GENIPIN-CROSSLINKED CHITOSAN HYDROGELS AS SCAFFOLDS FOR MAMMALIAN CELL GROWTH

1KWONGYUEW CHUNG, 2MARK A. BIRCH, 3KATARINA NOVAKOVIC

1School of Engineering, Temasek Polytechnic, 21 Tampines Avenue 1, Singapore 529757, Singapore
2Division of Trauma and Orthopaedic Surgery, Department of Surgery, University of Cambridge, Cambridge, CB2 2QZ, UK
3School of Engineering, Newcastle University, Newcastle, NE1 7RU, UK
E-mail: 1chungky@tp.edu.sg, mab218@cam.ac.uk, 3katarina.novakovic@ncl.ac.uk

Abstract - While hydrogels can take many forms, covalently crosslinked structures are favoured when enhanced stability and increased mechanical properties are required. One element of particular relevance to covalently crosslinked hydrogel structures is the crosslinking agent involved in the scaffold formation, where at present, non-toxic options are limited. Recent advances in research suggest that genipin is a beneficial crosslinking agent to consider. When such hydrogels are intended as scaffolds for mammalian cells in tissue engineering applications, further consideration must be given to biocompatibility, porosity and pore interconnection, so that appropriate cell activity within the bulk of the scaffold is achieved. Therefore, in this work, the viability of genipin-crosslinked chitosan-polyvinylpyrrolidone hydrogels for culturing non-differentiated adult mesenchymal stem cells is studied. Cell viability was observed through fluorescent microscopy and cell number assessed using the MTT assay. Results confirmed that these hydrogels are benign to studied cells. Furthermore, it was shown that cell proliferation propagates beneath the gel surface via interconnected pores within the hydrogels.

Keywords - Biomaterials, cell growth, chitosan, genipin, hydrogels, polyvinylpyrrolidone

I. INTRODUCTION

Many novel materials have been investigated with the aim of enhancing the development of artificial environments for controlled cell proliferation and differentiation; in particular, mechanoresponsive and mechatronicsensitive cells, such as adipocytes, chondrocytes, fibroblasts and osteoblasts have been studied for use in tissue engineering [1-3]. Soft materials identified as promising for this purpose are smart polymer hydrogels, since in addition to their biocompatibility, large water content and porous structure, they are also able to alter their conformation in response to relatively small external changes in the environmental conditions [20], making them promising for applications in many fields, especially in the biomedical field as tissue engineering scaffolds and implants, biosensors, biomimetic materials and drug delivery systems [20-23].

This paper focuses on an ionically crosslinked chitosan hydrogel, which exhibits pH-sensitive swelling [14,17,18,24]. The sensitivity to a wide range of pH fluctuations, together with other positive medical and pharmaceutical attributes derived from the properties of chitosan, suggest strong potential use of such hydrogels in cell culture and tissue engineering applications. Chitosan is a polycationic biopolymer feasible yet significantly less toxic crosslinking agent compared to the commonly used glutaraldehyde, which is used in some applications at present [13,14]. Hydrogels are three-dimensional macromolecular networks of randomly crosslinked hydrophilic polymer chains that are highly swollen in water [15-18]. The multiphase structure of an ionically crosslinked hydrogel usually embodies a solid crosslinked polymer network matrix or mesh, with the interstitial space filled up with water or fluid, and a third ionic phase consisting of ionizable groups bound onto the polymer chains, which give rise to the ionic and electrostatic interactions that affect the degree of swelling or de-swelling of the hydrogel [19]. These characteristics led to the development of polymer networks that are highly responsive to physical and/or chemical environmental stimuli called smart polymers, and hydrogels synthesized from these polymers known as smart hydrogels. These hydrogels are capable of undergoing large reversible physical and/or chemical changes in response to relatively small external changes in the environmental conditions [20].

This paper offers initial evaluation of smart genipin-crosslinked chitosan-polyvinylpyrrolidone hydrogels as an artificial environment for cultivating non-differentiated adult mesenchymal stem cells (MSCs). Recent advances have shown that genipin is a...
Chitosan, being polycationic, can react with negatively charged ions or molecules, leading to the formation of ionic bridges between polymeric chains during the synthesis of polymer hydrogel. The density of these ionic bridges or crosslinks and their ionic interactions influence the porosity and swellability of the hydrogel. An increase in crosslinking density will generally induce a decrease in swelling and pH-sensitivity of the hydrogel by tightening crosslinking and reducing pore size. At the same time, it will improve the stability of the hydrogel network [30]. The swelling and deswelling of the gel is, respectively, enabled by the protonation and deprotonation of free ammonium groups in chitosan [24].

To strengthen the chitosan hydrogel, a second water-soluble polymer can be incorporated to create a hybrid polymer network. Of interest to work reported here is polyvinylpyrrolidone (PVP), a hydrophilic polymer made from the monomer N-vinylpyrrolidone, which enhances water uptake, and creates a semi-interpenetrating polymer network with chitosan offering greater mechanical strength, and providing more sensitive and predictable swellability in water and ionic solutions [14,24]. PVP has significant uses in the pharmaceutical industry, for example, as a binder in many pharmaceutical tablets to enhance mechanical strength, and added to iodine to form povidone-iodine complex that possesses disinfactant properties [31]. Recent studies have also seen PVP applied in the field of cell biology with use as a cryoprotective agent in the freezing of stem cells, in stimulating clone-forming efficiency of stromal precursor cells, and increasing the recovery rate of oocyte-granulosa cell complexes during in vitro growth of primordial germ cells [32-34]. Its known haemocompatibility property [35] and its safety use in consumption drugs, make PVP a promising partner with chitosan as a hydrogel constituent for biomedical applications.

Additionally, genipin is chosen as the crosslinker to provide the ionic interactions for the chitosan-PVP hybrid polymer network [17], that will yield a hydrogel with promising swelling characteristics [36,37]. The characterization of the polymerization and pH-dependent cross-linking reactions between chitosan and genipin has been studied extensively [38-41], as the blue pigment of fluorophores produced when genipin crosslinks with chitosan makes fluorogenic observation of gel crosslinking convenient [14,42,43], and which give the hydrogel its characteristic dark-blue colour. Interest in genipin is increasing in recent years due to its pharmacological and biochemical significance for use in the therapies of a number of diseases [44], and in the manufacture of food commodities, which have shown its potential as a safe crosslinking agent [7,45]. Genipin can be extracted from geniposide present in the fruit of Gardenia jasminoides, an evergreen flowering plant [45]. It is an excellent natural crosslinker with recognised low acute cytotoxicity [46]. While the pH-responsive behaviour of the genipin-crosslinked chitosan-PVP hydrogel has been documented [14,17,18,37], investigation into the viability of this hydrogel in supporting mammalian cell growth has not yet been reported, and the study reported here aims to address this.

Adult mesenchymal stem cells (MSCs) are multipotent connective and structurally supportive tissue cells (stromal cells) that are able to differentiate into various types of specialized cell types, such as osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells), and adipocytes (fat cells), among others [47,48]. The study of these MSCs and their mechanisms for tissue formation has been widely explored in the context of many new therapeutic technologies and applications in tissue engineering [9,49]. This paper describes a study conducted on culturing non-differentiated adult mesenchymal stem cells on genipin-crosslinked chitosan-polyvinylpyrrolidone hydrogels, with the aim to obtain preliminary assessment of the viability of eukaryotic mammalian cells on these hydrogels, that is, whether the cells are capable of living or developing normally within the environment of the hydrogels. Also important is the assessment of whether the hydrogels can support cell proliferation, allowing cells to actively multiply in numbers instead of merely achieving quiescence [50]. Quiescent cells that have withdrawn from the cell-division cycle due, for example, to a lack of exposure to growth factors, do not proliferate, although they are viable.

In this study, cell viability was observed directly by microscopy using a fluorescent dye to identify cells. When bound to DNA within the cells, the staining agent produces a blue fluorescence (460 nm) that can be visualized under a microscope [51]. Cell number was assessed through the use of the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cytotoxicity assay [52]. Yellow MTT, a synthetic organic compound, is reduced to purple formazan in the mitochondria of living cells [53]. Mitochondrial dehydrogenases (reductase enzymes) of viable cells are capable of cleaving the tetrazolium ring of MTT, yielding purple MTT formazan crystals which are insoluble in aqueous solutions, but which can be dissolved in dimethyl sulfoxide (DMSO),
which is a polar solvent capable of dissolving both polar and non-polar compounds and is miscible in a wide range of organic solvents as well as water, forming a homogeneous purple solution. Since reduction of MTT can only take place when the mitochondrial reductase enzymes are active (in living cells), therefore, the amount of converted formazan quantified by the absorbance values in spectrophotometry directly relates to the number of viable cells in the assay. Corollary to this is that an increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance detected spectrophotometrically. The above techniques offer insights into the suitability of these hydrogel materials in providing a benign environment that promotes healthy cell growth.

II. MATERIALS AND METHODS

2.1. Hydrogel synthesis
Chitosan powder (from Sigma-Aldrich) of medium molecular weight (190 to 310 kDa) at 75% to 85% of deacetylation was dissolved at room temperature (25°C) in aqueous 1% (v/v) acetic acid solution to produce a 1.5% (w/v) (g/ml) pale yellow, viscous solution after 24 hours of continuous mechanical stirring. PVP powder of average molecular weight (40 kDa) was dissolved in distilled water after 1 hour of continuous mechanical stirring at 85°C to yield a 5% (w/v) (g/ml) colourless, homogenous solution. A clear, transparent solution of genipin (0.5% (w/v) (g/ml)) was produced by dissolving genipin in distilled water at room temperature through 15 minutes of ultrasonde palpitations in a sonication bath. 1 mL of the above chitosan solution was mixed with 0.2 mL of PVP solution in a low-density polyethylene (LDPE) specimen vial (Ø14 mm ID), and 100 µL of genipin solution was added to the mixture, which was then thoroughly mixed by continuous mechanical stirring over a period of 30 minutes at room temperature. Samples were incubated at 37°C over 24 hours to undergo polymerization, forming a dark blue gel upon successful crosslinking of the gel constituents over the gelation period. The gel samples were subsequently frozen at ~20°C for 1 hour and were slowly thawed and stored at 4°C before use. Each hydrogel was removed from the specimen vial by inserting a cork borer through the open end of the vial after cutting off its base. The resulting fixed diameter (14 mm) cylindrical gel was cut into a disc of 7 mm thickness. Using a smaller diameter cork borer, 10 mm diameter gels were also prepared.

2.2. Cell culture on the hydrogels
All the gels were immersed in 70% ethanol for 90 minutes to kill off any micro-organisms, such as bacteria, and then transferred into multi-well tissue culture plates. The hydrogels in the plates were washed with phosphate-buffered saline (PBS) buffer solution to remove any residual ethanol. Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS; Gibco), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (all InVitrogen) was then added to the gels in the wells to provide extracellular matrix biomolecules to help support cell adhesion and nutrients for cell growth. The gels were incubated at 37°C for 24 hours. Nine 14 mm-gels were divided out in sets of three and placed within three separate 24-well microplates (Costar® 3524) with a well diameter size of 15.6 mm, where each plate would be checked for cell growth under fluorescence microscopy over three distinct time periods; after 24 hours, 7 days and 14 days, respectively. Three circular cover glasses (Ø15 mm) were also placed in the wells of each microplate alongside the gels, and served as controls for comparison of results. Similarly, fifteen 10 mm-gels were divided out in sets of five and placed within three separate 48-well microplates (Costar® 3548) with a well diameter size of 11 mm, where each plate would be tested with MTT cell proliferation assay over the same three checkpoint periods. Human MSCs (Lonza) were routinely cultured in DMEM as described above and plated onto the gels to give 20,000 cells per well in the 24-well plates and 10,000 cells per well in the 48-well plates. All the plates were subsequently incubated at 37°C in 5% CO2 and taken out for observation and testing at the three time-check points.

2.3. Observation with fluorescence microscopy
At each of the 24th hour, 7th day and 14th day time points after seeding the cultured cells onto the hydrogels, the gels or glass cover slips were fixed in 4% (w/v) paraformaldehyde in Dulbecco’s Phosphate Buffered Saline (DPBS) solution for 30 minutes. The samples were then washed with 0.1% Tween-20 in DPBS and 50 µg/ml phalloidin-TRITC (tetramethylrhodamine B isothiocyanate) (Sigma-Aldrich) in DPBS was added to the samples for 1 hour in a dark chamber. The glass discs (with cells adhering on them) were carefully taken out from the control wells and a drop (approximately 25 µL) of VECTASHIELD® (Vector Labs) mounting medium with DAPI (4',6-diamidino-2-phenylindole) fluorescent staining agent was added to each disc. The disc was then flipped over onto a microscope glass slide with cells sandwiched between the glass disc and the microscope glass slide, and viewed under a microscope. The hydrogels (with cells adhering on them) were carefully taken out of the wells from the second column of the microplate and placed onto microscope glass slides with the gel surfaces containing cells facing up. A drop of VECTASHIELD® mounting medium with DAPI was added to each gel, and a cover slip was placed on top of each gel, keeping the cells covered, and viewed under a microscope. Cross-sectional cuts were also
made on the hydrogels and the sectioned surfaces viewed under the fluorescence microscope to observe the depth of migration made by the cells into the hydrogels.

2.4. MTT cell proliferation assay

2.8 mg of MTT was dissolved with 5.6 mL of DMEM. The solution was filtered to remove any bacteria present with a filter pore size of 0.22 \( \mu \)m, and warmed to 37°C. A 48-well microtitration plate that contained cultured cells on hydrogels corresponding to the relevant time-check point was taken out of the incubator and the DMEM in the cell culture wells was aspirated off, which also removed the cells that were not adhered to the well walls and the hydrogels. 0.5 mL of the MTT solution was introduced into each of the wells; 5 control wells in the first column and 5 with hydrogels in adjacent column. The plate was then covered up and incubated at 37°C for 3 hours. At the end of the incubation period, the microtitration plate was taken out and the MTT solution removed from the wells. The wells were then washed and filled to about half with DPBS. The hydrogels in the second column wells were carefully moved over to adjacent wells on the third column that was also half-filled with DPBS. 0.5 mL of DMSO was then added to each of the relevant wells in the first three columns. The solution turned purple in the wells, indicating that the MTT formazan crystals had dissolved in the DMSO. The microplate was covered up and placed into a laboratory shaker at 37°C for 24 hours to solubilize formazan out of the hydrogels. Subsequently, the sample solutions from all the wells were pipetted out and transferred into a fresh microplate, and the samples were scanned with the spectrophotometer of a Tecan® microplate reader at 570 nm absorbance wavelength to quantify the amount of viable cells. The above procedures were repeated for samples in each of the three time-check periods: after one day, after seven days and after fourteen days of cell incubation.

III. RESULTS AND DISCUSSION

3.1. Cell viability on hydrogel

Assessment of cell activity on the hydrogels was performed by fluorescence microscopy over a period of two weeks. Viable cells appear blue under the lighting of the microscope from fluorescent staining of their nuclei with DAPI (Fig. 1). The phalloidin conjugated TRITC highlights the actin cytoskeleton of the cells as red/pink and are indicative of the extent of the interactions that the individual cells are having with their external environment. Cells adhering to the glass discs and grown in control wells were seen to be surviving over the two-week period of observation (Fig. 1).

Cells were found surviving on the hydrogels after one week of seeding (Fig. 2). They formed small neighbourhood clusters of cells and anchored themselves within the pores of the gels, as well as on the interconnecting polymer network support between pores, demonstrating that this hydrogel can enable cell adhesion and cell organization on its soft material surface.

Not only had the cells survived into the second week, their numbers had also increased as observed through fluorescence microscopy (Fig. 3), a positive sign that the hydrogel was encouraging cell viability and proliferation. Greater formation of cytoskeletal structures was seen in the second week of growth, as evidenced by the vivid pink hues around the blue-stained cells (Fig. 3). This is an indication of interconnection and linkages set up between cells in their clusters that encourage cell growth.

Cross-sectional views of cut hydrogels show evidence of cell migration into the gels via the interconnected pores one week after cell seeding, to a depth of about 150 \( \mu \)m (Fig. 4a). After two weeks, deeper migration of cells beyond 500 \( \mu \)m into the hydrogels is captured (Fig. 4b). In both cases, the majority of cells remained near to the surface. This might suggest that the conditions within the gel do not support cell growth, due to a lack of nutrients and growth factors at these depths compared to the top surface of the gel. This
pattern of cell growth is typical for a scaffold that is neither dynamically seeded or cultured in vitro[54,55].

The above observations illustrate that the genipin-crosslinked chitosan-polyvinylpyrrolidone hydrogel supports cell adhesion, cell organization, cell growth and cell migration. Cell anchorage on a substrate is facilitated through the cell’s contractile machinery, whereby the actin stress fibres are contracted by the myosin molecular motors, which enable probing and connection to cell-extracellular matrix (ECM) receptors anchoring to the substrate [56]. Upon sensing the mechanical properties of its environment, a cell positions and orients itself and strengthens its contact and cytoskeleton, and this may include neighbouring cells, organizing into cell clusters. The clusters of adherent cells pull on the soft material environment through cell-matrix interactive traction forces to drive directional migratory movements [57,58], both across and into the hydrogel surface. The formation of ECM during cell activity aids in cell migration, as cells are able to generate and regulate the forces applied to the ECM-bound integrins to enable the cells to pull themselves forward [59,60]. Integrins, which are transmembrane receptors that form the bridges for cell-cell and cell-ECM interactions, anchor the actin cytoskeleton of the cells to their ECM at the cell-matrix junctions [61]. The resulting linkage of the cytoskeleton to the ECM provides the stability of the cell-matrix junctions. The matrices of interconnected pores in the hydrogel also facilitate access of the cells within the gel.

It is interesting to note that although cells tend to be more able to extend their biological processes on a stiff medium (such as the glass substrate in Fig. 1) compared to the softer gels [5,62,63], the viability of cell growth and cell migration exhibited on the genipin-crosslinked chitosan-polyvinylpyrrolidone hydrogel (Fig. 2 to 4) suggests that the structural, mechanical, chemical and polymeric properties of the hydrogel are sufficiently conducive to support human mesenchymal cell viability [6]. The use of naturally derived polymers such as chitosan and genipin to synthesize the hydrogel is attractive, because such polymers form biomaterials that are biochemically similar to tissues, and can be considered as good substitutes for hydrogels that are composed primarily of synthetic polymers, such as poly(ethylene glycol) (PEG) or polyacrylamide (PA) hydrogels [5,6,64].

3.2. Cell proliferation on hydrogel

The purple colour formed upon DMSO addition to the microplate wells (Section 2.4) indicates the presence of viable cells (Fig. 5). The dark purple solution in the control wells (Fig. 5, first well column) suggests the presence of a large number of viable cells that had originally adhered to the bottom of the plate. Lighter purple colour that can be seen in the second column, where hydrogels were kept prior to being moved to the third column, indicates existence of cells that were not adhered to the hydrogels but to the well walls, suggesting that cells can still survive in the presence of the hydrogels. The third column (Fig. 5) contains hydrogels and cells mechanically disrupted from the hydrogels on which they previously adhered to. It can be noted that this solution has the lightest purple colour among the three columns, suggesting the smallest number of viable cells.
Genipin-crosslinked Chitosan Hydrogels as Scaffolds for Mammalian Cell Growth

hydrogels while multiplying and growing (blue curve) and those unattached to the hydrogels but which were growing in the presence of the gels (red curve), were continuously growing and increasing in numbers (proliferating). Their combined growth curve is shown as the orange dashed line.

Fig. 6. (a) Cell growth curves over a period of 14 days for (i) the control group of cells from column one of the microplate in Fig. 5, (ii) cells that did not attach themselves to the hydrogels but were still viable in the gels’ presence (from column two of microplate), (iii) cells that had been adhering to the hydrogels while growing (from column three of microplate), and (iv) combined cells proliferating in the presence of hydrogels (sum of graphs ii and iii). (b) Data for control group of cells and cells growing on hydrogels are presented as mean ± standard deviation. The above results demonstrate that the genipin-crosslinked chitosan-polyvinylpyrrolidone hydrogels, although preliminary, have demonstrated the non-toxicity and suitability of this hydrogel material in supporting viable and multiplicative cell growth. Results revealed that genipin-crosslinked chitosan-PVP hydrogels are promising bioactive materials that have potential to mimic biological processes and promote cellular functions such as cell adhesion, growth, proliferation and migration, offering capacity for tissue engineering applications. The reported findings lay the foundation for subsequent work where the responsive nature of these smart materials is to be employed to both stimulate and control the rate of cell growth. While increased gel porosity and pore size has been shown to promote greater cell growth, it also resulted in diminished mechanical strength of the gel and accelerated degradation of the scaffold material [66]. The key is to obtain a functional limit for porosity and pore size, which can be achieved through appropriate selection of the genipin concentration to alter the degree of cross-linking [67,68]. Furthermore, in order to sustain and enhance cell growth on the hydrogel, suitable growth factors should be considered for encapsulation in the hydrogel with cell-binding motifs incorporated on the surface of the gel to improve cell adhesion. The environment around the hydrogel should also be improved and better controlled to provide suitable physiological and nutritional requirements that facilitate greater cell activity, including better aeration, toxic waste removal and nutrients supply through the use of appropriate bioreactors.

CONCLUSIONS

The experimental outcomes of this study from the seeding of cultured mesenchymal stem cells, that have the potential to differentiate into many mechanosensitive and mechanoresponsive cell types, onto the genipin-crosslinked chitosan-polyvinylpyrrolidone hydrogels, although preliminary, have demonstrated the non-toxicity and suitability of this hydrogel material in supporting viable and multiplicative cell growth. Results revealed that genipin-crosslinked chitosan-PVP hydrogels are promising bioactive materials that have potential to mimic biological processes and promote cellular functions such as cell adhesion, growth, proliferation and migration, offering capacity for tissue engineering applications. The reported findings lay the foundation for subsequent work where the responsive nature of these smart materials is to be employed to both stimulate and control the rate of cell growth. While increased gel porosity and pore size has been shown to promote greater cell growth, it also resulted in diminished mechanical strength of the gel and accelerated degradation of the scaffold material [66]. The key is to obtain a functional limit for porosity and pore size, which can be achieved through appropriate selection of the genipin concentration to alter the degree of cross-linking [67,68]. Furthermore, in order to sustain and enhance cell growth on the hydrogel, suitable growth factors should be considered for encapsulation in the hydrogel with cell-binding motifs incorporated on the surface of the gel to improve cell adhesion. The environment around the hydrogel should also be improved and better controlled to provide suitable physiological and nutritional requirements that facilitate greater cell activity, including better aeration, toxic waste removal and nutrients supply through the use of appropriate bioreactors.

ACKNOWLEDGEMENTS

The authors wish to acknowledge support by the UK Engineering and Physical Sciences Research Council (EPSRC) Grant Number EP/H003908/1 and the
School of Engineering, Newcastle University and Temasek Polytechnic, for funding this research.

REFERENCES


[40] Butler, M.F.; Ng, Y.-F.; Pudney, P.D.A. Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. Journal of Polymer Science 2003, 41, pp. 3941-3953.


