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Injectable 3D hydrogel scaffold with tailorable porosity post-implantation

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Abstract

Since rates of tissue growth vary significantly between tissue types, and also between individuals due to differences in age, dietary intake and lifestyle-related factors, engineering a scaffold system that is appropriate for personalized tissue engineering remains a significant challenge. In this study, we developed a gelatin-hydroxyphenylpropionic acid/carboxymethylcellulose-tyramine (Gtn-HPA/CMC-Tyr) porous hydrogel system that allows the pore structure of scaffolds to be altered in vivo after implantation. Crosslinking of
Gtn-HPA/CMC-Tyr hydrogels via horseradish peroxidase oxidative coupling was examined both in vitro and in vivo. Post-implantation, further alteration of the hydrogel structure was achieved by injecting cellulase enzyme to digest the CMC component of the scaffold; this treatment yielded a structure with larger pores and higher porosity than hydrogels without cellulase injection. Using this approach, the pore sizes of scaffolds were altered in vivo from 32–87 µm to 74–181 µm in a user-controlled manner. The mechanical properties of the hydrogel are similar to those of soft tissues. Biocompatibility of the hydrogel was demonstrated using African green monkey kidney (COS-7) cells, and faster cell growth was observed in hydrogels after cellulase digestion. The new hydrogel system developed in this work provides clinicians with the ability to tailor the structure of scaffolds post-implantation depending on the growth rate of a tissue or an individual’s recovery rate, and could thus be ideal for personalized tissue engineering.

Introduction

Porosity, pore size and pore interconnectivity are critical parameters determining the performance of scaffolds in tissue engineering. Pore size affects cell attachment, migration, morphology and proliferation; porosity and pore structure also have a strong influence on the mechanical properties of the matrix, the supply of nutrients and oxygen, and the removal of waste products. Different cells and tissues have been shown to require scaffolds of different pore size for maximum tissue growth and development: reports of the optimal average pore size range from as small as 5 µm for neovascularization to 400–500 µm for bone and cartilage regeneration. In general, relatively small pores are considered advantageous initially to maximize cell attachment and interactivity, while highly porous materials provide less diffusional resistance for nutrient and gas exchange. As porous scaffolds become increasingly occluded due to tissue deposition and development, the ability to
increase the pore size and porosity \textit{in situ} during cell culture offers significant advantages for improving nutrient, growth factor and oxygen penetration into the matrix for faster and better tissue growth.

The methods commonly used to fabricate porous scaffolds, such as application of porogens,\cite{7} fiber meshes, particulate leaching, gas foaming, electrospinning, freeze-drying and photo-patterning,\cite{8} typically do not allow the porosity or pore size to be tuned once the scaffold is created. \textit{In situ} degradation by hydrolysis or oxidation reactions (through enzyme and cellular activity) to yield lower molecular weight compounds may alter the scaffold porosity; however degradation rates are difficult to control as they depend not only on the composition and crosslink density of the scaffold\cite{3} but also on the prevailing physical, chemical and biological conditions. In particular, once pre-formed porous scaffolds are implanted \textit{in vivo}, very little or no further control can be exerted on the scaffold structure. In this situation, the use of scaffolds that do not degrade fast enough results in restricted cell migration and proliferation and nutrient and oxygen limitations within the developing tissue. On the other hand, a scaffold that degrades too fast can compromise the mechanical and structural integrity of the implant before the tissue is sufficiently well developed. In both cases, tissue regeneration is inhibited due to a mismatch between the rates of tissue growth and scaffold degeneration. Because different tissues have widely different regeneration rates,\cite{3} and as cell and tissue growth can also vary significantly between individuals due to differences in age, dietary intake, and lifestyle-related factors,\cite{9-11} engineering a scaffold system that is most appropriate for different tissues and individuals is very challenging.

In this work, we address the problem of pore size and porosity control in scaffolds by developing a hydrogel system that allows the pore structure to be altered \textit{in vivo} after
implantation. This new approach gives users the ability to tailor the scaffold architecture to match the specific growth rate of a tissue or an individual’s recovery rate. This was accomplished by synthesizing two polymers: phenol containing gelatin, namely gelatin-hydroxyphenylpropionic acid (Gtn-HPA), as the hydrogel backbone, and phenol containing cellulose, namely carboxymethylcellulose-tyramine (CMC-Tyr), to induce phase separation as well as acting as a sacrificing polymer. The two polymers were crosslinked via horseradish peroxidase-mediated oxidation to yield a porous hydrogel. The Gtn-HPA conjugate was first developed by Kurisawa and coworkers as a precursor to form injectable hydrogel scaffolds for tissue engineering: Gtn-HPA hydrogel was crosslinked via oxidative coupling of phenol moieties catalysed by horseradish peroxidase (HRP) and dilute hydrogen peroxide (H₂O₂).[12] The backbone of this hydrogel contains gelatin that can be degraded by a number of proteases, thereby rendering the Gtn-HPA hydrogel biodegradable.

Injectable hydrogels can also be produced by other methods, for example, physical crosslinking by ionic interactions or sol-gel transition, or chemical crosslinking by Michael-type addition reaction, disulfide bond formation or aldehydes. However, the gelation time of these hydrogels is usually controlled by varying the crosslinker or gel precursor concentration, which inevitably changes the mechanical strength of the resulting matrix.[12] In contrast, peroxidase-catalyzed crosslinking gives rise to hydrogels with mechanical strength and gelation rates that are independently tunable.[12] The gelation time (ranging from 1 s to 20 min) of Gtn-HPA hydrogel can be tuned solely by the HRP concentration, while the mechanical strength can be tuned solely by the H₂O₂ concentration.[14] Peroxidase-catalysed cross-linking is an attractive strategy as the enzymatic coupling can be carried out at room temperature and in an aqueous environment without the use of organic solvents or toxic crosslinkers: in situ cell immobilization can therefore take place by simply mixing the cells
Gtn-HPA hydrogels have been demonstrated as a promising scaffold material for tissue engineering. For instance, neurogenesis and myogenesis of human mesenchymal stem cells (hMSCs) can be controlled by simply tuning the Gtn-HPA hydrogel stiffness without the use of additional biochemical signals.\cite{14, 17} Peroxidase-mediated coupling has also been employed to crosslink various other phenol-containing polymers and is well known to yield \textit{in situ} hydrogels that are biocompatible with encapsulated cells.\cite{15, 18-20} For example, Kurisawa and coworkers have demonstrated that hydrogels produced using peroxidase-mediated oxidation support neural and mesenchymal stem cell viability at levels greater than 90\%.\cite{21, 22} Peroxidase-mediated oxidation has also been shown to produce \textit{in situ} hydrogels that are biocompatible \textit{in vivo}.\cite{23-25} However, such injectable hydrogels do not contain any micropores or macropores (See supplementary information, \textbf{Figure S1}); in addition, the porosity or pore size of the gel cannot be tuned once the hydrogel is created. Herein, we describe a new method to increase the pore size and porosity of tissue engineering scaffolds post-implantation using cellulase injection to digest the sacrificing polymer, cellulose, in hydrogels. In addition, as the hydrogel is crosslinked by horseradish peroxidase, it is injectable and suitable for applications in laparoscopic surgery.

\textbf{Materials \& Methods}

\textit{Preparation of Gtn-HPA/CMC-Tyr hydrogel}

CMC-Tyr conjugates were synthesized using a general carbodiimide/active ester mediated coupling reaction in distilled water. Briefly, CMC (5 g) and Tyr (0.864 g) were dissolved in milliQ water (250 ml). To this solution, N-hydroxysuccinimide (NHS) (0.573 g) and 1-ethyl-
3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (0.955 g) were added. The mixture was stirred overnight at room temperature and pH 4.7. The products were dialyzed against 100 mM NaCl, 25% ethanol and water in sequence for 2 days each, and then lyophilized. Conjugation of tyramine to CMC was confirmed by $^1$H NMR (D$_2$O); chemical shifts were detected at $\delta$ 6.8 and 7.2 indicated the presence of aromatic protons of tyramine on CMC. Gtn-HPA conjugates were prepared according to Wang et al.$^{[17]}$. To synthesize fluorescently labelled Gtn-HPA, the -COOH group of Gtn-HPA conjugates (10 g) were activated using NHS/EDC at pH 4.8 for 15 min. Fluoresceinamine isomer I (Sigma) dissolved in methanol (0.23 µM) was added dropwise to the Gtn-HPA conjugates while stirring and the reaction was allowed to proceed overnight in the dark. The products were purified by dialysis and then lyophilized.

Lyophilized Gtn-HPA and lyophilized CMC-Tyr were each dissolved in separate PBS solutions at a concentration of 5% (w/v). Hydrogel precursor solution was prepared by mixing the Gtn-HPA and CMC-Tyr solutions using a 80:20 weight ratio, respectively. Crosslinking was initiated by adding HRP to give three different final concentrations (3.9, 7.7 and 15.5 unit L$^{-1}$) and diluted H$_2$O$_2$ to give a final concentration of 49.8 µM.

**Adjusting the pore size in situ**

Cellulase from Trichoderma longibrachiatum (Sigma; $\geq$ 1.0 unit mg$^{-1}$) was dissolved in PBS at a concentration of 0.1 mg ml$^{-1}$ and sterilized by syringe filtration. Hydrogel precursor solution with added HRP and H$_2$O$_2$ was injected between two parallel glass plates clamped together with 1 mm spacing. The crosslinking reaction was allowed to proceed at 37 ºC. Round hydrogel disks of diameter 1.5 cm were then cut out using a circular mold. The hydrogel disks were immersed in PBS at 37 ºC for 24 h to reach swelling equilibrium. The
Swollen hydrogels were then immersed in cellulase solution (0.1 mg ml\(^{-1}\) in PBS). Spent solution was replaced by cellulase solution daily. The time-course of hydrogel digestion was examined over a period of up to 9 days by measuring the dry weight of residual hydrogel after oven drying at 50–60 °C to constant weight. Disks of Gtn-HPA hydrogel without cellulose content were used as controls. The pore sizes and porosities of lyophilized hydrogels were determined using a mercury porosimeter (Autopore II 9220, Micromeritics) and are reported as mean ± standard deviation. The Z-test (n > 30) was used to compare the results statistically before and after cellulase digestion.

**Swelling ratio measurement**

The swelling ratio of hydrogel was evaluated using a classical gravimetric method. Hydrogel disks prepared using 49.8 \(\mu\)M of \(\text{H}_2\text{O}_2\) and 3 different HRP concentrations (3.9, 7.7 and 15.5 unit L\(^{-1}\)) were swollen in PBS at 37°C for 2 days to reach swelling equilibrium. To obtain cellulase-digested hydrogel disks, these hydrogels were immersed in cellulase solution (0.1 mg ml\(^{-1}\) in PBS) at 37°C for 24 h. Spent solution was replaced by 1 ml of fresh cellulase solution daily for a period of 3 days. The cellulase-treated hydrogels were rinsed with MilliQ water for 2 days to remove residual cellulase. The surface of the swollen disks with or without cellulase treatment were gently blotted with Kimwipe and weighed immediately to obtain the swollen weight. The swollen disks were then lyophilized and the dry weight of the lyophilized disks was measured. The swelling ratio was calculated according to the equation (1) below:

\[
\text{Swelling ratio} = \frac{W_s - W_d}{W_w} \times 100\% 
\]

where \(W_s\) and \(W_d\) are the weights of swollen and dry hydrogels, respectively.
Measurements of gelation time, loss modulus and storage modulus of hydrogel prior to cellulase digestion were performed according to Wang et al.\cite{14}. To assess the mechanical properties of hydrogel after cellulase digestion, Gtn-HPA/CMC-Tyr hydrogel precursor solution prepared using three different concentrations of HRP (3.9, 7.7 and 15.5 unit L\(^{-1}\)) and a fixed concentration of H\(_2\)O\(_2\) (49.8 µM) was injected between two parallel glass plates clamped together with 1 mm spacing. After crosslinking, round hydrogel disks of diameter 1.5 cm were cut out using a circular mold. The hydrogel disks were immersed in 1 ml of cellulase solution (0.1 mg ml\(^{-1}\) in PBS) at 37°C for 24 h. Spent solution was replaced by 1 ml of fresh cellulase solution daily for a period of 3 days. The storage modulus of the swollen hydrogels was measured using a Haake MARS (Modular Advanced Rheometer System, Germany) at 37°C in the dynamic oscillatory mode with a constant deformation of 0.5 % and a frequency of 0.5 Hz.

**Cytocompatibility and cell proliferation assays**

The cytocompatibility of the hydrogel was evaluated using COS-7 cells. Lyophilized Gtn-HPA and CMC-Tyr were dissolved in 500 µl of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, and 50 units ml\(^{-1}\) penicillin-streptomycin, and sterilized immediately by syringe filtration through a 0.2 µm filter. African green monkey kidney (COS-7) cells at a density of 1 × 10\(^4\) cells ml\(^{-1}\) were mixed with the sterilized solution prior to crosslinking. Crosslinking was initiated by adding diluted H\(_2\)O\(_2\) and different concentrations of HRP and allowed to proceed at 37 °C in a humidified 5% CO\(_2\) incubator. To adjust the pore size \textit{in situ}, the hydrogel was incubated in medium containing cellulase (0.1 mg ml\(^{-1}\)) at 37 °C; spent medium was replaced by fresh cellulase-containing medium daily. COS-7 cells were harvested at different time points over a period of 7 days by digesting the hydrogel using trypsin and cellulase. Cell number was
determined by quantifying the DNA content using a Quant-iT PicoGreen dsDNA assay kit (Invitrogen) according to the manufacturer’s protocol. The total DNA content in each hydrogel sample was determined by measuring the fluorescence using a SpectraMax M2 microplate reader (Molecular Devices) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The number of cells in each sample was calculated by correlating the total DNA content with a known number of COS-7 cells. These experiments were performed using triplicate gels for each time point and HRP concentration.

**Subcutaneous formation of hydrogel in vivo**

The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC0092), Nanyang Technological University, and all experiments were carried out in compliance with the necessary regulations. All animals were maintained in pathogen-free conditions. Two milliliters of hydrogel precursor solution containing CMC-Tyr, fluorescently labelled Gtn-HPA, 49.8 µM H2O2 and different concentrations of HRP (3.9, 7.7, and 15.5 unit L⁻¹) were administered by dorsal subcutaneous injection to female Lewis rats (6–8 weeks old). Six rats were used for each HRP concentration. The hydrogels were allowed to gelify in situ. To adjust the pore size of the hydrogels, 0.3 ml of 0.1 mg ml⁻¹ cellulase in PBS were injected into the animals at the same position as the scaffold 2 h after the initial injection of hydrogel. Triplicate rats were injected with cellulase for each HRP concentration, leaving triplicate control animals for each HRP concentration without cellulase treatment. Six hours after the initial hydrogel injection, the animals were anesthetized and then sacrificed by intra-cardiac injection of an overdose of pentobarbital. The skin was incised to expose the hydrogel scaffolds, which were excised and fixed in 4% formalin. In order to investigate the effect of introducing cellulase at later time points, another set of in vivo experiments was carried out using the above procedure, except that 3 doses of cellulase (0.3 ml of 0.1 mg ml⁻¹ cellulase in...
PBS, 24 h between injections) were injected into the animal starting from day 4 after the initial injection of hydrogel. The animals were sacrificed 7 days after the initial hydrogel injection. The cross-sectional morphologies of the harvested hydrated hydrogels were visualized using LSCM. Samples were also lyophilized for SEM analysis. Pore sizes were measured from the LSCM images and are reported as mean ± standard deviation (n = 25–50). One-way analysis of variance (ANOVA) was used to compare multiple groups of data; the Student’s t-test was used to compare two groups of data.

**Results & Discussion**

**Hydrogel formation**

The scaffold fabrication process described in this study is simple and biocompatible. Two phenol-containing polymers, gelatin-hydroxyphenylpropionic acid (Gtn-HPA)\(^{[17]}\) as the hydrogel scaffold backbone, and carboxylmethylcellulose-tyramine (CMC-Tyr) (Figure 1a) as a sacrificing polymer were synthesized. The Gtn-HPA component contains Arg-Gly-Asp (RGD) peptides that act as cell adhesive ligands. The two polymer conjugates were dissolved together in phosphate buffered saline (PBS) and appear as a clear solution; horseradish peroxidase (HRP) and diluted hydrogen peroxide (H\(_2\)O\(_2\)) were then added to the solution. At this stage, for *in vivo* applications, the hydrogel may be injected to fill any irregularly shaped defect. The polymer conjugates were allowed to crosslink to form a polymer network via peroxidase-catalyzed oxidative coupling of the HPA and Tyr moieties (Figure 1b). It is well known that crosslinking occurs at the C–C and C–O position of phenols:\(^{[26]}\) this reaction proceeds rapidly in aqueous solution at room temperature\(^{[27]}\) so that living cells can be incorporated during crosslinking for *in situ* immobilization. Figure 1c summarizes the procedures involved in preparing the porous hydrogel. A porous polymer network or hydrogel scaffold was obtained after crosslinking, with pore formation driven by phase separation.
between Gtn-HPA and CMC-Tyr. The former biopolymer contains gelatin that is known to contain both hydrophilic and hydrophobic peptides,[13] while the latter biopolymer is rich in carboxylic acid residues and thus more hydrophilic than Gtn-HPA. The resulting hydrogel appears optically opaque upon crosslinking; this opaque appearance is a typical characteristic of hydrogels formed via phase separation.[28-30] The mechanisms of phase separation relevant to this system are discussed in more detail in the literature.[31-33]

To alter the hydrogel structure post-implantation, diluted cellulase enzyme (0.1 mg ml\(^{-1}\) in PBS) was injected into the hydrogel scaffold to digest the sacrificing polymer, CMC, to glucose or low-molecular-weight CMC via hydrolysis of the glucosidic linkages in CMC (Figure 2). These low-molecular-weight molecules are able to diffuse out of the porous hydrogel network to yield a material with higher porosity and larger pore size compared with hydrogel before cellulase treatment. The cellulase used was derived from *Trichoderma longibrachiatum*, has a long history of safe use in the food industry, has been affirmed by the US Food and Drug Administration (FDA) to be Generally Recognized As Safe (GRAS).[34] Since the rate and extent of cellulose digestion can be controlled by varying the amount and frequency of cellulase injection, it is possible for clinicians using this system to alter the hydrogel pore structure in a controllable manner post-implantation.

**In vitro pore size and porosity**

The hydrogels were further characterized using mercury porosimetry to measure the *in vitro* pore size and porosity of the scaffolds with and without cellulase digestion. These properties varied depending on the HRP concentration used (Table 1): hydrogels produced using the highest HRP concentration (15.5 unit L\(^{-1}\)) exhibited the smallest pore size and porosity, while hydrogels produced using the lowest HRP concentration (3.9 unit L\(^{-1}\)) exhibited the largest
pore size and porosity. The results demonstrate that HRP concentration is a critical factor affecting the pore structure of the hydrogel, most likely through its effect on phase separation during hydrogel formation. Despite both proteins and polysaccharides being soluble in aqueous solution, it has been reported that these macromolecules normally have limited compatibility at the molecular level, so that each macromolecule tends to be surrounded by its own species in mixed solution, thereby causing the mixture to separate into different liquid phases.\textsuperscript{[35]} The mixture of Gtn-HPA and CMC-Tyr is initially homogenous and miscible; however, upon crosslinking, further phase separation occurs as the fraction of molecules with high molecular weight increases, causing the morphology of the mixture to separate into discrete domains. Reducing the concentration of HRP and therefore the rate of crosslinking probably allows time for these discrete domains to increase in size and undergo a phenomenon known as spinodal decomposition\textsuperscript{[31]} so that a porous matrix with relatively large voids is formed.

Cellulase treatment was performed \textit{in vitro} to alter the hydrogel pore structure post-fabrication. \textbf{Figure 3} shows representative SEM images of lyophilized hydrogel with or without cellulase treatment. These images show that pore sizes in the hydrogel increased as the concentration of HRP decreased. It can also be observed that the pore sizes increased after cellulase treatment. These observations will be discussed in detail in later sections. Cellulase digestion for 24 h resulted in an increase in mean pore size of 31\% ($p < 0.0001$) and 61\% ($p = 0.003$) for hydrogels produced using 3.9 and 15.5 unit L$^{-1}$ HRP, respectively (\textbf{Table 1}); the 8\% increase in pore size measured using 7.7 unit L$^{-1}$ HRP was not statistically significant ($p = 0.376$). Hydrogel porosity was also enhanced after cellulase treatment: the mean porosity increased by 11\% ($p = 0.010$) and 48\% ($p < 0.0001$) after cellulase digestion of hydrogels
produced using 7.7 and 15.5 unit L\(^{-1}\) HRP, respectively; the measured increase in porosity for the 3.9 unit L\(^{-1}\) HRP samples was not statistically significant (\(p = 0.426\)).

**Weight loss induced by cellulose digestion**

To quantify the extent of cellulose digestion, Gtn-HPA/CMC-Tyr hydrogels produced using different HRP concentrations were treated with cellulase; hydrogels composed of pure Gtn-HPA only were used as controls. Whereas the Gtn-HPA/CMC-Tyr hydrogels were digested as indicated by weight loss measurements, the Gtn-HPA matrix remained unaffected (**Figure 4**). This result confirms the specificity of cellulase for digestion of cellulose in the CMC polymer, thus allowing Gtn-HPA in the Gtn-HPA/CMC-Tyr hydrogels to function as the cell adhesive structure that remains intact during cellulase treatment. The substrate specificity of cellulase also means that newly formed extracellular matrix (ECM) secreted by cells in the hydrogel is unlikely to be affected by cellulase treatment. In general, the rate of digestion observed for the Gtn-HPA/CMC-Tyr hydrogels increased as the HRP concentration used to produce the scaffolds decreased (**Figure 4**). This is consistent with the larger pore sizes and higher porosities observed at lower HRP levels (**Table 1**), which would facilitate more rapid penetration of cellulase into the hydrogel structure. These experiments demonstrate that it is possible to quantify cellulose digestion of the scaffolds and to generate data useful for tailoring the pore structure of scaffolds post-implantation to match the growth rate of specific tissues.

**Swelling ratio**

**Figure 5a** and **5b** show the swelling ratios of hydrogel before and after cellulase digestion, respectively. Swelling ratio represents the amount of water that can be absorbed by the hydrogel; this is an important property as high swelling ratios can facilitate diffusion of...
nutrients, oxygen, and metabolites.\textsuperscript{[28]} It is well known that the presence of open pores in hydrogels allows water to be absorbed by convection.\textsuperscript{[36]} Before cellulase digestion, the hydrogels prepared using three different HRP concentrations exhibited high swelling ratios reflecting the presence of porous structures. The swelling ratio was found to increase with decreasing HRP concentration: hydrogels prepared using lower HRP concentration (i.e. 3.9 unit ml\textsuperscript{-1}) contained larger pores, thus facilitating better water infiltration into the porous network. After cellulase digestion, the swelling ratios of the resulting hydrogels were enhanced, as the pores were opened up by cellulase digestion, thus allowing more water infiltration. After cellulase digestion, the swelling ratios continued to increase with decreasing concentration of HRP, consistent with the increase in pore size and porosity of the hydrogels with decreasing HRP concentration.

\textit{Rheology and mechanical properties of hydrogels}

The physical, mechanical and biocompatibility properties of the hydrogel were first characterized in vitro. Hydrogels were prepared using the same polymer composition and H\textsubscript{2}O\textsubscript{2} concentration but with different concentrations of HRP enzyme for catalyzing the crosslinking reaction. The formation of Gtn-HPA/CMC-Tyr hydrogels was investigated using oscillatory rheometry techniques. The typical evolution with crosslinking time of the storage modulus (G') and loss modulus (G'') of hydrogels is shown in the supplementary information section (\textbf{Figure S2a-c}). The crossover of the two moduli is defines as the gel point at which the viscoelastic liquid transforms into a viscoelastic solid, and the time required to reach the gel point is defined as the gelation time. The gelation rate was found to increase with increasing HRP concentration: gelation times of ~ 597, 330 and 110 s were obtained for hydrogels prepared using HRP concentrations of 3.9, 7.7 and 15.5 unit L\textsuperscript{-1}, respectively. However, the corresponding values of G' were similar at 3741, 3898 and 3933 Pa,
respectively. The control group prepared with 0 unit L\(^{-1}\) of HRP did not gelify. As with other HRP-mediated crosslinked hydrogel systems,\(^{[14, 23]}\) the mechanical properties of the Gtn-HPA/CMC-Tyr hydrogel were independent of gelation rate\(^{[14]}\) and similar to those of soft tissues and organs.\(^{[37]}\)

Pore size and porosity have little effect on the storage modulus of Gtn-HPA/CMC-Tyr hydrogels, as the mechanical properties of the hydrogels were measured using swollen samples, in which the hydrostatic effect of water entrapped in the scaffold pores reduces the deformability of the scaffold\(^{[38]}\) has also reported that the storage modulus was less dependent on pore size and porosity in starch/poly(L-lactic) acid scaffolds that were immersed under physiological conditions. In contrast, the storage modulus was dependent on pore size and porosity for samples measured in a dry state. Since the Gtn-HPA/CMC-Tyr hydrogels in the present study are intended for in vivo applications, it is more appropriate to perform the measurement of mechanical properties using wet samples immersed in physiological simulated solution.

The storage modulus of hydrogels after cellulase digestion was also evaluated. As expected, G’ decreased after cellulase digestion: the values of G’ were 2788, 3041, and 3362 Pa for hydrogels prepared with HRP concentrations of 3.9, 7.7 and 15.5 unit L\(^{-1}\), respectively. The values of G’ decreased with decreasing HRP concentration, which is likely due to the larger mass of cellulose lost during enzymatic digestion of hydrogels prepared with lower HRP concentration (Figure 4). A reduction in mechanical strength associated with scaffold degradation is expected in a biodegradable scaffold, and can be beneficial for tissue regeneration, as softer scaffolds allow new tissue to more easily infiltrate the scaffold.
**In vitro cytocompatibility**

To assess the cytocompatibility of the scaffolds, COS-7 cells were immobilized in Gtn-HPA/CMC-Tyr hydrogels *in situ* prior to crosslinking with HRP/H$_2$O$_2$. Mixing cells suspended in culture medium with polymer conjugates before crosslinking yields homogeneously seeded scaffolds and overcomes the difficulties associated with introducing cells and achieving uniform cell distributions within already gelified structures. The viability of COS-7 cells remained high after *in situ* immobilization (see supporting information). The number of cells inside the scaffolds was quantified by measuring the DNA content, as DNA is the cellular component that most accurately reflects cell number.\[^{39}\] The DNA content, and thus the number of cells, in the scaffolds increased over the 7-day culture period (Figure 6), indicating that the encapsulated cells were viable and capable of proliferation, and confirming that the Gtn-HPA/CMC-Tyr hydrogel is biocompatible for tissue engineering applications.

Cell growth was faster as the HRP concentration used for crosslinking was reduced; hydrogels with larger pores and porosities (produced using lower HRP concentrations; Table 1) provide entrapped cells with better access to nutrients and oxygen and more space for migration and proliferation than scaffolds with restricted pore structure. However, growth of cells in hydrogels without cellulase treatment showed signs of slowing down after about 4 days (Figure 6). In contrast, when the hydrogels were digested with cellulase starting on day 1 of the culture, significant further cell growth occurred after 4 days and the final cell numbers obtained were significantly higher than those achieved under the same conditions without cellulase digestion. For each HRP concentration, the final cell numbers obtained from hydrogels digested by cellulase starting from day 4 were similar to those found after digestion by cellulase starting from day 1 (Figure 6c).
As well as demonstrating the benefits of manipulating the scaffold pore size and porosity post-inoculation, these results show that cellulase treatment has no toxic effects on the cells. The approach to hydrogel fabrication and manipulation developed in this work allows scaffolds with smaller pore sizes and porosities to be used at the beginning of tissue engineering cultures when achieving cell attachment is critical,[1] while providing the opportunity to tailor the scaffold and enlarge the pores as required at the desired time to improve cell proliferation, transport of nutrients, and tissue formation.

**Subcutaneous formation of hydrogels**

To examine the ability of the Gtn-HPA/CMC-Tyr hydrogels to crosslink *in vivo*, and to demonstrate the feasibility of tuning hydrogel pore size and porosity post-implantation, Gtn-HPA and CMC-Tyr hydrogel precursors were injected into the dorsal subcutis of female Lewis rats (Figure 7a–b). Fluorescein isothiocyanate (FITC)-labelled Gtn-HPA was used to assist visualization. The gelation time for formation of gelified lumps of size 0.8–1.3 cm within the subcutaneous layer depended on the HRP concentration applied consistent with the *in vitro* results. Post-gelation, cellulase was injected directly into each lump to alter the pore size and porosity of the hydrogel *in vivo* (Figure 7b). Animals with no cellulase treatment were used as the control group (Figure 7a). After the animals were sacrificed, the hydrogel implants were detected easily within the subcutaneous space. No pathological reactions were observed within the implants or the subcutaneous wall.

The microscopic morphologies of the *in vivo* hydrogel implants in the control groups were investigated. Figure 8a-c shows typical micrographs of cross-sections of the hydrated implants obtained using laser scanning confocal microscopy (LSCM); these implants were composed of CMC-Tyr and FITC-labelled Gtn-HPA without cellulase treatment. Figure 9a-c
shows typical cross-sections observed using scanning electron microscopy (SEM) after lyophilization of hydrogel implants harvested 6 h after initial injection of hydrogels without cellulase treatment. The LSCM and SEM images reveal the presence of a homogenous interconnected macroporous structure in both hydrated and lyophilized hydrogel implants. Such networks of interconnected pores are important for supporting cell proliferation and deposition of ECM during tissue regeneration, and for facilitating exchange of oxygen and nutrients throughout the hydrogel. The LSCM images revealed that as the HRP concentration decreases, thus increasing the gelation time, a coalescence of Gtn-HPA-rich domains can be visualized in the hydrogel. Such morphologies are typically observed in ternary biopolymer systems (consisting of two biopolymers and a solvent) that undergo phase separation by spinodal decomposition.\cite{31}

Similar to the in vitro results (Figure 3, Table 1), pore sizes in the in vivo hydrogels varied with the concentration of HRP used for polymer crosslinking: visually in Figure 8a-c and Figure 9a-c, the pores were larger in hydrogels produced using the lowest HRP concentration (Figure 8a and Figure 9a) and smaller in hydrogels produced using the highest HRP concentration (Figure 8c and Figure 9c). As indicated in the LSCM images of the hydrated scaffolds (Figure 8a-c), pore sizes in the in vivo hydrogels were distributed bimodally: many small micropores were located within the polymeric material bridging between larger macropores. The diameter of the larger pores decreased as the concentration of HRP applied for crosslinking was increased ($p < 0.0001$) and were (mean ± standard deviation) $87 ± 2.1$, $51 ± 5.2$ and $32 ± 1.3 \mu m$ for hydrogels prepared using $3.9$, $7.7$ and $15.5$ unit L$^{-1}$ HRP, respectively. The diameter of the smaller pores also decreased with increasing HRP concentration ($p < 0.0001$) and were $19 ± 3.4$, $11 ± 7.2$, and $9 ± 8.2 \mu m$ for HRP concentrations $3.9$, $7.7$ and $15.5$ unit L$^{-1}$, respectively. Compared to hydrogels harvested 6 h
post-implantation, the diameter of both the larger (p < 0.0001) and smaller pores (p < 0.01) increased in hydrogels harvested 7 days post-implantation (Figure 8g-i and Figure 9g-i), probably due to the presence of in vivo proteases, which may also contribute to in vivo hydrogel degradation.

Adjusting the pore size post-implantation

Figure 8d-f shows typical LSCM micrographs of cross-sections of hydrated hydrogel implants after in vivo treatment with cellulase 2 h post-implantation. Figure 9d-f shows the corresponding SEM micrographs of cellulase-treated hydrogels after lyophilization. In vivo cellulase digestion resulted in an increase in pore size and porosity: the cellulase-digested hydrogels have a more open and accessible pore structure compared with untreated samples produced using the same HRP concentration. The homogeneity of the porous networks after cellulase treatment indicates that cellulase penetrated throughout the entire volume of the hydrogel lumps to digest CMC at all locations within the scaffolds. The cellulase-digested hydrogels continued to exhibit a bimodal distribution of pore sizes: micropores were present within the polymer surrounding much larger macropores (Figure 8d-f). For hydrogels treated with cellulase and harvested after 6 h, the diameters of the larger pores decreased with increasing HRP concentration (p < 0.0001) and were 181 ± 7.7, 135 ± 2.6 and 74 ± 8.3 µm for hydrogels prepared using HRP concentrations of 3.9, 7.7 and 15.5 unit L⁻¹, respectively (Table 2). These macropore sizes after cellulase digestion represent enlargements of 2.1-fold (p < 0.0001), 2.6-fold (p < 0.0001) and 2.3-fold (p < 0.0001), respectively, relative to the corresponding pores in scaffolds without cellulase treatment. In contrast, the average diameter of the smaller pores in the digested scaffolds was not dependent on HRP concentration (p = 0.183), being similar at 12 ± 2.7, 10 ± 7.4 and 9 ± 6.2 µm for 3.9, 7.7 and 15.5 unit L⁻¹ HRP, respectively. Although micropores in the 3.9 unit L⁻¹ HRP hydrogels were reduced in size
after cellulase treatment \( (p < 0.0001) \), cellulase digestion had no effect on micropore dimensions in scaffolds prepared using 7.7 and 15.5 unit L\(^{-1}\) HRP \( (p = 0.630 \text{ and } p = 0.716, \text{ respectively}) \). This reflects the relative inaccessibility and lower interconnectivity of the smaller pores for cellulase diffusion, so that the main effect of cellulase treatment was enlargement of the scaffold macropores. As larger pores enhance mass transfer and are preferred during the later stages of tissue regeneration for ECM deposition, an improvement in culture performance and tissue engineering outcomes can be expected after \textit{in situ} cellulase digestion of implanted hydrogels. Whereas the gelatin component of the Gtn-HPA/CMC-Tyr hydrogels is already biodegradable and capable of being adsorbed gradually within the body, directed application of cellulase enzyme allows control to be exercised over the rate of degradation of the cellulose (CMC) component of the polymer matrix.

Depending on the rate of tissue formation \textit{in vivo}, multiple applications of cellulase may be used to further enlarge the hydrogel pores as more space is required in the scaffold. To test this, we studied the effect of introducing cellulase at later time points (cellulase was introduced at days 4, 5, and 6). \textbf{Figure 8j-1} shows typical LSCM micrographs of cross-sections of hydrated hydrogel implants after multiple \textit{in vivo} cellulase treatments. \textbf{Figure 9j-1} shows the corresponding SEM micrographs of the cellulase-treated hydrogels after lyophilization. \textbf{Table 2} summarizes the pore sizes of \textit{in vivo} hydrogels obtained with or without cellulase digestion. As expected, for each HRP concentration, the mean diameters of the macropores in hydrogels with multiple cellulase treatments were larger than those in hydrogels treated with a single application of cellulase. Interestingly, the mean diameters of the micropores in hydrogels with multiple cellulase treatments were also larger than those in hydrogels with a single cellulase treatment at the corresponding HRP concentration. This suggests that cellulase can penetrate the micropores if multiple cellulase treatments are given,
as larger macropores provide better accessibility for cellulase diffusion into the micropores, thereby allowing the micropores to open up during subsequent cellulase treatment. In addition, the longer implantation time (7 days) also allowed in vivo proteases to penetrate and degrade the hydrogels to open up the pores. Consistent with the LSCM results in Figure 8g-l, the SEM images in Figure 9g-l revealed that hydrogels harvested after 7 days were highly porous with interconnected pores. The hydrogel scaffolds harvested after 7 days (Figure 9g-l) appeared to be more collapsed compared to those harvested after 6 h (Figure 9a-f), which is likely due to the reduced mechanical strength caused by cellulase digestion and protease degradation. It is expected that the reduction in mechanical strength associated with scaffold degradation allows new tissue to easily infiltrate the scaffold.

Conclusion

In conclusion, the pore structure of an injectable hydrogel scaffold was modified post-implantation in this proof-of-concept work. Using the prepared hydrogel, the properties of the scaffold were tuned in vivo by simply injecting a digestive enzyme into the implantation site. In untreated scaffolds, average macropore sizes in vivo ranged from 32 to 87 µm depending on the conditions applied for polymer crosslinking. After tuning of the pore structure using a single cellulase injection, macropore sizes were increased to 74–181 µm, while multiple cellulase injections produced macropore sizes in vivo of 108–236 µm. The hydrogel system developed is biodegradable, biocompatible and suitable for cell culture and tissue engineering applications. The mechanical properties of the biomaterial are similar to those of soft tissues and organs; an additional advantage is that the gelation rate and mechanical properties of the hydrogel can be tuned independently. Using the approach and methods described here, the pore size and porosity of scaffolds can be tailored in situ after implantation to match the
growth rate of the tissue being regenerated and in response to the recovery conditions of the patient. The system is therefore ideal for personalized tissue engineering.

Supporting Information

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

Acknowledgements

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a. CMC + Tyramine → CMC-Tyr conjugates

\[ R = H \text{ or } \text{HO-CO} \]

b. Gtn-HPA conjugates + CMC-Tyr conjugates → Gtn-HPA/CMC-Tyr hydrogel

\[ \text{HRP/H}_2\text{O}_2, \text{PBS or medium} \]
Figure 1. (a) Synthesis scheme of CMC-Tyr conjugates; (b) CMC-Tyr and Gtn-HPA conjugates were crosslinked by peroxidase-catalyzed oxidation. (c) Preparation of 5 % Gtn-HPA/CMC-Tyr hydrogel. Lyophilized Gtn-HPA and lyophilized CMC-Tyr were first dissolved to form a homogeneous solution at room temperature. HRP and diluted H$_2$O$_2$ were then added to the solution to crosslink Gtn-HPA and CMC-Tyr to form an opaque gel at room temperature.
Figure 2. Schematic representation of the formation of injectable hydrogel by HRP-catalyzed oxidation with *in situ* pore size control using cellulase digestion.
<table>
<thead>
<tr>
<th>HRP (unit/L)</th>
<th>Before cellulase digestion</th>
<th>After cellulase digestion</th>
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<tr>
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<td><img src="image5.png" alt="Image" /></td>
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</table>

**Figure 3.** Typical cross-sectional SEM images of lyophilized *in vitro* hydrogels prepared using 3.9, 7.7 and 15.5 unit L\(^{-1}\) HRP. For cellulase digestion, hydrogel disks were digested in cellulase solution (0.1 mg ml\(^{-1}\) in PBS) for a period of 3 days, followed by rinsing and lyophilization. Scale bars represent 50 µm.
Figure 4. Scaffold weight loss during enzymatic digestion of Gtn-HPA/CMC-Tyr and Gtn-HPA hydrogels in the presence of 0.1 mg ml\(^{-1}\) cellulase at 37 °C. The hydrogels were prepared using different concentrations of HRP. The error bars represent standard deviation (n = 3).
Figure 5. Swelling ratios of (a) Gtn-HPA/CMC-Tyr hydrogels prepared using 3.9, 7.7 and 15.5 unit L\(^{-1}\) HRP without cellulase treatment, and (b) cellulase-digested Gtn-HPA/CMC-Tyr hydrogels prepared using 3.9, 7.7 and 15.5 unit L\(^{-1}\) HRP. The error bars represent standard deviation \((n = 3)\).
Figure 6. Proliferation of COS-7 cells in vitro in (a) Gtn-HPA/CMC-Tyr hydrogels without cellulase treatment, (b) Gtn-HPA/CMC-Tyr hydrogels with cellulase treatment (0.1 mg ml\(^{-1}\) in medium) started from day 1, and (c) Gtn-HPA/CMC-Tyr hydrogels with cellulase treatment (0.1 mg ml\(^{-1}\) in medium) started from day 4. During cellulase treatment, spent medium was replaced by fresh cellulase solution daily. The error bars represent standard deviation (\(n = 3\)).
Figure 7. (a) Female Lewis rats were injected subcutaneously with Gtn-HPA/CMC-Tyr precursor solution, diluted H$_2$O$_2$ and HRP. In situ gelation of the hydrogel within the subcutaneous layer can be observed. (b) After injection with hydrogel solution and crosslinking, rats were also injected with cellulase solution into the hydrogel for digestion of CMC and modification of the scaffold pore size.
Figure 8. (a), (b) and (c) Representative LSCM images of hydrated fluorescently labelled \textit{in vivo} hydrogels prepared using 3.9, 7.7 and 15.5 unit L$^{-1}$ HRP, respectively, without cellulase treatment. These hydrogels were harvested 6 h after injection. (d), (e) and (f) Representative LSCM images of hydrated fluorescently labelled \textit{in vivo} hydrogels prepared using 3.9, 7.7 and 15.5 unit L$^{-1}$ HRP, respectively, after digestion by cellulase 2 h after hydrogel injection. These hydrogels were harvested 6 h after the initial hydrogel injection. (g), (h) and (i) Representative LSCM images of hydrated fluorescently labelled \textit{in vivo} hydrogels prepared using 3.9, 7.7 and 15.5 unit L$^{-1}$ HRP, respectively, without cellulase treatment. These hydrogels were harvested 7 days after injection. (j), (k) and (l) Representative LSCM images of hydrated fluorescently labelled \textit{in vivo} hydrogels prepared using 3.9, 7.7 and 15.5 unit L$^{-1}$ HRP, respectively, after digestion by cellulase at day 4, 5, and 6. These hydrogels were harvested 7 days after the initial hydrogel injection. The scale bars represent 200 µm.
Figure 9. (a), (b), and (c) Typical cross-sectional SEM images of lyophilized in vivo hydrogels prepared using 3.9, 7.7 and 15.5 unit L⁻¹ HRP, respectively, without cellulase treatment. These hydrogels were harvested 6 h after injection. (d), (e), and (f) Typical cross-sectional SEM images of lyophilized in vivo hydrogels prepared using 3.9, 7.7 and 15.5 unit L⁻¹ HRP, respectively, after cellulase digestion 2 h after hydrogel injection. These hydrogels were harvested 6 h after the initial hydrogel injection. (g), (h), and (i) Typical cross-sectional SEM images of lyophilized in vivo hydrogels prepared using 3.9, 7.7 and 15.5 unit L⁻¹ HRP, respectively, without cellulase treatment. These hydrogels were harvested 7 days after injection. (j), (k), and (l) Typical cross-sectional SEM images of lyophilized in vivo hydrogels prepared using 3.9, 7.7 and 15.5 unit L⁻¹ HRP, respectively, after cellulase digestion at day 4, 5, and 6. These hydrogels were harvested 7 days after the initial hydrogel injection. The scale bars represent 200 μm.
Table 1. Mean pore size and porosity of *in vitro* Gtn-HPA/CMC-Tyr hydrogels with and without cellulase digestion. For cellulase digestion, hydrogel disks were digested by cellulase solution (0.1 mg ml⁻¹ in PBS) for a period of 3 days; spent solution was replaced with fresh cellulase solution daily. Results are shown as mean ± standard deviation.

<table>
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<tr>
<th>HRP concentration (unit L⁻¹)</th>
<th>Mean pore size before cellulase digestion (µm)</th>
<th>Mean pore size after cellulase digestion (µm)</th>
<th>Increase in pore size after cellulase digestion (%)</th>
<th>Mean porosity before cellulase digestion (%)</th>
<th>Mean porosity after cellulase digestion (%)</th>
<th>Increase in porosity after cellulase digestion (%)</th>
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<td>8</td>
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* At the same HRP concentration, the difference between hydrogel before cellulase digestion and hydrogel after cellulase digestion is statistically significant at p < = 0.01.
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<th>Mean size of macropores without cellulase digestion (µm)</th>
<th>Mean size of micropores without cellulase digestion (µm)</th>
<th>Mean size of macropores with cellulase digestion (µm)</th>
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<td>3.9</td>
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<td>19 ± 3.4</td>
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</tr>
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<td>135 ± 2.6*</td>
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<tr>
<td>15.5</td>
<td>32 ± 1.3</td>
<td>9 ± 8.2</td>
<td>74 ± 8.3*</td>
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<table>
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<tr>
<th>HRP concentration (unit L⁻¹)</th>
<th>Mean size of macropores without cellulase digestion (µm)</th>
<th>Mean size of micropores without cellulase digestion (µm)</th>
<th>Mean size of macropores with cellulase digestion at days 4, 5, and 6 (µm)</th>
<th>Mean size of micropores with cellulase digestion at days 4, 5, and 6 (µm)</th>
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<tbody>
<tr>
<td>3.9</td>
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<td>36 ± 0.4</td>
<td>236 ± 3.6*</td>
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<td>15.5</td>
<td>48 ± 1.0</td>
<td>19 ± 0.2</td>
<td>108 ± 6.3*</td>
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</tr>
</tbody>
</table>

* At the same HRP concentration and for hydrogels harvested at the same time, the difference between the mean pore size before cellulase digestion and the mean pore size after cellulase digestion is statistically significant at p < 0.01.

**Table 2.** Mean macropore and micropore sizes in **in vivo** Gtn-HPA/CMC-Tyr hydrogels with and without cellulase digestion. Results are shown as mean ± standard deviation.

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The table of contents entry
This study demonstrates a hydrogel system that allows the pore structure to be altered in vivo after implantation. This new approach gives users the ability to tailor the scaffold architecture to match the specific growth rate of a tissue or an individual’s recovery rate. This system can be ideal for personalized tissue engineering

Keyword
Injectable hydrogel; tissue engineering; porous scaffolds; in vivo implantation; tunable porosity

Aswan Al-Abboodi, Jing Fu, Pauline M. Doran, Timothy T.Y. Tan*, Peggy P.Y. Chan*
Corresponding Author*

Title
Injectable 3D hydrogel scaffold with tailorable porosity post-implantation

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Supporting Information

for Adv. Healthcare Mater., DOI: 10.1002/adhm.((please add manuscript number))

Title ((Injectable 3D Hydrogel Scaffold with Tailorable Porosity Post-implantation))

Author(s), and Corresponding Author(s)* ((Aswan Al-Abboodi, Jing Fu, Pauline Doran, Timothy T.Y. Tan*, Peggy P.Y. Chan*))

((Please insert your Supporting Information text/figures here. Please note: Supporting Display items, should be referred to as Figure S1, Equation S2, etc., in the main text…)

Figure S1. Representative environmental SEM (ESEM) images showing the morphology of 5% (w/v) Gtn-HPA hydrogel in (a) hydrated state (scale bar 500 µm), and (b) lyophilized state (scale bar 200 µm).
Figure S2. Typical evolution of the storage modulus (G’) and loss modulus (G’’) of hydrogels prepared using (a) 3.9, (b) 7.7 and (c) 15.5 unit L\(^{-1}\) HRP.
Cell Viability

To investigate the potential cytotoxic effect of Gn-HPA/CMC-Tyr hydrogel, COS-7 cells were immobilized in Gtn-HPA/CMC-Tyr hydrogels in situ during crosslinking. The viability of the cells was examined according to the procedure described in [22] using a LIVE/DEAD staining kit (Life Technologies Pty Ltd, Australia). Stained cells inside the hydrogels were imaged using LSCM. Cells in nine randomly selected fields of view were counted. After 3 days of cultivation, the viability of COS-7 cells were found to be 93±6.5, 92±5.7, and 91±4.1 % for hydrogels prepared using HRP concentrations of 3.9, 7.7 and 15.5 unit L⁻¹, respectively.

In a separate experiment, hydrogels were prepared according to the above procedure followed by cellulase digestion for 1 day. The cells were cultivated inside the hydrogels for a further 24 h. Cell viability was assessed using a LIVE/DEAD staining kit (Life Technologies Pty Ltd, Australia) and the stained cells inside the hydrogels were imaged using LSCM. Figure S3 shows representative images of the morphology of COS-7 cells immobilized in situ in Gtn-HPA/CMC-Tyr hydrogel. Most of the cells remained viable (stained with Calcein-AM) inside the hydrogels. It was observed that both the cell number and the cell size were smaller in hydrogels prepared using higher HRP concentrations, which is likely due to the smaller pore size of the hydrogel.
Figure S3. Fluorescence images of COS-7 cells cultivated in Gtn-HPA/CMC-Tyr hydrogel for 24 hr after cellulase digestion. The live cells were stained by Calcein AM (scale bar 20 µm).