increment and the media was then centrifuged to collect any detached cells in a cell pellet, which was disaggregated through repetitive pipetting, and the cells were counted using a Neubauer haemocytometer. It was found that flow rates of less than 20 ml/min were not sufficient to prompt full cell sheet detachment (few if any cells detached with flux rates of less than 10ml/min and only small numbers of cells

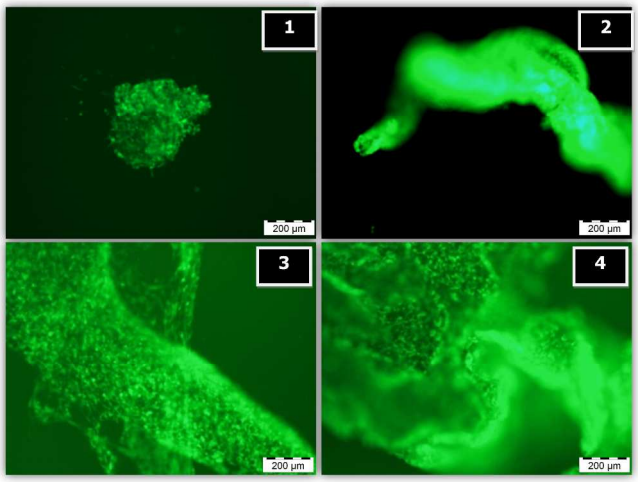


Fig. 4 Cell detachment from hydrogels type 1 to 4 after 6 days incubation followed by mechanical agitation. A full cell sheet was recovered from types 3 and 4 where-as smaller clumps or smaller cell sheets were detached from types 1 and 2 where cell growth had not reached confluence. Few cells had grown on the VP control and no detachment was observed. The scale bar denotes a distance of 200 µms.

were detached with 10 and 12,5 ml/min), but when the flow was 20 ml/min, a large and confluent cell sheet detached which could be seen detaching visually.

These results indicate that while mechanical agitation successfully initiates cell sheet detachment, a significant amount of agitation is needed. Therefore, and we can confirm from our own experience of moving the hydrogels while observing the growing cells e.g. from the incubator to the culture hood or incubator to microscope, it is possible to transport the cells for normal experimental needs without the cells detaching. When desirable it is possible to initiate cell detachment by the repetitive pipetting of the cell culture media, or by removing the cell media and adding new media quickly followed by pipetting.

The cells harvested using the flow rate of 20 ml/min were reseeded (2,500 cells/cm^2) in a TCP flask, to assess their ability to proliferate as compared to cells detached through conventional means. Cells attached and proliferated to confluence within 5 days, in the same way as cells would proliferate in a typical cell passage. The cells assumed morphology was typical of this type of adherent dependent cell line when 'healthy', **Fig. S7 C and C'**. Furthermore, an appropriate volume of the cell suspension was dedicated to a Trypan blue viability assay. The cells were deemed to be >95 % viable, which compares favorably with cell detached from established thermoresponsive systems.[7-8] These results confirm that healthy cells can be cultured, detached and reseeded in the manner described, similar to typical cell culture practice, without the need to employ traditional, harsh cell detachment methodologies.

Considering the desirable outcome outlined above, we sought to investigate the suitability of these hydrogels for further tissue engineering type applications. To this end, we studied the

employment of the hydrogels as confluent cell sheet transplantation vehicles. For this purpose, we chose the best hydrogels (in terms of cell proliferation), types 3 and 4 and seeded them with cells and incubated for 6 days. The hydrogels cell hosts were then transferred upside-down into bigger TCP

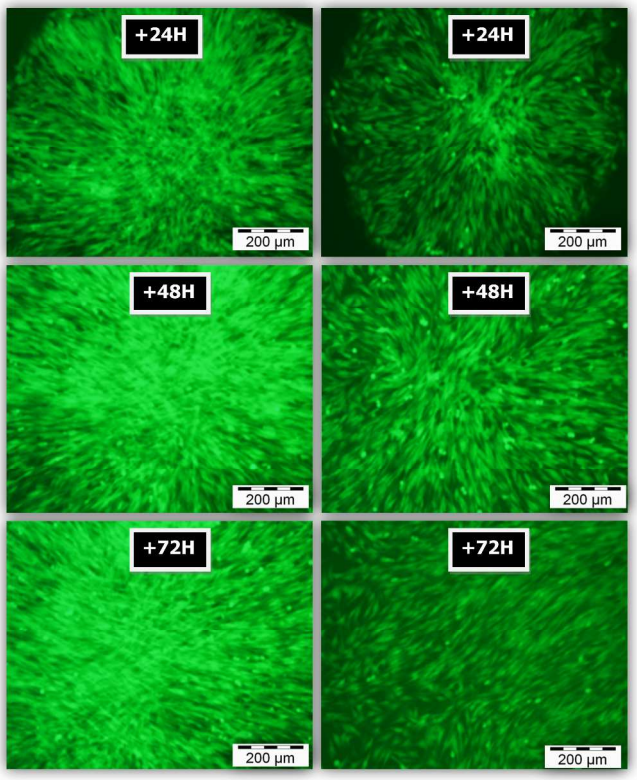


Fig. 5 Images showing the transplanted monolayers on TCP post hydrogel removal. Cells had detached from the hydrogels and preferentially attached onto the TCP below and assumed a morphology which is characteristic for this cell line when attached to a substrate. Upon re-incubation for another 24 H the cells continued to proliferate (middle images) and even more so after 48 H. The left most row of images and right most row of images show cells transplanted from hydrogel type 3 and 4 respectively and the time of incubation is given at the top of each row of pictures. The scale bar denotes a distance of 200 µms.

wells (diameter 34.6 mm) and re-incubated for 24 H. In this way, the hydrogel cell substrates now became transplant superstrates. Post incubation, the hydrogels were removed and it was possible to clearly observe a circular cell monolayer, the size of the now removed hydrogels, now attached to the TCP. This important result confirms successful cell sheet transplantation, see **Fig. 5**. These TCP dishes were then re-incubated to assess if the cells would continue to spread out on the TCP. As can be observed in Fig. 5 cells continued to thrive and expand when re-incubated over another 24 and 48 H. Further images of transplantation and subsequent growth from all pseudo-DNs are given in SI, **Fig. S8.** The cells were then trypsinized and re-plated on TCP and cells attached and proliferated as normal. Furthermore, a number of cells were dedicated to a Trypan blue viability count which indicated that the cells retained over 95 % viability in the cases of cells transplanted from both hydrogel types.

The results outlined above, indicate that the photopolymerized pseudo-DN-hydrogels are superior to their thermopolymerized counterparts, in terms of their ability to host cells to confluence in the least amount of time. This may be related to the differences in thicknesses i.e. the thermally initiated hydrogels were significantly thicker than their photo-initiated cousins, (several mm thick and less than 1 mm, respectively). Furthermore, within the range described here, the degree of crosslinking (*M/C1* ratio), the relative differences in water uptake or the mechanical properties, does not seem to have a clear influence on the cell response. Additionally, our investigations revealed that cell growth on the single network SN_{V2} was poor compared to the pseudo-DNs. Smith *et al*. studied cell interactions with crosslinked hydrogels of vinylpyrrolidone. They found that when cells were cultured in close proximity, (indirect contact), to the vinylpyrrolidone hydrogels, there was no negative influence on cell viability and in some cases cell viability was actually promoted. In contrast, cells placed in direct contact with the vinylpyrrolidone hydrogels failed to attach and grow, which is in agreement with the results reported here.[31]

Protein adsorption and cell adhesion are processes associated with a high level of complexity which are influenced by factors such as surface hydrophobicity, stiffness, surface charge *etc.*. It is likely that the very different mechanical properties of the vinyl-pyrrolidone SNs, (SN_{V2}), compared to the pseudo-DNs also have an influence on the poor cell adhesion outcome.

Another factor which could have contributed to the favourable cell adhesion outcome, seen for the pseudo-DNs compared to the SN_{V2} hydrogels, may be the presence of an anionic charge. Previous studies have shown that the presence of an anionic component can encourage improved cell adhesion. It should be noted, that this is a complicated issue though, with many other studies reporting that positive surface charge promotes cell adhesion as the cell membrane holds a negative charge which is naturally attracted to the counter-ion.[32-34] Tan *et al*. studied cell adhesion on hydrogels composed of anionic vinyl phosphonic acid (VPA) and acrylamide both in supplemented media and serum-free media.

Their results indicate that cell adhesion is superior when the anionic component content is ≥50 % when compared with hydrogels containing less or no VPA. They infer that this is due to elevated interactions between specific culture media serum proteins and the anionic constituents of the hydrogels. The uptake of these specific serum proteins, in-turn, mediate increased cell adhesion and consequent proliferation. [32] This may account for why the anionic pseudo-DNs are capable of hosting cells, where the SN counterparts do not.

It may be that this same anionic feature causes the cells to weakly interact with the underlying substrate, due to cell membrane and surface charge repulsion. In this way, cell-cell interactions are favoured more than cell-substrate interactions, and upon sufficient mechanical agitation the cell sheet detaches with cell-to-cell interactions maintained. The transplantation of the cell sheets to TCP requires that the cells preferentially attach to the new host substrate. Therefore the cell attachment must be stronger on the new host than the old, which further supports the hypothesis that the cell sheet is weakly attached to the hydrogel surfaces.

The results reported here-in suggest that these hydrogels could be proposed as candidates to compete with the thermoresponsive

systems currently employed. Furthermore, as well as sharing the advantages of pNIPAm mediated cell detachment over conventional cell detachment techniques there are advantages to this approach over pNIPAm based methods: Hydrogel production, via the technique described, is simplistic and inexpensive compared with complex and laborious grafting techniques. Rapid cell detachment via simple media agitation circumvents the need to induce a temperature change in order to achieve a cell or cell sheet detachment outcome. The fact that the hydrogels are not tethered to an underlying substrate unlike grafted pNIPAm surfaces, allows for the hydrogels to be easily moved to a transplantation site of choice. Additionally, since the cells are transferred directly from the hydrogel to the transplantation site there is no need to develop or employ a superstrate to prevent the cell sheet from folding in on itself.

Subsequent investigations will center on the better understanding of why and how cells attach and grow on the pseudo-DNs to confluence (and do not on the single network analogues) and why cell sheets can be easily detached/transplanted. Additional studies will also involve testing the cell compatibility of the hydrogels across a range of cell lines and the multi-layering of cell sheets through transplantation For now, we believe the development of these new types of materials presents exciting new avenues in terms of materials chemistry as well as regenerative biomedicine.

Conclusions

This body of work presents a fresh and simple approach to preparing materials with favourable mechanical properties. Moreover, the materials produced here, have shown themselves to be biocompatible and present notable promise in terms of possible applications, particularly for cell and cell sheet regeneration, which is generating very considerable interest in the ever-expanding field of cell sheet therapies.

Pseudo-double network hydrogels based on vinyl pyrrolidone and anionic methacrylic units were prepared, for the first-time, via a simple one step procedure using thermal or photo initiation. These networks showed improved mechanical properties, in the hydrated state, compared with their single network cousins. The prepared pseudo-DNs hydrogels were capable of hosting cells to confluence, but the most surprising aspect of this investigation was that cell sheets could then be rapidly detached (in a matter of seconds) by simple hydrogel mechanical agitation. Furthermore, the cell sheet can be transplanted simply by growing the cells on the surface of the hydrogels (until the desired level of confluence has been reached), followed by overturning the hydrogel onto the desired transplantation surface. The cells preferentially attach to the new (tissue culture plastic) substrate. After a suitable amount of time, to allow cells to attach to the 'new' site, the hydrogel is removed and the cell sheet remains on the transplantation site. Future work will centre on a better understanding of cell growth and detachment on and from the pseudo-DN hydrogels, but here we report on the development of these novel hydrogels and the unexpected cell sheet detachment and transplantation from conception, through characterization to application.

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Notes and references

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