Thermally reversible colloidal gels for three-dimensional chondrocyte culture

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Healthy cells are required in large numbers to form a tissue-engineered construct and primary cells must therefore be increased in number in a process termed ‘expansion’. There are significant problems with existing procedures, including cell injury and an associated loss of phenotype, but three-dimensional culture has been reported to offer a solution. Reversible gels, which allow for the recovery of cells after expansion would therefore have great value in the expansion of chondrocytes for tissue engineering applications, but they have received relatively little attention to date. In this study, we examined the synthesis and use of thermoresponsive polymers that form reversible three-dimensional gels for chondrocyte cell culture. A series of polymers comprising N-isopropylacrylamide (NIPAM) and styrene was synthesized before studying their thermoresponsive solution behaviour and gelation. A poly(NIPAM-co-styrene-graft-N-vinylpyrrolidone) variant was also synthesized in order to provide increased water content. Both random- and graft-copolymers formed particulate gels above the lower critical solution temperature and, on cooling, re-dissolved to allow enzyme-free cell recovery. Chondrocytes remained viable in all of these materials for 24 days, increased in number and produced collagen type II and glycosaminoglycans.

Keywords: physical gels; poly(N-isopropylacrylamide); graft copolymer; three-dimensional cell culture; thermally responsive

1. INTRODUCTION

One of the main challenges in the development of the cartilage tissue engineering process is the limited availability of healthy, differentiated cells for populating a tissue-repair construct. In order to eliminate the risk of immune rejection, the preferred cells are autologous chondrocytes from healthy donor tissue or autologous stem cells. Each of these is available in limited supply, so a proliferation step is required in order to ‘expand’ the population of cells prior to seeding a tissue-culture scaffold. Currently, cells are expanded routinely in two-dimensional monolayer on tissue-culture polystyrene (TCPS) and recovered by the action of the proteolytic enzyme trypsin in combination with ethylenediaminetetraacetic acid (EDTA). Proliferating cells in this way has a number of associated problems: the use of trypsin–EDTA has been associated with cell injury [1–4] and there are concerns regarding the use of animal-derived products in the tissue engineering process owing to the risk of disease transmission. Additionally, culturing chondrocytes in monolayers has been shown to alter their phenotype, leading to de-differentiation [5]. This change in phenotype characterized by a relative increase in collagen type I production accompanied by a decrease in collagen type II and glycosaminoglycan (GAG) synthesis may limit the potential of cells to form functional tissue and is therefore an important limitation of current monolayer culture techniques. Crucially, de-differentiation during two-dimensional monolayer culture has also been reported for a range of biological substrates, including collagen type I, collagen type II and aggrecan [6]. This indicates that the problem is not specific to TCPS and may be due to the two-dimensional nature of the culture system per se.

On the contrary, it has also been reported that chondrocytes could be re-differentiated by transferring them to three-dimensional gel culture systems. Experiments using agarose [7], alginate [8] and Matrigel [9] raise the possibility of a three-dimensional method that retains a chondrogenic phenotype, but recovering cells from these materials is challenging. A solution is the use of a polymer system that forms a reversible gel in response to a small change in temperature. In an ideal system, cells could be entrapped in a three-dimensional structure at 37°C and subsequently recovered by cooling the construct below a lower critical solutions temperature (LCST). It is well known that poly(N-isopropylacrylamide) (PNIPAM) passes through a coil-to-globule transition in aqueous media and two-dimensional surfaces based on this technology have already been demonstrated as culture substrates for a range of eukaryotic cells [10–16]. Cooling such substrates below the LCST results in cell detachment, thus, providing
a non-enzymatic process for cell recovery. In addition, Hopkins et al. [17] showed that highly branched PNIpAM with glycine–arginine–glycine–aspartic acid–serine (GRGDS) peptide end-groups could lift cells from TCPS surfaces. However, truly reversible three-dimensional systems are rare. Gutowska and co-workers developed a gelling system for chondrocyte propagation comprising a PNIpAM copolymer with 2 per cent acrylic acid [18–21]. They reported that physical gels comprising 100 per cent PNIpAM exhibited syneresis (water separation) whereas those incorporating a small proportion of acrylic acid did not. Also chondrocytes continued to proliferate in the three-dimensional gels and maintained a chondrogenic phenotype. However, the incorporation of hydrophilic monomers, such as acrylic acid, leads to an increase in the LCST and brings the significant disadvantage that such materials quickly become soluble when the temperature is dropped below 37°C. Hydrophilically modified materials rapidly liquify when removed from an incubator unless suitable engineering controls are introduced to maintain the temperature of the culture vessel. Such controls bring additional complexity to the cell culture process.

An alternative approach to improving water content and diffusion is to modify PNIpAM by the incorporation of hydrophilic grafts. This separates the thermoresponsive and hydrophilic functions and allows each property to be tuned independently. This concept was demonstrated by Yoshioka et al. [22], who developed a tissue culture scaffold based on poly(NIPIAM-graft-PEG) [23–25]. These publications also report the modification of the LCST by incorporating butyl methacrylate into the polymer backbone in order to shift the LCST to a lower temperature. In subsequent cell culture work, the authors reported a system with a gelling temperature of 7°C in a cell culture medium [26]. Thermoresponsive polymers with a hydrophobic component are known to form particulate gels in aqueous media above the LCST when the concentration exceeds a critical minimum. This phenomenon is due to the aggregation of particles and, following our work on the thermally responsive flocculation of poly(styrene-graft-PNIpAM) particles [27], Li & Ngai [28] used poly(styrene-co-NIPIAM) core-shell particles to form particulate gels above the LCST of the shell. Thermodialysis responsive particulate gels with poly(ε-caprolactone) cores and shells composed of a poly(ethylene glycol) (PEG) brush were also prepared recently. The micro-dispersions formed gels above the LCST of the PEG and were used as a thermally reversible cell culture system for mouse 3T3 fibroblasts [29] or C2C12 myoblasts [30]. An alternative approach to a three-dimensional hydrogel for the delivery of chondrocytes or mesenchymal stem cells used particulate cell carriers, formed from either collagen-coated polylactide [31] or gelatin [32,33]. The cells were immobilized on the particles and then blended with polymers that could be cross-linked and gelled by the action of sulphate radicals. Also, recently poly(ethylene glycol-block-propylene glycol) (pluronic) have been modified with the arginine–glycine–aspartic acid (RGD) peptide, which is known to bind to a wide range of integrin receptors, and biodegradable polyesters [34]. The authors suggest that the gels formed from these polymers could be used to support cells.

This paper reports the use of two types of particulate gel for the expansion of articular chondrocytes. Gels were produced from random copolymers of poly(NIPIAM-co-styrene) (PNS) and in the second system similar polymers were produced with poly(N-vinyl pyrrolidinone) (PNVP) pendant grafts (PNS-g-NV). PNVP grafts are attractive owing to their long-standing record of biocompatibility and, in addition, NVP hydrogels appear to have a stimulatory effect on cell proliferation [35,36].

2. EXPERIMENTAL

2.1. Materials

NIpAM (97%, Sigma-Aldrich, UK) was re-crystallized twice from n-hexane. Styrene (99%, inhibited with 10–15 ppm tert-butyl catechol, Sigma-Aldrich, UK) was washed three times with 10 per cent NaOH (aq) and once with deionized water before drying (MgSO4) and distilling. NVP (greater than 99%, Sigma Aldrich, UK) was distilled under reduced pressure. Isopropylbenzyl chloride (90%, Sigma Aldrich, UK) was distilled under reduced pressure. Isopropoxyethanol (99%, Sigma Aldrich, UK) and sodium hydride (greater than 99.5%, Sigma Aldrich, UK) were used as received. 2,2′-Azobis(isobutyronitrile) (AIBN; 97%, BDH) was re-crystallized from diethyl ether.

2.2. Cell culture media

Basic cell culture medium was comprised of Dulbecco’s modified Eagle’s medium (DMEM, sterile, Sigma-Aldrich, UK) supplemented with: 1 vol% 1X minimum essential medium (MEM) non-essential amino acids (1X, without l-glutamine, sterile, Sigma-Aldrich, UK); 10 000 units cm−3 penicillin; 10 000 μg cm−3 streptomycin; 20 mM L-alanylglutamine and 10 mM 4-(2-Hydroxyethyl)-1-piperazinencanesulphonic acid (HEPES) buffer. Complete medium was comprised of basic cell culture medium supplemented with 10 per cent foetal calf serum (FCS; heat inactivated, sterile, Biosera, UK). Cell expansion medium was comprised of: complete medium supplemented with 1 μl cm−3 basic fibroblast growth factor stock solution (bFGF; stock solution of 10 μl cm−3 human growth factor in phosphate-buffered saline (PBS) containing 1 mg cm−3 bovine serum albumin, sterile, PrepoTech, UK). Chondrocyte differentiation medium was comprised of complete medium supplemented with 1 μl cm−3 insulin stock solution (from bovine pancreas, stock solution of 1 μl cm−3 human growth factor in phosphate-buffered saline (PBS) containing 1 mg cm−3 ascorbic acid stock solution (50 mg ml−1 in DMEM).

All media were prepared immediately prior to use. All other reagents were used as received.

3. SYNTHESIS

3.1. Synthesis of poly(N-isopropylacrylamide-co-styrene) linear copolymers (PNS)

Eight copolymers were synthesized with different proportions of styrene and NIPAM (table 1). In a typical procedure, NIPAM (3.9597 g, 35.0 mmol), styrene (0.0201 g, 0.19 mmol) and AIBN (0.1216 g, 0.74 mmol) were dissolved in dioxane (12 cm3) and transferred to a
Table 1. Polymer compositions and molecular weight.

<table>
<thead>
<tr>
<th>ref</th>
<th>NIPAM (mmol)</th>
<th>styrene (mmol)</th>
<th>PNVP-VB (mmol)</th>
<th>AIBN (mmol)</th>
<th>% styrene (NMR)</th>
<th>conversion (%)</th>
<th>Mn (g mol⁻¹)</th>
<th>Mw (g mol⁻¹)</th>
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<tr>
<td>PNS 4.7</td>
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<td>4.69</td>
<td>69</td>
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<td>304 600</td>
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<td>—</td>
<td>0.675</td>
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<td>61 800</td>
<td>311 000</td>
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<td>81</td>
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<td>0.737</td>
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<td>23 100</td>
<td>66 300</td>
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<td>—</td>
<td>0.335</td>
<td>9.30</td>
<td>76</td>
<td>28 700</td>
<td>121 100</td>
</tr>
</tbody>
</table>

3.2. Synthesis of poly(N-vinyl pyrrolidinone) macromonomers (PNV-VB)

Sodium hydride (11.5 mg, 0.288 mmol, 14.1 eq) was added to a dry flask and washed three times with dry diethyl ether to remove mineral oil before adding dry dimethyl formamide (DMF) (20 cm³). PNVP-OH (2.0487 g, 0.02 mmol, 1.0 eq) was dissolved in dry DMF (2 cm³) and added to the reaction mixture via a syringe. 4-Vinylbenzyl chloride (27.3 mg, 0.179 mmol, 8.75 eq) was diluted with dry DMF (5 cm³) with nitrobenzene (15 μl) before adding dropwise via a syringe. The mixture was stirred for 24 h in the dark before reducing the volume to ca 5 cm³ by evaporation and precipitating into rapidly stirred diethyl ether. The off-white precipitate was allowed to settle before decanting the ether and drying overnight under vacuum. The recovered oligomer was precipitated twice from methanol into diethyl ether before drying to a glassy solid under vacuum (recovery = 179.2 mg, 72%).

1H-NMR (500 MHz, CDCl₃): δ/ppm: 4.0–3.4 (m, br, styryl aromatic); 6.8–6.3 (br, NIPAM proton); 5.7 (d, d, vinyl proton); 5.2 (d, vinyl proton); 4.0–3.4 (br, backbone methine CH₂–CH(N)–CH₂); 3.4–2.9 (br, pyrrolidone N–CH₂–CH₂); 2.5–2.1 (br, pyrrolidone –C(=O)–CH₂–CH₂); 1.8–1.2 (br, backbone methylene –CH₂–CH₂–CH₂–CH₂–CH₂–).

Molecular Weight (GPC, tetrahydrofuran): Mn = 12 200; Mw = 36 100; PDI = 3.0.

3.3. Synthesis of poly(N-isopropyl acrylamide-co-styrene) with poly(N-vinyl pyrrolidinone) grafts (PNS-g-NVP)

Graft copolymers comprising NIPAM, styrene and PNVP were prepared using the same method as for linear (PNS) copolymers: NIPAM (3.5592 g, 31.5 mmol), poly(N-vinyl-pyrrolidinone-vinylbenzyl) (PNVP-VB) (0.4351 g, 0.036 mmol),¹ styrene (0.364 g, 3.5 mmol) and AIBN (0.055 g, 0.34 mmol) were dissolved in dioxane (12 cm³) and this solution was transferred to a flame-sealable glass tube, which was evacuated using the freeze–thaw technique. The mixture was polymerized and purified as above to yield a white, glassy solid. (3.3117 g, 76%).

1H-NMR (500 MHz, CDCl₃): δ/ppm: 7.5–6.8 (m, br, styryl aromatic); 6.8–5.8 (br, NIPAM proton); 3.95 (br, isopropyl CH₂–CH₂–CH₂); 3.2 (br, PNVP backbone CH₂–CH₂–CH₂–).

Molecular Weight (DMF, GPC): Mn = 28 700; Mw = 121 100; PDI = 4.2.

3.4. Characterization

NMR spectra were obtained using a Bruker DRX-500 spectrometer. Molecular weight data for PNS copolymers and PNS-g-NVP graft copolymers were obtained using gel permeation chromatography (GPC) with

¹The above polymer was formulated to give a graft copolymer with a backbone ratio of NIPAM: styrene = 90:10 and an overall NVP content NVP/(NIPAM + styrene + PNVP) of 10 mol%. The above feed ratios are based on a graft Mᵣ = 12 200 g mol⁻¹ and therefore an average degree of polymerization of 109 monomer units per graft.
DMF eluent, a Polymer Laboratories LC1150 pump and Viscolec TDA model 300 refractive index detector. The stationary phase comprised 3 × 30 cm Polymer Laboratories PL gel mixed-B columns. For PNVP oligomers, GPC was performed using: tetrahydrofuran, with 2 × 40 cm Polymer Laboratories PL gel mixed-E columns. The instruments were calibrated using poly(methyl methacrylate) standards (Polymer Laboratories, UK) and analysis was performed using Cirrus software.

3.4.1. Matrix-assisted laser-desorption-ionization time-of-flight mass spectrometry. Matrix-assisted laser-desorption-ionization time-of-flight mass spectra (MALDI-ToF MS) were obtained for PNVP oligomers using a Micromass ToFSpec 2E spectrometer. The instrument was operated in positive ion reflectron mode with an accelerating potential of 20 kV. Spectra were acquired by averaging at least 100 laser shots. An external two-point calibration was performed using angiotensin II (1046.54 g mol⁻¹) and insulin (5734.61 g mol⁻¹). Mass spectra were also used to estimate the functionalization of PNVP-VB macromonomers. The proportion of functionalized end-groups for a particular s-mer (%F) was calculated using the following equation:

\[
%F_i = \frac{h_{\text{VB}}}{h_{\text{VB}} + h_{\text{OH}} + h_{\text{AIBN}}} \times 100,
\]

where, \( h_{\text{VB}} \), height of the peak corresponding to vinylbenzyl-end groups
\( h_{\text{OH}} \), height of the peak corresponding to hydroxyl-end groups
\( h_{\text{AIBN}} \), height of the peak corresponding to AIBN-end groups.

Peak heights were measured using the Data Reader tool in the Micralocal Origin software. Heights were calculated for all peaks in the spectrum that could clearly be measured above the baseline noise and an overall %F was calculated as a weighted arithmetic mean.

3.5. Characterization of solution–gelation behaviour of thermally responsive polymers

3.5.1. Lower critical solution temperatures. Lower critical solution temperatures were measured by turbidimetry using a Varian CARY3-bio UV–visible spectrophotometer with a temperature-controlled cell chamber. Samples (0.1 wt% in ultrapure water) were subjected to a temperature ramp of 1°C min⁻¹ and absorbance was monitored at a wavelength of 500 nm. Turbidity data were collected for both heating and cooling cycles, and LCSTs were defined as 1 per cent of normalized turbidity during the heating cycle. The temperature at which turbidity returned to 1 per cent during the cooling cycle was also recorded to allow for calculation of hysteresis.

3.5.2. Gel formation and gelation temperatures using cone and plate viscometry. The rheological behaviour in aqueous solution was investigated using a cone and plate viscometry using a T.A. instruments Rheology Advantage AR-G2 cone and plate viscometer with heated Peltier plate and a Julabo F24 temperature-controlled water bath, equipped with 40 mm cone. Temperature sweeps of 1°C min⁻¹ were performed at a fixed strain rate of 0.5 Hz and a fixed strain of 10 per cent. Samples were 5 wt% polymer compositions in ultrapure water which had been through at least one heating and cooling cycle before measurements were taken. \( G' \) and \( G'' \) data were collected for both heating and cooling cycles. Gelation temperatures were defined as the temperature at which \( G' = G'' \) during the heating cycle.

3.5.3. Minimum gel concentration. Minimum gelation concentrations for thermo-responsive polymer solutions in pure water were measured using the following self-supporting structure test. Polymer solutions (15 mg in 0.75 cm³) were prepared by stirring rapidly in a glass sample tube under ice. The solution was transferred to a water bath (37°C) and allowed to precipitate to form a full-volume colloidal gel structure. The tube was then removed from the bath and inverted to confirm the formation of a self-supporting structure. The outcome was recorded. The procedure was repeated until either three consecutive ‘passes’ or three consecutive ‘fails’ were recorded. When a solution passed the above test, the concentration was decreased by addition of 100 µl aliquots of ultrapure water before repeating. The minimum gelation concentration (\( C_{\text{gel}} \)) was defined as the lowest-recorded concentration at which a self-supporting structure was formed.

3.5.4. Equilibrium water content at 37°C. Equilibrium water contents (EWC37) of thermoreversible gels were measured using 5 wt% solutions. Polymers (40 mg) were dissolved in either ultrapure water or DMEM (7.6 cm³) by stirring rapidly in a pre-weighed tube under ice. The solutions were transferred to an incubator (37°C) and maintained for 72 h to allow the mixture to reach a gelled equilibrium state. Following this, any liquid extruded by the gel was removed and the weight of gel was recorded (Masswet). The gels were then transferred to an oven (50°C) and dried to constant mass (ca 24 h) before a dry weight was recorded (Massdry). EWC37 was calculated as follows:

\[
\text{EWC}_{37} = \frac{\text{Masswet} - \text{Massdry}}{\text{Masswet}} \times 100.
\]

3.5.5. Chondrocyte isolation and expansion. Chondrocytes were harvested from bovine metacarpophalangeal joints and expanded as described previously [37]. In brief, chondrocytes were isolated using two enzymatic treatments: trypsin (0.25% w/v in PBS, 37°C for 30 min), then collagenase (2 mg cm⁻² bacterial collagenase in complete medium, 37°C for 15 h with agitation). The cells were passaged twice before encapsulating in three-dimensional gels.

3.6. Chondrocyte encapsulation and three-dimensional culture

3.6.1. Chondrocyte encapsulation. Polymers were freeze dried from ethanolic solutions (10% ethanol/ultrapure water) and stored in tightly sealed tubes to maintain sterility. Sterility was confirmed by the lack of
bacterial/fungal infections after 24 days in culture. Three-dimensional cell culture constructs were formed by combining a suspension of chondrocytes (P3) with a polymer solution at 4°C to give a final cell density of 2 million cells cm⁻³ and a final polymer concentration of 5 wt%. The combined suspension was aliquoted into non-cell-adhesive 24-well plates (1 cm² per well) and stored in an incubator (37°C, 5% CO₂) overnight to form individual three-dimensional cell culture constructs (final dimensions approx. 5 mm diameter × 2 mm depth). Agarose controls with an equal cell-density were prepared by mixing a suspension of chondrocytes in basic medium with a solution of agarose (Type XII) in sterile PBS at 38°C. The resulting mixture gelled on cooling to give constructs with a final cell concentration of 2 million cm⁻³ in 2 wt% agarose. Following encapsulation constructs were transferred to 9 cm non-cell-adhesive culture dishes (three constructs per dish with 25 cm² of culture medium).

3.6.2. Assessment of chondrocyte activity using alamarBlue. Constructs were transferred to 12-well TCPS plates in triplicate and each was immersed in 3 cm³ of 10 vol% cell-free controls to eliminate interference arising from material–dye interactions. Statistical analysis was performed using the GraphPad Prism software (v. 5) via a one-way ANOVA with a Tukey post hoc test.

3.6.3. Assessment of chondrocyte proliferation in three-dimensional culture. The proliferation of chondrocytes in the three-dimensional constructs over time was quantified by cell counting. Following alamarBlue analysis, the theroesponsive polymer constructs were dissolved in 5 cm³ sterile PBS (16 h at 4°C) and agitating to give a homogeneous solution. Chondrocyte numbers were determined by counting eight samples using a haemocytometer.

3.6.4. Preparation of samples for histological staining and immunolocalization of collagens. Whole constructs were removed from the culture medium and halved using a scalpel. Samples were embedded, cut section face-up, in optimum cutting temperature (OCT) compound before cutting 8 µm sections using a Leica CM3050S cryostat and transferred to glass slides coated with 3-aminopropyltriethoxy silane (APES). Slides were fixed using 4 wt% paraformaldehyde/PBS, pH 7.4 and stored at 4°C. Haematoxylin and eosin (H&E) staining was used to determine the distribution of cells within the three-dimensional constructs. Staining was done using an automated line (Shandon, Runcorn, UK) before mounting with a coverslip.

3.6.5. Toluidine blue staining. One drop of 1% (wt/vol) toluidine blue in 0.5 wt% sodium borate was added to fixed sections for 5 s. Slides were washed with distilled water and dried overnight before mounting.

3.6.6. Immunolocalization of collagens type I and II. The accumulation of collagens type I and II was observed by immunohistochemical staining using a VECTASTAIN Elite ABC kit (Cambridge Bioscience, UK). Samples were washed in PBS before treating firstly with 10 mg cm⁻³ hyaluronidase/PBS (30 min at 37°C), then 2 mg cm⁻³ pronase/PBS (30 min at 37°C). Following a wash in TBS, the sections were blocked with 3 per cent BSA in 0.05 vol% Tween20/TBS to prevent non-specific staining. Samples were then treated for 1 h with the relevant primary antibody mixture, to detect either collagen type I or collagen type II, or with an antibody-free control, before applying the secondary antibody and ABC reagent as directed by the kit. Collagens were visualized using a DAB substrate.

4. RESULTS

4.1. Polymer synthesis

The PNS series were synthesized by conventional radical copolymerization and PNS-g-NVP was prepared using a macromonomer approach, as shown in scheme 1. PNVP was prepared using a radical polymerization with transfer to solvent (2-isopropoxyethanol) and a chain transfer constant (Ct) as 0.017 at 70°C was calculated using the method of Ranucci et al. [38]. The MALDI-TOF mass spectrum for the PNVP-VB macromonomer is shown in figure 1. The spectrum of PNVP-VB comprised two series of peaks, each having a repeat unit of ca 111 g mol⁻¹. The main series was described by m/z = 242.33 + n111.33 (where n = degree of polymerization). The residual mass (m/z = 242 ± 1 Da) is derived from the (vinylbenzyl)isopropoxyethoxy and H end groups plus a single sodium cation. The minor series was described approximately by m/z = 308.8 + n111.4. In this series, the residual mass (309 ± 2 Da) indicates that the end groups were (vinylbenzyl)isopropoxyethoxy group derived from termination by solvent-derived radicals and the cyanoisopropyl radical, which is produced by initiation from radicals derived from the thermolysis of AIBN. No other end groups could be identified, so that the data indicated that essentially all of the chains were functionalized with the polymerizable 4-vinylbenzyl groups. Approximately, 90 mol% of the oligomers had the 4-vinylbenzyl group at the α-chain end and H at the ω-chain end. The other 10 mol% was composed of oligomers that had the 4-vinylbenzyl group at the α-chain end and the cyanoisopropyl group at the ω-chain end.

The structure of PNS-g-NVP was confirmed by 1H NMR spectroscopy (figure 2a,b). Three additional peaks were present in the NMR spectrum compared with spectra obtained for PNS copolymers (figure 2c). These peaks (at 2.4, 3.2 and 3.8 ppm) matched those for PNVP, and the overall pattern of chemical shifts was consistent with that reported by Dincer et al. [39] for poly(NIPAM-co-NVP). The final polymer composition had an estimated styrene content of 9.3 ± 0.5 mol%. This figure is comparable to the feed composition, indicating that the incorporation of styrene was not significantly affected by the presence of the NVP macromonomer. Quantification of the final proportion of

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NVP grafts could not be achieved with confidence owing to the significant overlap between the pyrrolidone protons and those of the polymer backbone. An estimated proportion of 8–10 mol% NVP was calculated based on the comparison with the NIPAM isopropyl methine proton (figure 2 ‘peak u’) and assuming a normally distributed unimodal peak for the pyrrolidone CH2 protons (‘peak c’). PNS materials were synthesized by free-radical solution polymerization and recovered in good yields (table 1). An example of NMR spectrum for PNS is given in figure 2c. Polymer compositions could be tailored by varying the monomer feed ratio, which also affected the molecular weight: increasing the proportion of styrene led to a decrease in both $M_n$ and $M_w$.

4.2. Characterization of thermoresponsive polymers

The effect of the incorporation of styrene on the cloud point was investigated by turbidimetry and the data
derived from heating and cooling are reported in table 2. Figure 3 shows data for a range of polymer compositions in both pure water and DMEM (both heating and cooling cycles). As the proportion of styrene was increased, the onset of turbidity shifted towards lower temperatures. These results are consistent with the literature, where increasing fractions of hydrophobic monomer are reported to lead to a decrease in the LCST. The progress of transitions also changed in line with polymer composition: increasing the mole fraction of styrene led to a more gradual increase in turbidity, rather than a sharp change (over $1^\circ C$) as seen in PNIPAM. This behaviour reflects an increased heterogeneity in the composition of the polymers. Hysteresis ($\Delta_c = \text{cloud point by heating} - \text{cloud point by cooling}$) in the turbidimetry was larger when the polymer was composed of an increasing fraction of styrene. This can be explained by considering the slow dissolution times of styrene-rich domains, which are increasingly prevalent as the overall proportion of styrene is increased. It should be noted that there are differences in both molecular weight and polydispersity in the above polymers. The significant hysteresis has important implications when considering techniques for measuring the LCST. Turbidimetry is widely used to measure the transition, but it is common practice to measure only the heating cycle. This practice may give a representative understanding for homopolymers in very dilute solution but it is unlikely to do so for copolymer systems or cases where homopolymer chains exist for extended periods of time in the collapsed state [40]. A small shift in the LCST ($ca 3^\circ C$) was observed when the polymers were dissolved in DMEM in comparison with pure water.

Figure 2. 1H NMR of (a) PNS-g-NVP, (b) PNVP-VB and (c) PNS.
Table 2. Cloud points, gelation temperatures, minimum gel concentrations and equilibrium water contents of PNS and PNS-g-NVP. $C_{gel}$ minimum gelation concentration.

<table>
<thead>
<tr>
<th>ref</th>
<th>% ST (NMR)$^a$</th>
<th>% NVP (NMR)$^b$</th>
<th>cloud point (UPW) (°C)</th>
<th>cloud point (DMEM) (°C)</th>
<th>gelation temperature ($G' = G''$) (°C)</th>
<th>$C_{gel}$ (wt%)</th>
<th>EWC (wt%)</th>
</tr>
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<tr>
<td>PNS 4.7</td>
<td>4.69</td>
<td>—</td>
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<td>4.5</td>
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<tr>
<td>PNS 9.6</td>
<td>9.57</td>
<td>—</td>
<td>24.0, 12.0</td>
<td>12.0</td>
<td>21.0, 11.5</td>
<td>9.5</td>
<td>0.76</td>
</tr>
<tr>
<td>PNS-g-NVP</td>
<td>9.30</td>
<td>8–10</td>
<td>25.0, 10.9</td>
<td>14.1</td>
<td>21.5, 9.8</td>
<td>11.7</td>
<td>1.55</td>
</tr>
</tbody>
</table>

$^a$Proportion of styrene estimated by NMR by comparing the isopropyl peak from NIPAM (1 proton, 4.1–3.9 ppm) and the aromatic multiplet from styrene (2 protons, 2.7–2.2 ppm).

$^b$Proportion of NVP estimated by NMR by comparing the isopropyl peak from NIPAM (1 proton, 4.1–3.9 ppm) and the aromatic multiplet from styrene (4 protons, 7.4–6.8 ppm).

$^c$The gel collapsed under strain and a cooling curve could not be measured.

The gelation of PNS and PNS-g-NVP in aqueous media was investigated using cone and plate viscometry. This technique was used previously by Zeng et al. [41] to demonstrate the formation of physically cross-linked networks in aqueous solutions of PNIPAM. Figure 4 shows modulus–temperature plots for PNS-4.7 and PNS-g-NVP at 5 wt% in ultrapure water. Both polymer solutions displayed a crossover of the elastic ($G'$) and loss moduli ($G''$) as the temperature increased. This was associated with an increase in viscosity and a change in appearance from a transparent, colourless solution to an opaque, white solid. Table 2 shows that the gel point obtained as the temperature was increased ($G' = G''$) correlated well with the cloud point data for each of the PNS and PNS-g-NVP. It was possible to measure re-dissolution temperatures using the lower concentrations employed in the cloud point experiments and these cooling experiments allowed us to measure the apparent hysteresis. However, the polymers in the PNS series, other than PNS-4.7, produced only weak gels, and it was not possible to derive meaningful cooling data from viscometry owing to gross phase separation. Both $G'$ and $G''$ in the PNS-4.7 solution were independent of temperature between 15°C and 30°C and $G'' > G'$: consistent with a viscous polymer solution. Between 30°C and 34°C, there was a large increase in both $G'$ and $G''$ (four orders of magnitude) and a crossover in these parameters so that, above 33°C, $G' > G''$. This process was both reversible and reproducible on subsequent heating cycles. Heating and cooling of the PNS-4.7 solution gave a hysteresis, $\Delta_T$, of approximately 4°C. The behaviour of PNS-g-NVP was similar to the PNS-4.7 copolymer during the heating cycle (figure 4b). However, on cooling the PNS-g-NVP, gel did not liquefy until 15°C. Figure 4b and table 2 show that this is a substantial $\Delta_T$ and similar to that obtained from turbidimetry.

The present copolymers formed self-supporting structures at low concentrations (less than 1 wt%). However, within the range of concentrations assessed (up to 2.5 wt%), the strength of the structure was observed to decrease substantially with decreasing concentration. Examples of this type of study are rarely reported in the literature, but Liu et al. [42] reported that the minimum gel concentration (resistant to inversion with ‘vigorous shaking by the hand’) of quaternized poly(DMAEMA-graft-NIPAM)s was 6.4 wt%. The concentration reported for cell culture studies is typically 5–10 wt% [19,20,26].

It is well established that the water contained in hydrogels exists in at least two distinct states: water that is closely associated or ‘bound’ to the polymer by hydrogen bonding or hydrophobic interactions and that which is
used previously as a matrix for maintaining chondrocytes in three-dimensions. However, the recovery of entrapped cells is impossible by thermal liquefication owing to the high temperature required. Enzymatic degradation of the gels to yield viable cells has also not been achieved in the literature. In this work, agarose was used as a positive control owing to its known biocompatibility, availability and easily-controlled gelling behaviour. In preliminary studies using live/dead staining, chondrocytes remained viable in three-dimensional agarose gels (7.5 mm diameter, 5 mm depth) for up to 12 weeks and visibly increased in number, indicating proliferative potential.

Three synthetic polymers were selected for cell culture testing: PNS-5.0, PNS-7.7 and PNS-g-NVP. Both PNS-5 and PNS-7.7 are linear copolymers which dissolved readily in DMEM below 15°C, formed three-dimensional colloidal gels when incubated at 37°C and had EWCs of greater than 55 per cent at 37°C in DMEM. PNS copolymers with a greater proportion of styrene were difficult to redissolve after gelation and were therefore considered inappropriate for use as reversible cell-culture systems. PNS-g-NVP was included to study the effects of adding hydrophilic NVP grafts. This polymer had the highest proportion of styrene in the polymer backbone, but also the highest EWC owing to the hydrophilic NVP grafts. These grafts were expected to improve diffusion into the centre of materials by overcoming the ‘hydrophobic skin’ effect [45,46].

Graft copolymers with a lower proportion of styrene (not reported) did not form colloidal gels. An initial polymer concentration of 5 wt% was chosen to form colloidal gels, informed by colloidal gel experiments, limits of solubility and mechanical properties (as determined by viscometry).

4.4. Chondrocyte viability and proliferation in three-dimensional gels

4.4.1. Comparison of respiration rates over first 14 days in three-dimensional culture using alamarBlue. Chondrocyte viability in the new three-dimensional cell culture materials was initially assessed during short-term three-dimensional culture. Chondrocytes were entrapped before culturing for 14 days in chondrocyte expansion medium. The change in construct viability over time is given in figure 5a. After 3 days in three-dimensional culture, there was no significant difference in cell activity between the agarose and PNS-5.0 constructs whereas activity in the PNS-g-NVP constructs was significantly lower (p < 0.001). Very little activity was recorded in the PNS-7.7 constructs and the response of cells to this material was consistently poor throughout this experiment. With the exception of PNS-7.7, an increase in construct respiratory activity was seen in all constructs after 14 days. Both the agarose and PNS-g-NVP cultures increased in respiratory activity by ca 250 per cent whereas activity in the PNS-5.0 cultures increased by 56 per cent. This result provides strong evidence that chondrocytes were viable in the experimental materials PNS-5.0 and PNS-g-NVP in addition to the agarose controls. Moreover, after short periods in culture, viability in the PNS-5.0
materials was as good as in agarose, indicating that the LCST-driven entrapment process was no more detrimental to cell viability than the low-temperature gelation process used in agarose culture.

4.4.2. Change in respiration over extended culture periods using alamarBlue. A further study was performed, which additionally investigated the change in cell numbers over 24 days in culture. Chondrocytes were maintained in an expansion medium for the first 10 days before culturing in chondrocyte differentiation medium for a further two weeks. This regime was employed to encourage the synthesis of extracellular matrix macromolecules, which are commonly used to assess chondrocyte phenotype. Figure 5b shows the change in chondrocyte activity over 24 days in culture. After encapsulation (4 days), significant differences could be seen between the respiratory activity in all samples. The collective ‘construct viability’ decreased in the order: PNS-5.0 > agarose > PNS-g-NVP > PNS-7.7. For agarose, PNS-g-NVP and PNS-7.7, this order was consistent throughout the 24 days. Respiratory activity in agarose constructs increased steadily over 24 days, with fluorescence intensity rising from ca 250 to ca 450. PNS-g-NVP showed a small but significant increase in respiratory activity (p < 0.01) over the same period. No significant change was recorded for PNS-7.7. Activity in the PNS-5.0 constructs remained constant over the first 10 days, yet decreased significantly (p < 0.01) between days 10 and 24. This drop in activity is coincident with the change in culture medium from expansion- to chondrocyte differentiation medium after day 10, but this is most likely to be indicative of the gradual dissolution of the material over time. Activity in PNS-g-NVP also peaked after 10 days in culture then remained constant for the remaining 14 days, whereas agarose constructs continued to increase in respiratory activity.

4.4.3. Quantitative study of cell recovery after extended culture times. Following the assessment of cell activity using alamarBlue, constructs were dissolved (16 h, 4°C) to allow cell counting. All constructs showed a modest increase in cell number after 24 days in three-dimensional culture (figure 6). The largest increase was seen in PNS-5.0 (+98%), followed by PNS-7.7 (+54%) and PNS-g-NVP (+24%). Population profiles for each material were consistent with the alamarBlue data. For example, PNS-g-NVP demonstrated an increase in cell numbers between days 4 and 10, but no significant change occurred between day 10 and day 24. The initial increase in cell numbers was consistent with the use of chondrocyte expansion medium over the encapsulation period (days 1–3) and the first subsequent week (days 4–10). Examination of recovered cells in the light microscope showed no evidence of cell injury or other temperature-mediated effects, and cells were further protected by immersion in culture medium throughout the whole process. These results are comparable to those from similar studies reported in the literature (An et al. entrapped P1 chondrocytes from rabbit scapula in a copolymer gel comprising poly(NIPAM-co-acrylic acid) and reported an increase in cell number of approximately 180 per cent after 28 days [19]. Au et al. [47] counted the number of P3 articular chondrocytes recovered from a poly(NIPAM-co-acrylic acid) hydrogel after 14 days in three-dimensional culture.

Figure 5. AlamarBlue respiration data for chondrocytes in three-dimensional culture. (a) Proliferation after 14 days (expansion medium), (b) one week proliferation followed by two weeks differentiation. Arrow indicates switch in cell culture medium. Square, agarose; filled square, PNS-5.0; filled circle, PNS-7.7; filled triangle, PNS-g-NVP. Data shown are means ± standard error (n = 6) after 3 h incubation with alamarBlue.

Figure 6. Change in chondrocyte numbers recovered from thermo-responsive constructs over time. Filled square, PNS-5.0; filled circle, PNS-7.7; filled triangle, PNS-g-NVP. Arrow indicates switch in cell culture medium (expansion → differentiation). Data shown are means ± s.e., n = 12.
and, in the best case, recorded an increase of 85 per cent. Kwon & Matsuda [48] counted cell numbers in various NIPAM–PEG–NIPAM block- and star-copolymers after culturing for 7 days, finding that none of the materials showed an increase in cell number.

4.4.4. Histological staining and immunolocalization of collagens I and II. Extracellular matrix components can provide valuable information about chondrocyte phenotype and are fundamentally linked to the functional properties of the engineered tissue. Entrapped chondrocytes were observed in all three-dimensional culture systems following H&E staining, both as isolated cells and as larger cohesive colonies (figure 7). The number of cells and cell colonies observed in agarose culture were higher than in the experimental materials. This result is consistent with both of the above alamarBlue studies in which the respiratory activity in agarose gels was significantly higher. The cell density in the thermoresponsive materials appeared to decrease in the order PNS-5.0 > PNS-g-NVP > PNS-7.7, consistent with the results for the direct cell counting study. The presence of eosinophilic structures was limited to the immediate vicinity of entrapped cells in all cases, although this observation was less clear for the agarose and PNS-g-NVP samples, where background staining also occurred. Toluidine blue staining confirmed the presence of GAGs in all the three-dimensional constructs, with GAGs localized in the vicinity of individual cells and cell colonies. The intensity of staining associated with chondrocyte colonies was greater in the synthetic culture materials, where large stained colonies were detected. Staining in the agarose colonies appeared to be limited to the immediate vicinity of individual cells. It should be noted that very little background staining was seen for the PNS-5.0 sample, in contrast to the other materials owing to the solubility of the polymer in the aqueous washes employed after staining.

Immunolocalization confirmed the presence of both collagens type I and II in all of the three-dimensional cell culture materials (figure 8). However, differences in staining intensity were observed, indicating differential expression between constructs. In agarose, both type I and II collagens were detected in close association with cell colonies with little difference in intensity. In PNS-5.0, the density of collagen type I staining was similar in appearance to the agarose cultures but collagen type II staining appeared to be denser. Relatively little staining was seen in the PNS-7.7 cultures, except for a small number of large cell colonies, which stained predominantly for collagen type I. Both markers were seen in the PNS-g-NVP cultures with individual cells staining for both collagens type I and II. Larger colonies in this material were only visualized by collagen type II staining.

5. DISCUSSIONS AND CONCLUSIONS

The repair or replacement of damaged tissues, even relatively simple types such as cartilage, is an ambitious goal. Additional challenges arise when the role of biomaterials is extended from that of an extant, ‘static’ scaffold to one which is required to form an appropriate cell culture material on demand (by responding to a desired stimulus) and in the presence of a population of living cells. The data presented in this work demonstrate that compromises arise when trying to design a material for both the desirable thermoresponsive phase behaviour (LCST) and final material properties (mechanical stability, water content and diffusion) and consideration of other work in the field leads to the same conclusion [19,21,47,48]. Polymer PNS-5.0 from this work underwent a rapid phase-transition at 33°C and supported good cell viability and proliferation, yet it was mechanically poor at room temperature owing to a poor temperature tolerance. Increasing the mechanical stability by introducing more hydrophobic domains (and thus lowering the LCST and EWC) resulted in a decrease in respiratory activity and no significant cell proliferation in PNS-7.7. This may be the result of limited diffusion owing to the hydrophobic skin effect [45,46]. These compromises can be alleviated by separating the functions of transition temperature and permeability into different polymer blocks as discussed by Yoshioka et al. and Yasuda et al. when rationalizing their PEG-grafted system [23–26] and demonstrated by the present PNS-g-NVP system. This system was stable at room temperature and had good mechanical stability, but also a higher EWC than both PNS-5.0 and PNS-7.7.
In this study, a range of polymers were synthesized, which formed reversible and biocompatible three-dimensional gels in response to a change in temperature. By selecting appropriate polymer compositions, mechanically stable gels could be formed rapidly by incubating aqueous solutions at 37°C. The present hydrogel system could, with further work, also form the basis of a three-dimensional cell-delivery system for therapeutic tissue engineering, as described recently by Borden et al. [49]. However, this remains a challenging application that would require a comprehensive study of the fate of these polymers after in vivo implantation. The work presented here, therefore, focused on the more immediate problem of primary cell expansion.

Two linear copolymers and one graft copolymer were assessed as reversible three-dimensional cell-culture materials alongside an agarose control. Chondrocytes entrapped in these materials remained viable for 24 days and a direct cell-counting study showed an increase in cell number in PNS-5.0 and PNS-g-NVP. The materials could all be processed to produce frozen sections for histological staining or immunolocalization using routine procedures. Both collagen type II and GAG were detected, and the presence of these molecules, in close association with entrapped cells, was indicative of a chondrocytic phenotype. Chondrocytes were readily recovered from all three of the materials by lowering the temperature below the LCST and gently agitating the mixture. This demonstrates an important advantage over agarose constructs, which have previously been demonstrated as cell-culture matrices. These data indicate that three-dimensional thermoreversible gels with the right balance of hydrophobicity and water content are suitable for chondrocyte culture and are suitable for use as three-dimensional scaffold materials in cell expansion.

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REFERENCES


