University of Wollongong
Research Online

Faculty of Science - Papers (Archive)   Faculty of Science, Medicine and Health

2012

Gelapin, a degradable genipin cross-linked gelatin hydrogel

Damian Kirchmajer
University of Wollongong, dmk444@uow.edu.au

C Watson
University Of Wollongong

Marie Ranson
University of Wollongong, mranson@uow.edu.au

Marc in het Panhuis
University of Wollongong, panhuis@uow.edu.au

Follow this and additional works at: https://ro.uow.edu.au/scipapers

Part of the Life Sciences Commons, Physical Sciences and Mathematics Commons, and the Social and Behavioral Sciences Commons

Recommended Citation
Kirchmajer, Damian; Watson, C; Ranson, Marie; and in het Panhuis, Marc: Gelapin, a degradable genipin cross-linked gelatin hydrogel 2012, 1073-1081.
https://ro.uow.edu.au/scipapers/4701

Research Online is the open access institutional repository for the University of Wollongong. For further information contact the UOW Library: research-pubs@uow.edu.au

Gelapin, a degradable genipin cross-linked gelatin hydrogel

Abstract

The synthesis of genipin cross-linked gelatin (Gelapin) hydrogel materials is presented. Gelapin hydrogels were comprehensively characterised through chemical, mechanical and physical analysis techniques. It was found that the hydrogels could be cross-linked to up to 90% using a genipin concentration of 24.4% (w/ w). The hydrogels reach a stable swollen state and cease leaching of residual starting materials after 72 h in phosphate buffered saline solution at 37 uC. The proteolytic degradation of Gelapin by collagenase is tuneable through manipulation of the material composition with the rate of degradation ranging from 60 mg per day up to 500 mg per day. The mechanical characteristics (at 37 uC) are controllable through adjustment of the gelatin and genipin concentrations resulting in compressive stress-at-failure values ranging from 26 kPa to 300 kPa. Gelapin gels were found to become more elastic and ductile during proteolytic degradation up to 70% mass loss. The ability for vascularisation of these hydrogels was demonstrated using a chick embryo chorioallantoic membrane assay method.

Keywords

gelatin, hydrogel, gelapin, genipin, degradable, cross, linked, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Kirchmajer, D., Watson, C., Ranson, M. & in het Panhuis, M. (2012). Gelapin, a degradable genipin cross-linked gelatin hydrogel. RSC Advances, 3 (4), 1073-1081.

This journal article is available at Research Online: https://ro.uow.edu.au/scipapers/4701

RSC Advances

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Published on 13 November 2012. Downloaded by University of Wollongong on 14/07/2014 07:33:06.

Gelapin, a degradable genipin cross-linked gelatin hydrogel†

Cite this: *RSC Advances*, 2013, **3**, 1073

Damian M. Kirchmajer,[a] Clare A. Watson,[b] Marie Ranson[b] and Marc in het Panhuis*[a]

Received 12th November 2012,
Accepted 13th November 2012

DOI: 10.1039/c2ra22859a

www.rsc.org/advances

The synthesis of genipin cross-linked gelatin (Gelapin) hydrogel materials is presented. Gelapin hydrogels were comprehensively characterised through chemical, mechanical and physical analysis techniques. It was found that the hydrogels could be cross-linked to up to 90% using a genipin concentration of 24.4% (w/w). The hydrogels reach a stable swollen state and cease leaching of residual starting materials after 72 h in phosphate buffered saline solution at 37 °C. The proteolytic degradation of Gelapin by collagenase is tuneable through manipulation of the material composition with the rate of degradation ranging from 60 mg per day up to 500 mg per day. The mechanical characteristics (at 37 °C) are controllable through adjustment of the gelatin and genipin concentrations resulting in compressive stress-at-failure values ranging from 26 kPa to 300 kPa. Gelapin gels were found to become more elastic and ductile during proteolytic degradation up to 70% mass loss. The ability for vascularisation of these hydrogels was demonstrated using a chick embryo chorioallantoic membrane assay method.

1. Introduction

The engineering of tissue and organs in a laboratory is a comparatively cost effective and convenient alternative to human tissue and organ donations. The tissue scaffold, *i.e.* the material that reinforces the component's structure, and holds the cells in place during maturation, is a critical component of all *in vitro* tissue engineering strategies.[1] For engineering of soft tissues, hydrogel materials are the most appropriate scaffold materials because they have similar mechanical properties and chemical composition to natural tissues.[2,3]

Hydrogels are a class of hydrated polymer materials whose polymer fraction generally lies between 0.1%–10% (w/v), with the remainder comprising of water or an aqueous solution.[4] Typical examples of commercial hydrogel products include toothpaste, foods, contact lenses, cosmetics, drug capsules and medical creams and ointments. Over the past 30 years an extensive array of both naturally derived and synthetic hydrogels have been utilised in research and development for all manner of tissue engineering objectives.[2]

The study and use of gelatin hydrogels dates back centuries with the first documented use of gelatin materials for biomedical application as a hemostatic substance.[5] Since then it has also been utilised for surgical glues, sealants and wound dressings.[5–8] Gelatin is denatured, hydrolysed collagen which itself is the primary constituent of all the connective tissues of the human body (within skin, muscle, bones, cartilage, fat).[9,10] It is produced commercially *via* acid and alkaline hydrolysis and by proteolysis of collagen which can be derived from all manner of organisms including, but not limited to: pigs, cows, fish and rats.[9,10] Depending on the type and extent of hydrolysis/proteolysis and the originating species, different gelatins can be produced with a variety of isoelectric points, molecular weights and Bloom numbers (indicative of gel strength).[11] Gelatin hydrogels have the advantage of being biocompatible, non-toxic, non-immunogenic and biodegradable as well as being cheap and readily available,[12] making them ideal candidate materials for biomedical applications.[13] However, unmodified gelatin hydrogels are mechanically weak and dissolve at temperatures above 29 °C which are limitations that need to be addressed in order to develop them for practical applications.[9]

It has been demonstrated that the mechanical and thermal properties of gelatin hydrogels can be improved by cross-linking.[14,15] Examples of reagents and methods used for cross-linking gelatin include chemical cross-linking with gluteraldehyde, formaldehyde, diisocyanates, carbodiimides and acyl azides,[16] as well as physical cross-linking using dehydrothermal treatment, ultraviolet irradiation and gamma irradiation.[13] However, the physical treatments tend to achieve a very limited extent of cross-linking, and the chemical treatments have the potential to leave toxic residues in the gels which will release upon biodegradation *in vivo*.[13] Novel cross-linking

[a]*Soft Materials Group, School of Chemistry, Intelligent Polymer Research Institute, ARC Centre of Excellence for Electromaterials Science, AIIM Facility, University of Wollongong, Wollongong, NSW 2522, Australia. E-mail: panhuis@uow.edu.au; Tel: +61 24221 3155*
[b]*School of Biological Sciences, Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW 2522, Australia*

† Electronic supplementary information (ESI) available: schematic of genipin cross-linking gelatin, tabulated details of: swelling ratio of Gelapin hydrogels after soaking in pH 7.4 and 8.4; and release of gelatin from Gelapin. See DOI: 10.1039/c2ra22859a

This journal is © The Royal Society of Chemistry 2013

Published on 13 November 2012. Downloaded by University of Wollongong on 14/07/2014 07:33:06.

reagents that are both effective and non-toxic are required to improve the mechanical and thermal properties of gelatin whilst retaining its inherent advantageous properties.

Genipin is a potent yet non-toxic cross-linker of proteins such as chitosan, collagen and gelatin that has been recently demonstrated to bestow anti-inflammatory properties to the materials it is incorporated into.[13,17–19] It is produced both synthetically and naturally as the algycone of geniposide, an iridoid glycoside that is the major component of the fruit of the gardenia plant, *Gardenia jasmindides Ellis*.[20] Genipin was first proposed and demonstrated for use as a biological tissue fixative and gluteraldehyde alternative in 1999 where it was demonstrated to be 10 000 times less toxic and almost equally efficacious.[20] It has been established that genipin cross-linked gelatin *via* the epsilon amino group present in lysine and hydroxylysine residues (Fig. S1, ESI).[21] Genipin has been used in the preparation of cross-linked gelatin films and hydrogels,[13,22–24] and employed in nerve guiding conduits, wound dressings, and cartilage scaffolds.[6,25,26]

There is considerable interest in bioreactors for tissue engineering that can culture three-dimensional constructs in a dynamic fashion, and can also perfuse scaffold materials and stimulate their contents mechanically.[27–33] In particular, mechanical stimulation has been shown to be crucial in directing the differentiation and phenotype of many cell types towards phenotypes which can produce a functional extracellular matrix which will ultimately replace the scaffold and form the bulk of the new tissue component.[31,32,34–38] In consideration of this aspect of tissue engineering, it is obviously of benefit to have a thorough understanding of the mechanical and rheological properties of candidate materials.

In this paper, the preparation and characterisation of genipin cross-linked gelatin (Gelapin) is presented. The ability of genipin to act as a gelatin cross-linker was investigated, including the stability and retention of both gelatin and genipin in phosphate buffer saline solution at 37 °C. The degradation behaviour and mechanical characteristics of these hydrogels at 37 °C are discussed in detail and their ability for vascularisation is demonstrated.

2. Materials and methods

2.1 Preparation of hydrogels

All reagents used were AR grade unless otherwise stated and deionised (DI) water (resistivity 18.2 MΩ cm) was prepared using a combined ion exchange and osmosis filtration system (Millipore, Australia). Gelapin hydrogels were prepared with type A, porcine gelatin (Bloom number of 300, molecular weight of 87 500 Da, Sigma-Aldrich, USA) with concentrations of gelatin ranging 2%–10% (w/v) and genipin (Challenge Bioproducts, Taiwan) concentrations ranging 0%–19.5% (w/w). Throughout this report Gelapin hydrogels will be referred to using nomenclature that describes their composition in the format Gelapin-[gelatin %(w/v)]/[genipin %(w/w)]. A summary of the preparation regime is as follows: Gelatin was initially dissolved with gentle stirring in DI water at 50 °C on a magnetic stirrer/hot plate (Stuart CB162, UK). Subsequently, the appropriate amount of 20.3% (w/v) genipin in 60% (v/v) ethanol (Ajax Finechem, Australia) solution was added and stirred for a further 3 min at 50 °C. The hot solution was then poured into glass Petri dishes (60 mm diameter × 15 mm height, Schott, Australia) and allowed to cure for 24 h at 21 °C. Silanisation of the Petri dishes with Coatasil (Thermofisher Scientific, Australia) prior to use facilitated the easy removal of the hydrogels without damaging them.

2.2 Extent of cross-linking

The extent of cross-linking in Gelapin hydrogels was determined by spectrophotometric comparison of the number of epsilon amino groups present in cross-linked and uncross-linked gelatin. The method used was based on the colorimetric assay developed by Offner and Bubnis:[39,40] Gelapin samples were first lyophilised (LD Plus, Alpha, USA) and divided into 10–20 mg subsamples for individual analysis. Each subsample was combined with 4 mL of 4% (w/v) $NaHCO_3$ (Merck, Australia), 1 mL of 0.5% (w/v) 2,4,6-trinitrobenzenesulfonic acid (Sigma-Aldrich, USA) and heated in an oven (Binder-FD, USA) for four hours at 40 °C. The subsamples were then acidified with 3 mL of 6 M HCl (Ajax Finechem, Australia) and hydrolysed in an autoclave (Tomy ES-315, 121 °C, 186 kPa, 20 min). Three extractions using 20 mL diethyl ether (BDH, Belgium) were performed after addition of 5 mL of DI water. A 5 mL aliquot of the aqueous extract was taken, heated on a steam bath for 15 min and allowed to cool to 20 °C for 18 h. The samples were then made up to 20 mL and measured using a dual beam spectrophotometer (Cary 500 Scan, Varian, Australia) in 1 cm path length glass cuvettes at 346 nm.

The extent of cross-linking (E_C) was determined by comparing the cross-linked blank corrected sample absorbance (A) with the non-cross-linked blank corrected sample absorbance (A_0):

$$E_C = 1 - \frac{A}{A_0}. \quad (1)$$

2.3 Swelling behaviour

Gelapin hydrogels were placed into round plastic containers (60 mm diameter x 40 mm, Chanrol, Australia), filled with 40 mL of phosphate buffered saline (PBS) solution (pH = 7.4, 3 mM NaN_3) and stored in a controlled temperature/humidity chamber (TRH-150-SD, Thermoline, Australia) at 37 ± 1 °C for up to 168 h. The samples were removed from the PBS solutions, padded dry with filter paper, and weighed after 3, 6, 12, 24, 48, 72 and 168 h. The PBS solutions were replaced with fresh PBS solutions at 24, 48 and 72 h. The swelling ratio (S_W) was calculated:

$$S_W = \frac{W_t}{W_i} \times 100\%, \quad (2)$$

where W_i and W_t are the initial mass and the mass at different swelling times, respectively.

 This journal is © The Royal Society of Chemistry 2013

Published on 13 November 2012. Downloaded by University of Wollongong on 14/07/2014 07:33:06.

2.4 Genipin and gelatin leaching

Gelapin hydrogels were immersed in 40 mL PBS solution and stored in sealed round container (60 mm diameter × 40 mm, Chanrol, Australia) at 40 °C for up to 72 h. At 24, 48 and 72 h, the absorbance of the PBS solution (A_{24h}, A_{48h} and A_{72h}, respectively) was measured at 589 nm in the spectrophotometer in 1 cm glass cuvettes. After each 24 h period, the hydrogels were re-immersed in fresh PBS solution. Although the specific quantity of genipin could not be determined in this way, the amount of genipin released relative to the total leachable genipin (G_R) was calculated as:

$$G_R = \frac{A_t}{A_{24h} + A_{48h} + A_{72h}} \times 100\%, \quad (3)$$

where A_t is the absorbance measured at either 24, 48 or 72 h.

At 24, 48 and 72 h, the gelatin concentration in the same PBS solution was measured using a Coomassie Blue protein assay kit (Coomassie Plus, Thermofisher Scientific, Australia) in 96 well microplate format using a plate reader (Polarstar, BMG Labtech, Germany) with an absorption spectrophotometer (595 nm). The gelatin concentration was calculated from interpolation of the linear regression of a standard curve. Calibration standards were prepared at gelatin concentrations of 0, 75, 150, 300, 450, 750, 1125 and 1500 μg mL^{-1} in duplicate and samples were prepared in quadruplicate with separate blanks.

2.5 Proteolytic and hydrolytic degradation

Gelapin hydrogels were preconditioned by soaking in PBS solution in the temperature/humidity chamber at 37 ± 1 °C for 14 days.

Hydrogel samples were placed into tissue cassettes (model M512, Simport, Canada) before being immersed in either collagenase solution for the proteolytic degradation study, or PBS for the hydrolytic degradation study. The collagenase solution was prepared from a lyophilized mixture of collagenases isolated from *Chlostridium histolyticum* (Sigma, USA) in PBS to a concentration of 10 units mL^{-1} of 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA). One unit of FALGPA hydrolyses 1.0 mmole of FALGPA per min at 25 °C and pH 7.5 in the presence of calcium ions. The samples and solutions were kept at 37 ± 1 °C for the duration of the study and had the collagenase and PBS solutions changed regularly.

For each mass measurement, the samples in their cassettes were removed from the collagenase/PBS solution, dried and then weighed. The drying procedure involved drip-draining the majority of the solution from the cassettes, followed by blotting and wiping the solution away with tissue paper. The mass of the samples (m_s) were calculated by subtracting the mass of the cassettes (m_c) from the mass of the samples in their cassettes (m_t):

$$m_s = m_t - m_c. \quad (4)$$

The extent of degradation (D) was calculated every 24 h as:

$$D = \left(1 - \frac{m_t}{m_i}\right) \times 100\%, \quad (5)$$

where m_t is the mass of the sample at a particular point in time and m_i is the initial mass. The degradation rate and the induction period were determined from the slope and the intercept of the steady-state region in a plot of the extent of degradation *versus* time.

2.6 Rheological properties

The rheological properties of Gelapin hydrogels were examined using a rheometer (Physica MCR-301, Anton Paar, Australia) with a Peltier temperature controlled bottom platen and 15.0 mm parallel plate measuring system. All measurements were conducted at 37.0 ± 0.1 °C with 15.0 mm diameter samples; a gap length of 1.00 ± 0.01 mm and 1.0 ± 0.1 N of normal force. The following experiments were conducted sequentially (with at least one minute rest between experiments): a strain-sweep experiment, where the oscillating frequency was held constant at 1 Hz and the oscillating strain was varied from 0.01% up to 10%; a frequency-sweep experiment, where the oscillating strain was held constant at 0.05% and the oscillating frequency was varied from 0.8 Hz–80 Hz; a creep recovery experiment, where samples were subjected to a non-oscillating shear stress of 10 Pa for a period of 5 s and then allowed to relax. The linear viscoelastic (LVE) region was defined in these experiments as the strain for which the storage modulus deviates by more than 5% of the initial (low strain) value.

2.7 Compressive mechanical properties

The mechanical properties of the full compositional range of Gelapin hydrogels was assessed using a universal mechanical analyser (EZ-S, Shimadzu, Japan). Prior to analysis, the hydrogels were immersed in PBS solution within the temperature/humidity chamber 37 ± 1 °C for 72 h. The instrument was adapted to incorporate a heated bath of PBS solution held at 37 ± 2 °C, so that samples were immersed during analysis. These compression tests were performed at a strain rate of 3 mm min^{-1} with samples that had been cut with a scalpel to a rectangular prism geometry of ∼10 mm × 10 mm × 8 mm. Stress-strain data was used to determine the compressive failure strain (ε_{max}), compressive tangent modulus over 20%–30% strain (E_{tan}), compressive failure stress (σ_{max}) and compressive strain energy to failure (U).

The mechanical properties of Gelapin hydrogels with composition of 4% (w/v) gelatin and 9.7% (w/w) genipin were assessed during degradation *via* collagenase proteolysis. Prior to testing, hydrogels (20 mm diameter, 5 mm height) were allowed to equilibrate in PBS solution followed by immersion in the collagenase solution. Partially degraded samples were removed at regular intervals during the proteolytic degradation process and subjected to compressive mechanical testing.

2.8 Angiogenesis

To examine the ability of blood vessels to intrude into Gelapin hydrogels and form nascent blood vessels the chick chorioallantoic membrane (CAM) on-plant assay was conducted which utilises the highly vascularised CAM as an experimental model of angiogenesis *in situ*.[41] Grey rooster/white hen cross-bred fertile chicken eggs (Barter and Sons Hatchery, Australia) were incubated (HERAcell, Kendro Laboratory Products,

Published on 13 November 2012. Downloaded by University of Wollongong on 14/07/2014 07:33:06.

Germany) for three days at 38 °C and 60% relative humidity before separation of the shell from the embryo, yolk and albumin. The embryo was then incubated (37.8 °C, 5% CO_2) for a further three days before Gelapin on-plants were laid upon the surface of the vitelline. The embryos were then returned to incubate for a further four days, during which evidence of angiogenesis was monitored *via* optical microscopy (Z16, Leica, Germany).

Gelapin-6/9.7 on-plants were prepared in the absence (control) and presence of 0.5 μM phorbol-12-myristate-13-acetate (PMA) as a chemical stimulator of angiogenesis (Cayman Chemical, USA).[42] Partially cured gels were then dispensed into the cavity of the on-plant device consisting of the space between two 5 mm squares of sterile mesh (Nylon-6,6, 180 μm grid size, 47% open area, Sefar Pty Ltd, Australia) and a spacer (Nylon-6,6, internal diameter 5.3 mm, external diameter 11.0 mm, 1.0 mm thickness, RS Components Pty Ltd, Australia). The gels were then allowed to cure and solidify over 4 more hours before being immersed in PBS solution and rested for an additional 18 h to allow uncross-linked genipin to leach out and for the gel to shrink to its equilibrium size.

Macroscopic images were taken with a digital camera (Lumix DMC-FT1, Panasonic, Japan). Microscopic images were taken with the microscope with attached digital camera (DFC-290, Leica, Germany). Image analysis was performed with the Leica Application Suite (Leica, Germany). Assessment of the extent of angiogenesis, both in and around the on-plants were made on the basis of the number of blood vessels intersecting the on-plant boundary at 60°–120° in a spherical area with radius 10 mm from the centre of the on-plant. Other qualitative assessments were also made by inspecting above and below the on-plants and measuring the range and thickness of the surrounding blood vessels.

2.9 Statistical treatment of data

Dixon's Q-test (95% confidence) was used to confirm and justify the removal of spurious data. Unless otherwise stated, the data presented in this manuscript are the mean ± one standard deviation (SD).

3. Results and discussion

3.1 Extent of cross-linking

Hydrogels were prepared by cross-linking gelatin with genipin. The extent of cross-linking (E_C) is a measure of the effectiveness of genipin as the cross-linker for gelatin and was used to determine the amount of genipin required to obtain specific amounts of cross-linking in all subsequent experiments. The E_C ratio presents the percentage of cross-linking sites in gelatin that form part of a cross-link *versus* the total amount of potential cross-linking sites and is calculated as described in eqn (1). The ratio was found to increase with increasing genipin concentration (Fig. 1). The maximum extent of cross-linking attainable for Gelapin hydrogels was observed to be 84%–90% for all concentrations of gelatin examined. This data can be used to predict the concentration of genipin required to attain any E_C desired or *vice-versa*.

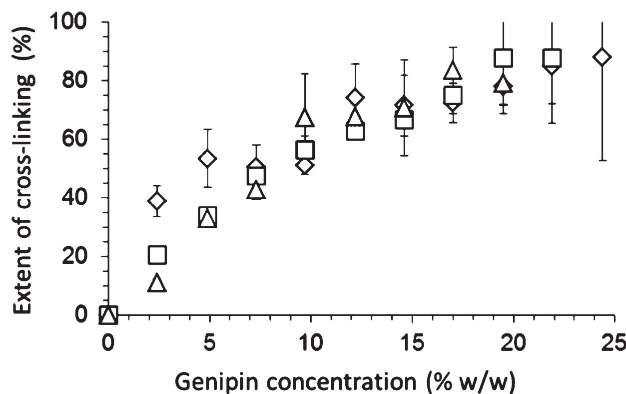

Fig. 1 Extent of cross-linking in Gelapin hydrogels as a function of genipin concentration. Diamonds, squares and triangles represent gelatin concentrations of 2%, 5% and 10% (w/v), respectively.

Our results are in agreement with those of Bigi and coworkers,[13] who suggested that genipin is unable to attain 100% E_C due to a shielding effect of the gelation tertiary structure, which prevents some of the lysine residues from reacting with genipin. Smaller and more flexible cross-linking molecules, such as gluteraldehyde have been reported to attain a maximum E_C of 100%.[16] Notwithstanding, genipin should still be considered an effective cross-linker of gelatin, especially when one considers that it has been demonstrated that genipin is not cytotoxic[20,43] and bestows anti-inflammatory character.[18,19]

3.2 Swelling behaviour

The swelling ratio of Gelapin hydrogels was observed to be dependent on both the gelatin concentration and the cross-linker concentration (Table S1, ESI†). The swelling profiles of all hydrogels could be described as an initial rapid decrease in size which slowed to a steady state where no further significant change in mass occurred (an example profile for the Gelapin-10/X hydrogels is presented in Fig. 2). The hydrogels all exuded water to varying extents, dependant on their composition

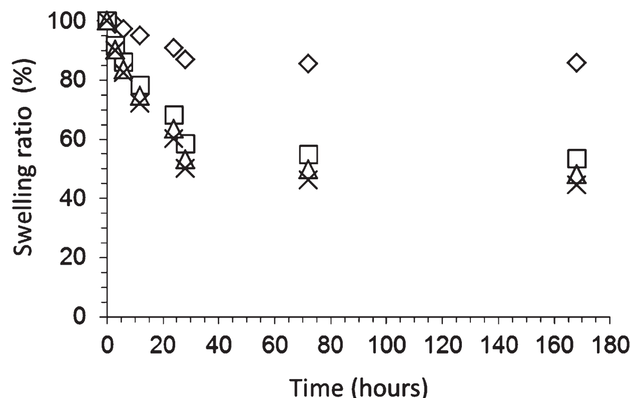

Fig. 2 Swelling ratio as a function of time for typical Gelapin hydrogels with gelatin concentration of 10% (w/v). Diamonds, squares, triangles and crosses indicate genipin concentrations of 4.9, 7.3, 12.2 and 19.5% (w/w), respectively.

 This journal is © The Royal Society of Chemistry 2013

Published on 13 November 2012. Downloaded by University of Wollongong on 14/07/2014 07:33:06.

(Table S1, ESI†). For 2%, 3% and 4% (w/v) gelatin hydrogels it took 48 h of soaking before the equilibrium swelling state was reached. For 5% and 10% (w/v) gelatin hydrogels, it took 72 h to reach the equilibrium swelling state. The extent of de-swelling was found to be inversely proportional to the gelatin concentration and directly proportional to the genipin concentration. This behaviour is consistent with the Flory–Rehner theory where hydrogel volume is affected by the balance between osmotic pressure and elastic restorative forces.[44] Hydrogels which contain more gelatin possess a greater osmotic pressure which acts to draw water into the gels and increases the equilibrium volume, while those with more extensive cross-linking possess a greater elastic restorative force which decreases the volume at equilibrium.

Some Gelapin hydrogels were not physically stable in simulated physiological conditions, PBS solution at 37 °C and pH 7.4. Hydrogels compositions comprising of 2% (w/v) gelatin and less than 7.3% (w/w) genipin; 3% (w/v) gelatin and less than 4.9% (w/w) genipin; and 4% (w/v) gelatin with 2.4% (w/w) genipin, dissolved completely over 24 h of soaking. These hydrogels possess relatively low concentrations of gelatin and genipin and therefore possess only a small amount of polymer entanglement and covalent cross-links. It is suggested that this causes them to dissolve when the temperature of the hydrogels is raised from the curing temperature (~20 °C) to the temperature (37 °C) at which all testing is carried out.

It is well known that proteinaceous hydrogels, such as Gelapin are particularly sensitive to changes in pH which affect the balance of charges on the polymeric backbone.[44] This was demonstrated by investigating the swelling behaviour in PBS soaking solutions (at 37 °C) of pH 8.2. Under these conditions it was observed that the S_W was higher compared to the corresponding gels swollen in PBS at pH 7.4.

3.3 Genipin and gelatin leaching

Gelapin hydrogels were immersed in PBS solution at 37 °C and pH 7.4 for several days during which the leaching of genipin and gelatin were monitored regularly. A noticeable amount of genipin in the form of partially reacted (not cross-linked) blue genipin pigments was observed to leach from the Gelapin hydrogels during 72 h of immersion in PBS solution. The blue pigments are commonly known as the edible blue food dye "Gardenia Blue" and are a result of interactions between genipin and gelatin.[17] The percentage of genipin released (G_R) was calculated according to eqn (3) at 24 h, 48 h and 72 h (data not shown). It was observed that the majority (>89%) of the genipin was released during the first 24 h for all of the Gelapin hydrogels.

Insignificant amounts of gelatin were released from Gelapin hydrogels whilst being immersed in PBS (Table S2, ESI†). The majority of the gelatin was released within the first 24 h of soaking with no detectable amount being released on subsequent days. The amount released was inversely proportional to the extent of cross-linking. This is likely to be a result of cross-links tethering gelatin molecules together as well as contributing to confinement and entanglement of non-covalently cross-linked gelatin molecules. This initial release may represent the superficial, unbound gelatin. These results demonstrate that by increasing the amount of cross-linking, the gelatin release can be slowed or extinguished altogether.

3.4 Proteolytic and hydrolytic degradation

The extent of proteolytic and hydrolytic degradation (D) of Gelapin hydrogels were obtained using mass measurements of samples immersed in PBS solution with and without collagenase, respectively, over 93 days. Samples of hydrogel which had been immersed with collagenase were observed to lose mass at three different rates (Fig. 3a, Table 1), referred to as the initial, steady state and tailing periods. In the initial (induction) period the infiltration of the hydrogel by collagenase commences and it begins to make scissions in the

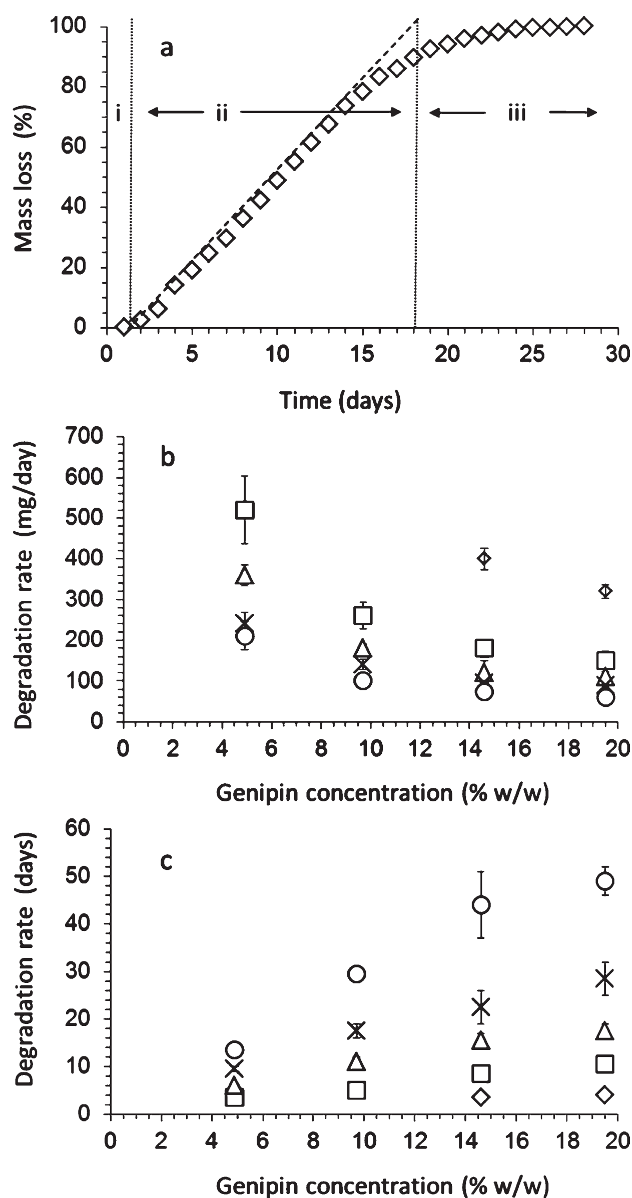

Fig. 3 (a) Typical proteolytic degradation profile for Gelapin-8/14.6. (i-iii) indicate the three period of the degradation, *i.e.* induction, steady state and tailing, respectively. (b) Proteolytic degradation rates of Gelapin hydrogels (± SD). (c) Degradation time for Gelapin hydrogels (± ½ range).

Published on 13 November 2012. Downloaded by University of Wollongong on 14/07/2014 07:33:06.

Table 1 The induction times (t_I), degradation rates (D) and degradation times (t_D) for proteolytic degradation and the hydrolytic mass change (ΔM) for Gelapin hydrogels after a 93 day hydrolytic degradation period. The nomenclature of Gelapin hydrogels describes their composition in the format Gelapin-[gelatin %(w/v)]/[genipin %(w/w)]

Sample	Proteolytic			Hydrolytic
	t_I/days	D/mg per day	t_D/days	ΔM/%
Gelapin-2/14.6	1.01 ± 0.06	400 ± 26	3.5 ± 0.5	−2 ± 2
Gelapin-2/19.5	1.0 ± 0.2	320 ± 17	4 ± 0	−2 ± 2
Gelapin-4/4.9	0.07 ± 0.06	520 ± 83	3.5 ± 0.5	+2.5 ± 0.6
Gelapin-4/9.7	0.83 ± 0.09	260 ± 33	5 ± 0	+2.7 ± 0.2
Gelapin-4/14.6	1.3 ± 0.1	180 ± 22	8.5 ± 0.5	+2.3 ± 0.2
Gelapin-4/19.5	2.1 ± 0.3	150 ± 22	10.5 ± 0.5	+2.9 ± 0.3
Gelapin-6/4.9	0.09 ± 0.03	360 ± 25	6 ± 0	+3.9 ± 0.3
Gelapin-6/9.7	0.84 ± 0.07	181 ± 9	11 ± 1	+3.44 ± 0.06
Gelapin-6/14.6	1.4 ± 0.3	120 ± 29	15.5 ± 1.5	+3.3 ± 0.2
Gelapin-6/19.5	1.5 ± 0.3	110 ± 12	17.5 ± 1.5	+5.6 ± 0.5
Gelapin-8/4.9	0.0 ± 0.1	240 ± 28	9.5 ± 0.5	+4.8 ± 0.4
Gelapin-8/9.7	0.73 ± 0.07	140 ± 13	17.5 ± 1.5	+5.9 ± 0.2
Gelapin-8/14.6	1.4 ± 0.2	94 ± 1	22.5 ± 3.5	+6.5 ± 0.4
Gelapin-8/19.5	1.5 ± 0.3	90 ± 12	28.5 ± 3.5	+6.8 ± 0.5
Gelapin-10/4.9	0.0 ± 0.2	210 ± 34	13.5 ± 0.5	+5.9 ± 0.1
Gelapin-10/9.7	0.9 ± 0.2	100 ± 15	29.5 ± 1.5	+7.41 ± 0.07
Gelapin-10/14.6	2.0 ± 0.3	74 ± 9	44 ± 7	+7.9 ± 0.2
Gelapin-10/19.5	2.14 ± 0.05	60 ± 3	49 ± 3	+9.0 ± 0.3

gelatin. During this period the hydrogel was being degraded but there was no significant change in mass loss until there were enough scissions made in the matrix to dislodge fragments from the bulk material. For samples immersed in collagenase, the induction period was found to be proportional to the amount of cross-linking in the Gelapin hydrogels, *i.e.* it was instantaneous for the least cross-linked hydrogels and up to 2 days for the hydrogels with the highest degree of crosslinking.

In the second (steady state) period, the degradation rate for the hydrogels immersed in collagenase solution ranged from 60 mg per day up to 500 mg per day and was observed to be dependent on both the gelatin concentration and the concentration of the genipin (Fig. 3b). Increasing the gelatin concentration increased the density of the hydrogel producing a less penetrable matrix for collagenase and partially degraded matrix to diffuse through. As a result, the degradation rate observed for high gelatin content hydrogels was lower than that of the low gelatin content hydrogels. Gels with higher concentrations of the genipin cross-linker exhibited slower degradation rates. It is suggested that the covalent cross-links formed with genipin molecules are resistant to degradation by collagenase and may be tying partially degraded gelatin molecules together and contributing to their entanglement for longer periods of time than less densely cross-linked Gelapin hydrogels.

In the third (tailing) period, degradation rate decreased until the entire material was degraded. During this period, degradation is limited by the available surface area of the sample which diminishes in proportion to the radius of the sample squared. Consequently, as the sample is degraded to a smaller size, the sample area and degradation rate decrease quickly. The total time it took samples to degrade *via* proteolysis ranged from 3.5 ± 0.5 to 49 ± 3 days and was proportional to the gelatin and genipin concentrations (Fig. 3c). Slower degradation rates were observed for higher gelatin content and more extensively cross-linked Gelapin hydrogels which had a direct effect on the overall time taken to degrade the component. In contrast, Gelapin hydrogel which was not exposed to collagenase did not show significant signs of degradation over 93 days, or a time period which is twice as long as it took for the most robust Gelapin hydrogels to degrade completely in collagenase. In fact, most samples increased in mass by a small amount which may be a result of swelling (Table 1). This suggests that in the absence of matrix metalloproteases such as collagenase, Gelapin hydrogels will not significantly degrade as a result of indiscriminate hydrolysis.

3.5 Rheological properties

Strain-sweep experiments were conducted to identify the linear viscoelastic (LVE) region which was observed to be up to 5% shear strain (data not shown). Hydrogels which possess a higher cross-linker concentration had shorter LVE regions as compared with hydrogels with smaller cross-linker concentration, which can be explained by the stiffening effect of cross-linking. For example, the shear strain values for the limit LVE region were 5% and 0.1% for Gelapin-4/4.9 and Gelapin-8/14.6 hydrogels, respectively. As expected, the values for the storage and loss moduli were larger for gels with a higher degree of cross-linking, consistent with a previous rheological study of cross-linked hydrogels using pollock gelatin.[23] For example, Gelapin-4/4.9 and Gelapin-8/14.6 hydrogels exhibited storage modulus values in the LVE region of 9 kPa and 90 kPa, respectively. The data from the frequency-sweep and creep recovery experiments indicated that the storage and loss moduli of Gelapin hydrogels were independent on frequency up to 80 Hz (data not shown).

3.6 Compressive mechanical properties

The mechanical properties of Gelapin hydrogels were examined in compression whilst immersed in PBS solution at 37 °C (Fig. 4). Compressive strain at failure (ε_{max}) values varied between 50 ± 3% and 74 ± 5% and were inversely proportional with increasing concentration of genipin

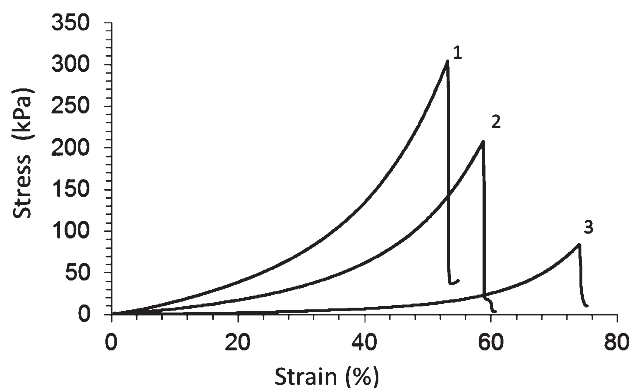

Fig. 4 Typical stress-strain curves for, Gelapin-8/14.6 (line 1), Gelapin-6/9.7 (line 2) and Gelapin-4/4.9 (line 3).

 This journal is © The Royal Society of Chemistry 2013

Published on 13 November 2012. Downloaded by University of Wollongong on 14/07/2014 07:33:06.

(Table 2). This can be explained by the increased density of covalent cross-linking in the hydrogels induced by a higher genipin concentration—with more covalent cross-links, comes a greater amount of elastic stiffness reducing the extent of deformation before the hydrogel fails. The gelatin concentration was also observed to have a similar effect on ε_{max} values. The increased gelatin concentration is accompanied by more polymer entanglement which acts as a comparatively weak cross-link.

Tangent modulus (E_{tan}) values were observed to increase significantly with increasing concentration of genipin (Table 2). E_{tan} was also observed to be proportionate to the gelatin concentration. It is suggested that these observations are a result of increased polymer entanglement and physical cross-link density. In other words, the gels are becoming stiffer but less ductile with increasing genipin and gelation concentrations.

Compressive stress at failure (σ_{max}) values ranged between 26 ± 6 kPa and 300 ± 60 kPa in proportion to the gelatin concentration. It is likely that this can be explained by realising that gels with higher amounts of gelatin have a higher overall density. Hence, they require more stress to deformation to the point of failure (Table 2). The cross-linker concentration (genipin) did not have a significant effect on σ_{max}. Compressive strain energy at failure (U) values ranged from 3 ± 1 kJ m^{-3} to 50 ± 10 kJ m^{-3}, and increased with increasing gelatin and genipin concentration. The compressive mechanical characteristics of our gels are in the same order of magnitude as those reported for a gelatin scaffold (100–140 kPa, cross-linked by immersion in a genipin solution) developed for cartilage tissue engineering.[26]

Our results demonstrate that Gelapin hydrogels are a versatile material which can have tuned mechanical properties through careful control over the gelatin and genipin concentration. Increasing the gelatin concentration increased the overall strength and toughness as indicated by the compressive stress and strain energy to failure, while increasing the genipin concentration increased the stiffness and toughness and limited the maximum strain attainable as reflected by the E_{tan}, U and ε_{max} values.

Table 2 Summary of mechanical properties for Gelapin hydrogels. Compressive strain at failure, compressive tangent modulus (20%–30% strain), compressive stress at failure and compressive strain energy to failure are indicated by ε_{max}, E_{tan}, σ_{max} and U, respectively. The nomenclature of Gelapin hydrogels describes their composition in the format Gelapin-[gelatin %(w/v)]/[genipin %(w/w)]

Sample	ε_{max} (%)	E_{tan}/kPa	σ_{max}/kPa	U/kJ m^{-3}
Gelapin-2/9.7	67 ± 7	13 ± 6	26 ± 6	3 ± 1
Gelapin-2/14.6	57 ± 5	22 ± 4	26 ± 8	3 ± 1
Gelapin-2/19.5	55 ± 7	40 ± 10	27 ± 9	4 ± 1
Gelapin-4/4.9	74 ± 5	18 ± 3	80 ± 40	11 ± 5
Gelapin-4/9.7	59 ± 5	90 ± 20	100 ± 20	16 ± 3
Gelapin-4/14.6	54 ± 2	130 ± 20	110 ± 30	18 ± 4
Gelapin-4/19.5	52 ± 5	160 ± 30	120 ± 30	18 ± 4
Gelapin-6/4.9	65 ± 6	50 ± 10	90 ± 30	14 ± 4
Gelapin-6/9.7	58 ± 5	150 ± 30	180 ± 50	26 ± 7
Gelapin-6/14.6	53 ± 5	200 ± 40	170 ± 70	30 ± 10
Gelapin-6/19.5	51 ± 5	270 ± 40	200 ± 60	30 ± 10
Gelapin-8/4.9	62 ± 5	170 ± 20	230 ± 70	40 ± 10
Gelapin-8/9.7	55 ± 6	250 ± 50	200 ± 100	40 ± 20
Gelapin-8/14.6	52 ± 3	320 ± 30	240 ± 60	38 ± 9
Gelapin-8/19.5	52 ± 5	350 ± 40	270 ± 73	40 ± 10
Gelapin-10/4.9	64 ± 6	160 ± 20	250 ± 90	40 ± 10
Gelapin-10/9.7	62 ± 8	240 ± 40	300 ± 100	50 ± 20
Gelapin-10/14.6	50 ± 3	380 ± 40	270 ± 60	40 ± 10
Gelapin-10/19.5	51 ± 5	400 ± 50	300 ± 60	50 ± 10

The effect of degradation on the mechanical characteristics was examined using a typical Gelapin hydrogel (Gelapin-4/9.7) which was exposed to collagenase for up to 10 days. Fig. 5 shows that strain-at-break values increase, while tangent modulus values increase during proteolytic degradation of up to 70% (by mass). In other words, the gels become more ductile and elastic as a result of the mass loss. Once the hydrogels had degraded by 70%, they became too fragile to handle and could not be subjected to mechanical testing.

3.8 Angiogenesis

Materials used as a tissue scaffold have traditionally relied upon diffusion of nutrients and oxygen to provide nourishment and address the respiratory requirements of the cells therein. However, diffusion is limited to only several hundred microns in most cases and this presents a challenge for tissue homeostasis.[45] This can be overcome by promoting blood vessel invasion into the scaffold.[46] Vascularisation of Gelapin hydrogels from existing vasculature was investigated using the chick embryo CAM as an experimental model of angiogenesis *in situ*. Gelapin-6/9.7 hydrogels materials were

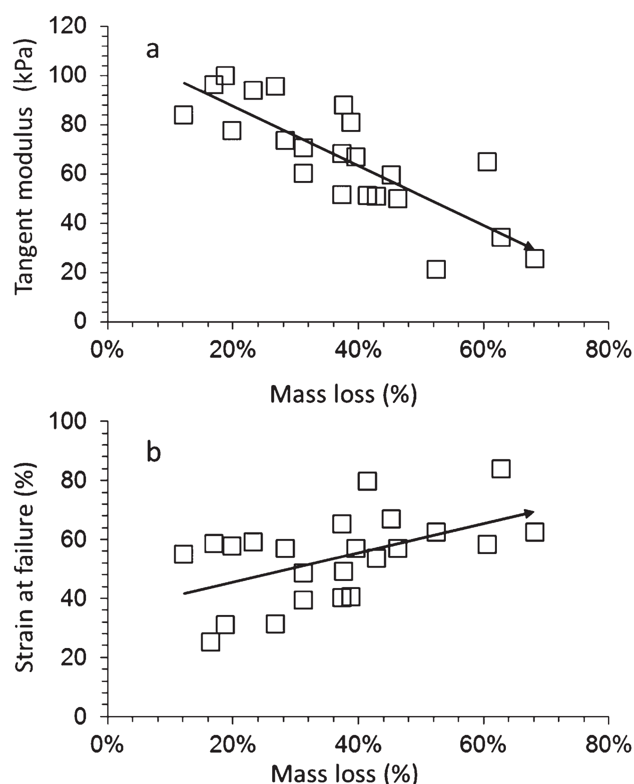

Fig. 5 (a) Tangent modulus (20%–30% strain) and (b) compressive stress at failure for typical Gelapin-4/14.6 hydrogels as a function of mass loss during proteolytic degradation. Arrows indicate trend with mass loss.

Published on 13 November 2012. Downloaded by University of Wollongong on 14/07/2014 07:33:06.

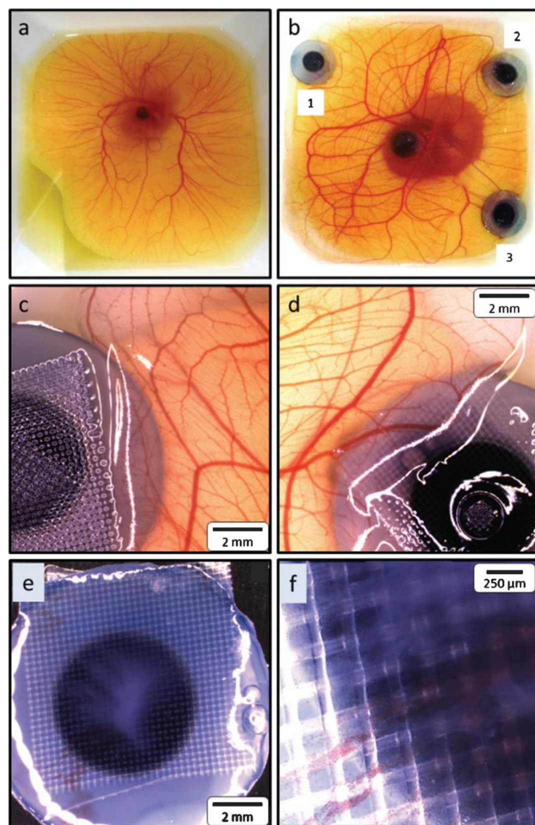

Fig. 6 Optical images of (a) typical chick embryo after 6 days prior to placement of the Gelapin-6/9.7 on-plants, (b) typical chick embryo after 4 days with on-plants prepared in the absence (1) or presence (2 and 3) of PMA in place, (c) enlargement of control on-plant, (d) enlargement of on-plant 2 prior to excision, and (e–f) enlargements of on-plant 2 after excision showing red blood cells within the confines of the on-plant.

selected for these studies as gels with this gelatin concentration (6% w/v) exhibit mechanical properties which are in the middle of the range of values of the investigated sample compositions (see Table 2). On-plants prepared using Gelapin-6/9.7 hydrogel materials in the absence or presence of the angiogenic stimulator PMA[42] were placed on developing chick embryos and observed for up to 4 days. The presence of the on-plants did not compromise the viability of the embryos and their ability to grow blood vessels and evidence of blood vessel intrusion and formation within Gelapin on-plants is shown in Fig. 6. Examination of the area surrounding the on-plants after 4 days of testing revealed that the number of blood vessels near both the control and PMA containing hydrogels was 19 ± 1. However, the blood vessels were generally very fine around the control on-plant and did not appear to extend into the hydrogel (Fig. 6c). In contrast, there were also large blood vessels evident in the PMA-containing on-plants which intruded into the hydrogel (Fig. 6d–f) suggesting that the Gelapin hydrogel acts as a vascularisable soft tissue scaffold for the *pro*-angiogenic effect of PMA.

4. Conclusions

We successfully prepared genipin cross-linked gelatin hydrogel materials which were characterised in terms of extent of cross-linking, swelling behaviour, degradability and mechanical characteristics as well as ability for vascularization. It was shown that soaking Gelapin in phosphate buffered saline solution (37 °C, pH 7.4) for 72 h resulted in the removal of 99% of the leachable gelatin and genipin. The swelling behaviour, mechanical properties and degradability of these hydrogels can be controlled through adjustment of the genipin and the gelatin concentrations. The degradation rate could be tuned between 50 mg per day and 500 mg per day. Compressive stress at strain value could be tuned over one order of magnitude (26 ± 6 kPa to 300 ± 60 kPa). Proteolytic degradation studies showed that typical Gelapin hydrogels became more ductile and elastic until they degraded by more than 70% (by mass). It was shown that typical Gelapin hydrogels have the ability to become vascularised with incorporation of *pro*-angiogenic factors. This paper contributes to the development of Gelapin hydrogels as a potential material for use in future tissue engineering applications.

Acknowledgements

This work was funded by the Australian Research Council Centre of Excellence and Future Fellowship (M. in het Panhuis) programs. The authors thank Prof P. Calvert (USA) and Dr R. Clark (USA) for useful discussions.

References

1 R. Langer and J. P. Vacanti, *Science*, 1993, **260**, 920–926.
2 P. X. Ma, *Adv. Drug Delivery Rev.*, 2008, **60**, 184–198.
3 K. Y. Lee and D. J. Mooney, *Surgery*, 2001, 101.
4 A. S. Hoffman, *Adv. Drug Delivery Rev.*, 2002, **54**, 3–12.
5 H. P. Jenkins and J. S. Clarke, *Arch. Surg.*, 1945, **51**, 253–261.
6 W. H. Chang, Y. Chang, P. H. Lai and H. W. Sung, *J. Biomater. Sci., Polym. Ed.*, 2003, **14**, 481–495.
7 T. Sasajima, M. Inaba, N. Azuma, K. Goh, S. Koshiko, Y. Kubo, K. Miyamoto, M. Tokita and T. Komai, *Artif. Organs*, 2008, **21**, 287–292.
8 Y. Nakayama and T. Matsuda, *J. Biomed. Mater. Res.*, 1999, **48**, 511–521.
9 M. Djabourov, J. Leblond and P. Papon, *J. Phys.*, 1988, **49**, 319–332.
10 M. Djabourov, J. Leblond and P. Papon, *J. Phys.*, 1988, **49**, 333–343.
11 K. B. Djagny, Z. Wang and S. Xu, *Crit. Rev. Food Sci. Nutr.*, 2001, **41**, 481–492.
12 S. Young, M. Wong, Y. Tabata and A. G. Mikos, *J. Controlled Release*, 2005, **109**, 256–74.
13 A. Bigi, G. Cojazzi, S. Panzavolta, N. Roveri and K. Rubini, *Biomaterials*, 2002, **23**, 4827–4832.
14 D. Hellio and M. Djabourov, *Macromol. Symp.*, 2006, **241**, 23–27.

This journal is © The Royal Society of Chemistry 2013

Published on 13 November 2012. Downloaded by University of Wollongong on 14/07/2014 07:33:06.

15 S. Fakirov, Z. Sarac, T. Anbar, B. Boz, I. Bahar, M. Evstatiev, A. A. Apostolov, J. E. Mark and A. Kloczkowski, *Colloid Polym. Sci.*, 1996, **274**, 334–341.
16 A. Bigi, G. Cojazzi, S. Panzavolta, K. Rubini and N. Roveri, *Biomaterials*, 2001, **22**, 763–768.
17 F.-L. Mi, H.-W. Sung and S.-S. Shyu, *J. Polym. Sci., Part A: Polym. Chem.*, 2000, **38**, 2804–2814.
18 H. J. Koo, K. H. Lim, H. J. Jung and E. H. Park, *J. Ethnopharmacol.*, 2006, **103**, 496–500.
19 H. J. Koo, Y. S. Song, H. J. Kim, Y. H. Lee, S. M. Hong, S. J. Kim, B. C. Kim, C. Jin, C. J. Lim and E. H. Park, *Eur. J. Pharmacol.*, 2004, **495**, 201–208.
20 H.-W. Sung, R.-N. Huang, L. L. H. Huang and C.-C. Tsai, *J. Biomater. Sci., Polym. Ed.*, 1999, **10**, 63–78.
21 M. T. Nickerson, J. Patel, D. V. Heyd, D. Rousseau and A. T. Paulson, *Int. J. Biol. Macromol.*, 2006, **39**, 298–302.
22 C. H. Yao, B. S. Liu, C. J. Chang, S. H. Hsu and Y. S. Chen, *Mater. Chem. Phys.*, 2004, **83**, 204–208.
23 B. Chiou, R. J. Avena-bustillos, J. Sheya, E. Yee, P. J. Bechtel, S. H. Imam, G. M. Glenn and W. J. Orts, *Polymer*, 2006, **47**, 6379–6386.
24 B. S. Liu, C. H. Yao, W. Wang, S. Lee, C. C. Lo, C. K. Liu and Y. S. Chen, *J. Med. Biol. Eng.*, 2008, **29**, 29–38.
25 Y. S. Chen, J. Y. Chang, C. Y. Cheng, F. J. Tsai, C. H. Yao and B. S. Liu, *Biomaterials*, 2005, **26**, 3911–3918.
26 S. M. Lien, W. T. Li and T. J. Huang, *Mater. Sci. Eng., C*, 2008, **28**, 36–43.
27 E. J. Lee and L. E. Niklason, *Tissue Eng., Part C*, 2010, **16**, 1191–1200.
28 B. David, D. Bonnefont-rousselot, K. Oudina, M.-C. Degat, M. Deschepper, V. Viateau, M. Bensidhoum, C. Oddou and H. Petite, *Tissue Eng., Part C*, 2011, **17**, 505–516.
29 T. Miki and A. Ring, *Tissue Eng., Part C*, 2011, **17**, 557–568.
30 R. J. McCoy and F. J. O'Brien, *Tissue Eng., Part B*, 2010, **16**, 587–15.
31 P. A. Galie and J. P. Stegemann, *Tissue Eng., Part C*, 2011, **17**, 527–536.
32 C. D. Maria, S. Giusti, D. Mazzei, A. Crawford and A. Ahluwalia, *Tissue Eng., Part C*, 2011, **17**, 1–8.
33 A. B. Yeatts and J. P. Fisher, *Tissue Eng., Part C*, 2011, 1–22.
34 N. Wang, J. P. Butler and D. E. Ingber, *Science*, 1993, **260**, 1124–1127.
35 L. Haglund, J. Moir, L. Beckman, K. R. Mulligan, B. Jim, J. A. Ouellet, P. Roughley and T. Steffen, *Tissue Eng., Part C*, 2011, **17**, 1011–1019.
36 A. A. Gassman, T. Kuprys, A. A. Ucuzian, E. Brey, A. Matsumura, Y. Pang, J. Larson and H. P. Greisler, *J. Tissue Eng. Regener. Med.*, 2011, **5**, 375–383.
37 K. A. Ahmann, S. L. Johnson, R. P. Hebbel and R. T. Tranquillo, *Tissue Eng., Part A*, 2011, **00**, 1–11.
38 A. R. Cameron, J. E. Frith and J. J. Cooper-White, *Biomaterials*, 2011, **32**, 5979–5993.
39 C. M. Ofner III and W. A. Bubnis, *Pharm. Res.*, 1996, **13**, 1821–1827.
40 C. M. Ofner III and W. A. Bubnis, *Anal. Biochem.*, 1992, **207**, 129–133.
41 C. Storgard, D. Mikolon and D. G. Stupack, *Angiogenesis Assays in the Chick CAM.* 1 ed. Cell Migration: Developmental Methods and Protocols (Methods in Molecular Biology), Ed. J.-L. Guan. **Vol. 294**. 2004, Humana Press Inc.: Totowa, NJ. 123–135.
42 N. E. Tsopanoglou, E. Pipili-Synetos and M. E. Maragoudakis, *J. Vasc. Res.*, 1993, **30**, 202–208.
43 C.-C. Tsai, R.-N. Huang, H.-W. Sung and H.-C. Liang, *J. Biomed. Mater. Res.*, 2000, **52**, 58–65.
44 N. A. Peppas, Z. Hilt, A. Khademhosseini and R. Langer, *Adv. Mater.*, 2006, **18**, 1345–1360.
45 M. Lovett, K. Lee, A. Edwards and D. L. Kaplan, *Tissue Eng., Part B*, 2009, **15**, 353–70.
46 T. Kaully, K. Kaufman-Francis, A. Lesman and S. Levenberg, *Tissue Eng., Part B*, 2009, **15**, 159–169.