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Hydrogels for Tissue Engineering and Regenerative Medicine

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Abstract

Injectable hydrogels have become an incredibly prolific area of research in the field of tissue engineering and regenerative medicine, because of their high water content, mechanical similarity to natural tissues, and ease of surgical implantation, hydrogels are at the forefront of biomedical scaffold and drug carrier design. The aim of this review is to concisely summarise current state-of-the-art in natural and synthetic hydrogels with respect to their synthesis and fabrication, comparing and contrasting the many chemistries available for biomedical hydrogel generation using both biologic and synthetic base materials. We then discuss these hydrogels in the specific instance of several pertinent areas of TERM which have been specifically selected to demonstrate how this versatile class of materials can be modified to augment damage and disease of a seemingly limitless array of adult tissues.

Injectable Hydrogels for Tissue Engineering and Regenerative Medicine

1. Introduction

As a consequence of their tunable mechanical properties hydrogels have been exploited in a variety of biomedical applications such as contact lenses in ophthalmology, absorbable sealants in general surgery, fillers for aesthetic correction of scars and other cosmetic applications, and as anti-adhesive coatings on meshes for abdominal wall and hernia repair[1-5]. In recent years, because of their ability to immobilize and release cells, genes, proteins and drugs, hydrogels have become ubiquitously appreciated throughout biomedicine[6-9].

Tissue engineering and regenerative medicine (TERM) are rapidly evolving interdisciplinary fields with the unified aim of creating systems to replace or restore tissue which is functioning sub optimally as a result of either through chronic disease or acute trauma. In TERM, a biomaterial often acts as a scaffold; mimicking extracellular matrix to provide mechanical, spacial and biological signals for regulating and guiding a tissue response. Hydrogels possess many properties which are attractive in biomedical scaffold design including cytocompatibility, tissue mimetic water content, support of cell migration and tissue integration, sustained release of growth factors, controllable physical properties and minimally invasive surgical delivery via injection in a liquid phase[10-11].

The objectives of this review are to iterate the promise of hydrogels in TERM via discussing their chemistries, origins and past, present and future applications.

2. Gelation of injectable hydrogels

An ideal TERM hydrogel should possess a low-viscosity prior to injection, and rapidly gel under the physiologic tissue environment where it is required. The most important component of this process is gelation (sol-gel transition) by cross-linking. Injectable hydrogels can be cross-linked either *in vitro* during their preparation or in situ (*in vivo*) after injection. There are a variety of hydrogel cross-linking mechanisms[213] which will be discussed further

throughout this review including: physical cross-linking, chemical cross-linking, enzyme-initiated cross-linking and ionic cross-linking.

2.1 Physical cross-linking

Physical cross-linking is triggered by pH, temperature and other physical stimuli. The advantages of physical cross-linking are relative ease of the process and no requirement of exogenous using cross-linking agents which may increase material toxicity[10,12].

Temperature-responsive hydrogels

Poly(N-isopropylacrylamide) (poly (NIPAAM)) is the most studied temperature-responsive hydrogel which undergoes a coil-to-globule transition on warming above a lower critical solution temperature (LCST) of ~32^{o}C. The main mechanism of thermal-induced crosslinking is an aqueous phase separation of poly (NIPAAM) which is induced by release of water molecules bound to the polymers isopropyl side groups. This results in increasing intra- and inter molecular hydrophobic interactions between the isopropyl groups above its LCST[13-14]. However, the high shrinking behaviour on gelation makes poly (NIPAAM) not practical for cell encapsulation and protein delivery. Hydrophilic segments such as acrylic acid (AAc) or PEG[15-16] have been incorporated into poly (NIPAAM) chains to counteract shrinking and improve cell compatibility. To introduce biodegradation, poly(NIPAAM) has been incorporated into the backbone of biodegradable polymers such as chitosan, gelation, hyaluronic acid and dextran[17-21] to produce a series biodegradable temperature-responsive hydrogels for TERM.

Another group of temperature-responsive injectable hydrogels with LCST are block copolymers, such as PEO-PPO-PEO (Pluronic), PLGA-PEG-PLGA, PEG-PLLA-PEG, PCL-PEG-PCL and PEG-PCL-PEG[22-26]. These amphiphilic copolymers can be produced by simply warming the polymer solutions that causes the block copolymerisation. The copolymers often have a hydrophobic block such as poly(lactide-co-glycolide) (PLGA), poly(propylene oxide) or polycaprolactone (PCL) and a hydrophilic block which is always comprised of poly(ethylene oxide) (PEO). At low temperature, the amphiphilic polymer chains assemble into micelles and bridged micelles; when temperature increases to LCST, more hydrophobic segments are exposed which increases hydrophobicity leading to micellar aggregation to form a hydrogel. These triblock copolymers have been widely used throughout research as injectable hydrogels for cell delivery.

Agarose represents a different type of temperature-responsive hydrogel whose cross-linking initiates due to the formation and subsequent aggregation of double helical structures after temperature decreasing from melting (~85^{o}C) to below the setting (ranging from 17-35^{o}C depending on chemical structures).

pH-Responsive hydrogels

Poly (NIPAAM) and block copolymer hydrogels can be made to respond to both temperature and pH by copolymerizing with pH-sensitive substituted acrylates[29-30] such as 2-(dimethylamino) ethyl methacrylate (DMAEMA) or 2-(diethylaminoethyl) methyl methacrylate. These dual-responsive polymers have been used to prolong release of proteins such as VEGF [31]. Due to concerns about biocompatibility and sufficient gel stability *in vivo*, pH-responsive hydrogels are not typically regarded as good choices as cell carriers.

Self-assembling peptides hydrogels

Hydrogels can form using weak and non-covalent interactions to bring short peptides together to form higher order structures by self-assembly. By adjusting the amino acid sequence, the secondary structure of peptides can be manipulated by hydrogen bonding, ionic, electrostatic, hydrophobic and van der Waals interactions to form β-sheets, β-hairpins and α-helices. These structures further spontaneously organize to form nanofibres which aggregate into 3D hydrogels[32-33]. Due to their ability to form scaffolds under physiological conditions, and their shear thinning property, self-assembling peptide hydrogels can be injected after subjecting the materials to stress and then gel with subsequent mechanical recovery in situ. For example, nanofibrillar gels have been developed[32, 34-35] using ionic self-complementary peptides spontaneously assembling to form well-ordered nanofibres ~10nm in diameter which further self-assemble to form scaffolds. Another approach synthesized self-assembling peptide amphiphiles (PAs) bearing biological signals such as an epitope of laminin; IKVAV[36], which have been used for 3D cell culture[32, 35-38].

2.2 Chemical cross-linking

Chemical cross-linked hydrogels are prepared through covalent bond formation between different polymer chains. The resulting hydrogel is generally more resistant to mechanical forces but undergoes greater volume changes than physically cross-linked networks.

Photocrosslinked hydrogels

The usual technique for chemical cross-linking is photopolymerization which enables in situ formation of crosslinked hydrogels at physiological pH and temperature. Photopolymerization is initiated by free radicals which are produced by decomposition of the photoinitiator upon exposure to visible light and UV. The free radicals react with hydrogel precursors bearing polymerizable groups, such as acrylate or methacrylate moieties to form a hydrogel. Because the mild gelation conditions allow cells to be encapsulated within photocrosslinked hydrogels and remain viable, and UV/visible light irradiation can be applied *in vivo* in a minimally invasive manner, photocrosslinked hydrogels have been widely developed for biomedicine. Many researchers are interested in exploiting the photo-crosslinkable poly(ethylene glycol)-diacrylate (PEGDA), poly(ethylene glycol)-dimethacrylate (PEGDMA), poly(propylene fumarate) (PPF) and oligo(poly(ethylene glyco) fumarate) (OPF)[39-43] for use in TERM. Recently, natural photo-crosslinkable hydrogels such as dextran, alginate, chitosan and hyaluronic acid[44-47] also have been synthesised through methacrylation or copolymerization with PEGDA/PEGDMA.

Michael-type addition reaction hydrogels

Unlike most addition reactions carried out in organic media, Michael-type addition between thiols and acrylates or vinyl sulfones can occur in aqueous medium, at room temperature, and at physiological pH. For example, thiol-modified HA and PEGDA crosslinked through Michael-type addition have been used to make hydrogels which support human adipose derived stem cell and fibroblast adhesion and proliferation[10, 48-49].

Schiff base – crosslinked hydrogels

A Schiff base reaction which forms a carbon-nitrogen double bond between amino and aldehyde groups can be used to achieve in situ crosslinking without chemical cross-linking agents. Aldehyde groups introduced by partially oxidized polysaccharides such as hyaluronic acid, dextran, gum arabic and chondroitin sulphate can react with amino groups in other natural or synthetic water soluble polymers such as N-Succinyl-chitosan to form hydrogels [50-53].

Other chemical cross-linking processes which have been used to generate hydrogels include: genipin induced gelation[54] and diels-alder reaction[55].

2.3 Enzyme-mediated cross-linking

Enzyme-initiated cross-linking mediated by either endogenous or exogenous enzymes can allow gelation in situ in a biologic environment. Due to the enzyme specificity, these cross-links avoid side reactions which limit toxicity concerns. The most utilized enzymes to catalyze hydrogel cross-linking for TERM are transglutaminases (including Factor Xllla) and horseradish peroxidases (HRP).

Transglutaminases are a wide family of thiol enzymes that catalyze formation of a covalent bond between free amine groups of lysine and the γ-carboxamide group of glutamine. The cross-linking reactions are relatively fast (5-20 minutes) and the resultant covalent bonds are highly resistant to proteolysis. Fibrin is a classic example of a transglutaminase catalyzed hydrogel prepared from fibrinogen and thrombin, the key proteins involved in blood clotting which is catalyzed by factor Xllla (plasma transglutaminase). Fibrin was among the first biomaterials used to prevent bleeding and promote wound healing. Using this mechanism, many novel injectable hydrogels, such as modified fibrin hydrogel with bioactive peptides[56], factor Xllla catalyzed star-shaped PEG hydrogels[57] and tissue transglutaminase catalyzed PEG hydrogels[58] have been explored and now have been used in a variety of biomedical applications.

Horseradish peroxidase (HRP), a member of the peroxidase family, is the most commonly used peroxidase in hydrogel formation. HRP catalyzes the conjugation of phenol and aniline derivatives in the presence of hydrogen peroxide by oxidization of hydroxyphenyl groups present in tyramine, tyrosine and 4-hydrophenyl acetic acid[59]. HRP mediated cross-linking has several advantages including adjustable reaction rates, mild cross-linking conditions and good cytocompatibility. Based on this mechanism, a variety of enzyme-mediated crosslinked hydrogels have been developed by engrafting tyramine groups into natural and synthetic polymers such as dextran, hyaluronic acid, alginate, cellulose, gelatin, heparin and PEG-PPO [60-66].

Other enzymes have been utilized to mediate hydrogel cross-linking including tyrosinase, phosphopantetheinyl transferase, lysyl oxidase, plasma amine oxidase, and phosphatases[67].

2.4 Ionic cross-linking

Ionic cross-linking occurs when a water soluble and ionizable polymer crosslinks with a soluble di- or tri-valent ion of opposite charge. The most common example utilized for in situ gelling applications is calcium-crosslinked alginate, which utilizes the divalent cation to

electrostatically interact with the anionic polycarboxylates to form a crosslinked network. Ionic crosslinking has the advantage over other noncovalent chemistries in that ionic bonds are relatively strong and thus fewer crosslinks are required to generate functional gels.

Some other examples of ionic crosslinked hydrogels are chitosan-polylysine, chitosan-glycerol phosphate salt and chitosan-alginate hydrogels[68-70].

3. Classes of hydrogel

Over the past 20 years, a variety of natural and synthetic materials have been utilized to prepare hydrogels for TERM. Natural hydrogels using biological polymers as building blocks have been widely used because of their inherent excellent biocompatibility, low toxicity and susceptibility to enzymatic degradation. However, synthetic hydrogels which lack biologic stimuli often require modification to introduce chemical and physical signals for instructive cell and tissue responses.

3.1 Natural hydrogels

The 2 main classes of natural materials used in hydrogel preparation are polysaccharides and fibrous proteins. Polysaccharides and fibrous structural proteins are components of extracellular matrix (ECM), which provides chemical, physical and biological signals to support cell responses including adhesion, differentiation and proliferation. For TERM applications, the most prolific natural materials to prepare hydrogels are chitosan, alginate, hyaluronic acid and collagen. Other natural polymers such as agarose[27-28], chondroitin sulfate [71-72], dextran[21, 73-74], fibrin[56-58, 75,76], matrigel[77-78] and silk[79-80] have also been applied to generate gels.

Chitosan is a linear polysaccharide which is a copolymer of glucosmine and N-acetylglucosamine derived from natural chitin by partial deacetylation. Chitosan is non-toxic, stable, biodegradable, and can be sterilized. These properties make chitosan a very versatile material with extensive applications in biomedicine and biotechnology. However, unmodified chitosan is insoluble at neutral pH due to its strong intermolecular hydrogen bonds, which limit its applications as an injectable hydrogel. Water-soluble chitosan derivatives can be prepared after formation of carboxylate salts, such as formate, acetate, lactate, malate, citrate, glyoxylate, pyruvate and glycolate[81]. Water-soluble chitosan derivatives have been used for

drug delivery and as cell carriers through glutaraldehyde crosslinking, UV irradiation, and thermal variations[12, 68-70, 82].

Alginate is a hydrophilic unbranched polysaccharide composed of (1−4′)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) moieties in varying composition, which is derived primarily from brown seaweed and bacteria. Simple gelation can be performed through G-rich blocks of the polymer binding to divalent or trivalent cations such as Ca^{2+} or Mg^{2+} to form ionic bridges[83]. The mild method of preparation makes these gels very suitable for encapsulation of cells and for controlled release of proteins and peptides.

Hyaluronic acid (HA) is a non-sulfated linear glycosaminoglycan (GAG) composed of repeating disaccharide units of D-glucuronic acid and N-acetylglucosamine, which is ubiquitous in cells and serum. Hyaluronic acid can be modified to form a hydrogel by photopolymerization[47], enzyme catalyzed crosslinking using an oxidation reaction through HA−tyramine conjugates[60], disulfide bond formation between HA-SH[10, 84], and Michael addition[48-49]. HA gels are incredibly attractive to TERM due to their intrinsic biocompatibility.

Collagen is the most abundant protein in the human body [ref]. The basic structure of collagen is 3 polypeptide chains, which wrap around one another to form a 3-stranded rope structure. Gelatin is formed by partial hydrolysis of collagen, breaking the natural triple helix into single-strand molecules. Collagen and gelatin have been investigated extensively as biomaterials due to advantageous properties which include high tensile strength, excellent biocompatibility and biodegradability. Collagen and gelatin hydrogels are mostly cross-linked using gluteraldehyde, genipin or water-soluble carbodiimides[85-87]. The hydrogels can also be formed non-covalently; cross-linking fibrillar collagen by entanglements of collagen fibres.

Recently, ECM hydrogels have become increasingly popular[88-90]. ECM hydrogels formed by partial digestion, solubilisation and polymerization in situ of decellularized tissues may retain some of the biologic activity of intact ECM. Thus, ECM hydrogels have complex biochemical compositions containing fibrous proteins (e.g. collagen, fibrin, elastin), proteoglycans (PGs), and glycosaminoglycans (GAGs) that more closely mimic a native tissue 3D environment vs. single component materials.

3.2 Synthetic hydrogels

Compared to natural polymers, synthetic materials have the advantage of more controllable and reproducible chemical and physical properties allowing preparation of a broader range of materials with specific properties. However, cell/material interaction and biocompatibility has to be taken into account throughout the design of these materials. One approach to improve cell-materials interactions of synthetic hydrogels is functionalization of the hydrogels with peptides or polysaccharides which specifically bind to cells by ligand-receptor interactions. The most widely used synthetic materials for hydrogel preparation are poly(ethylene glycol) (PEG), poly (NIPAAM)[13-21], poly(vinyl alcohol) (PVA), poly(propylene fumarate) (PPF), and poly(hydroxyethyl methacrylate) (PHEMA).

PEG, otherwise known as poly(oxyethylene) or poly(ethylene oxide) (PEO) is one of the most widely investigated systems. Different modification and copolymerization approaches have been explored to develop a variety of PEG hydrogels. For example, photocrosslinked PEG hydrogels have been synthesised by modifying each end of the polymer with either acrylates or methacrylates[39–41, 48] and temperature-responsive biodegradable PEG hydrogels have been obtained via copolymerization with degradable polymers such as poly(lactic acid) (PLA) and poly(lactide-co-glycolide) (PLGA)[23-26, 91]. Furthermore, enzyme-mediated crosslinked PEG hydrogels have been prepared by grafting enzyme sensitive molecules such as tyramine into the polymer[66, 92]. These PEG-based hydrogels are characterized by excellent biocompatibility, lack of toxicity and ease of processing, which makes them one of most popular choices for TERM hydrogels.

Similar to PEG, PVA is another synthetic hydrophilic polymer that has been widely developed for injectable hydrogels. PVA hydrogels can be formed by chemical crosslinking via γ-ray, e-beam irradiation or glutaraldehyde (GA) and by physical crosslinking via freezing-thawing cycles and self-assembly[93-95]. Similar to PEG, PVA does not support cell spreading and adhesion, but can be modified by conjugation with biological factors[96].

PPF is a novel degradable linear polyester based on fumaric acid which is a natural product of mammalian cell metabolism. PPF can form hydrogels when synthesized as a block copolymer with PEG and crosslinked either chemically or by UV exposure[97-98]. The injectability and biodegradation of fumarate-based polymers, coupled with the ease with which they can be modified, uniquely position fumarate-based macromers as excellent hydrogels for TERM[99].

Poly (hydroxyethyl methacrylate) (PHEMA) which has been use to produce contact lenses since 1955 is one of the most well-known and widely applied hydrogel biomaterials. In recent years, PHEMA has also been prepared as photocrosslinked hydrogels by using 2-hydroxyethyl methacrylate (HEMA) as monomer, polyethylene glycol dimethacrylate (PEGDM) as a crosslinking agent and benzoin isobutyle ether (BIE) as the UV-sensitive initiator. PHEMA hydrogels have been used for neural and cartilage tissue engineering[93, 100].

4. Application of hydrogels in TERM

Hydrogels present a fantastic regenerative medical tool, as stand-alone tissue scaffolds or vehicles to deliver drugs, growth factors or cell therapies, the broad cytocompatibility, ease of delivery and tunability to a 3D form mimetic of seemingly any tissue in the an adult organism means that both synthetic and biologic hydrogels are the subject of huge biomedical research interest[212, 215].

The list of tissues that hydrogels have been suggested for augmentation appears almost limitless including retina[101], ligament[102], adipose[103], kidney[104], muscle[105] and blood vessels[106-107]. However, for the purpose of this review we have selected to concisely consider the state of the art in 5 areas of hydrogel based tissue engineering and regenerative medicine which highlight the versatility of this class of materials; cardiac, neural, intervertebral disc, bone and cartilage.

4.1 Cardiovascular

Engineering of cardiac tissue is particularly pertinent to repair and regenerate damage which occurs as a consequence of myocardial ischemia. The intricate chemical engineering of a bespoke hydrogel, tailored to suit the physiology of a specific tissue has been elegantly demonstrated in the instance of ischemic myocardium by Garbern *et al*. In this study Garbern and co-workers designed and validated a poly(N-isopropylacrylamide-co-propylacrylic acid-co-butyl acrylate) hydrogel for which the gellation stimuli was the suppressed physiological pH (pH6.8) associated with ischemic tissue. The purpose of this hydrogel was a topical controlled release vehicle for bFGF to encourage the revascularisation of ischemic myocardium. The group confirmed by injection of gel or saline containing bFGF into cardiac tissue that the gel was superior at localising bFGF than the saline equivalent. Using western blot analysis it was demonstrated that bFGF remained at the injection site for 7 days in conjunction with the hydrogel, bFGF in a saline control however was not recoverable after

this time. This was presented this as western blot band density relative to a T_0 bFGF saline control. They reported band densities of 21%, 13% and 3% for bFGF in hydrogel after day 1, 2 and 7 respectively, vs. 2%, 1% and no bFGF recoverable for days 1, 2 and 7 in the saline control[108]. An additional study which utilised a rat infarct model delivered the hormone erythropoietin (EPO) via a biodegradable alpha-cyclodextrin/MPEG–PCL–MPEG by intramyocardial injection to locally exploit the anti-apoptotic and pro-angiogenic properties of EPO to improve function of infarct tissue. This study interestingly demonstrated a significant increase in stem cell (CD34+) homing towards the EPO containing gel compared to gel or EPO in isolation. This trend was also mirrored for vascular density, both around the infarct area and within the infarct itself, with greater vessel formation reported in EPO containing gels vs. gel or EPO alone[109].

Outside of infarct/ischemic cardiac tissues hydrogels have also shown promise in the regeneration or support of existing sub-optimal tissues associated with chronic heart failure (CHF). In one study an alginate hydrogel was used to restore appropriate geometry to the left ventricular wall in a canine model of CHF, improving cardiac function by significantly increasing ejection fractions ($26 \pm 0.4\%$ at Pre-implant to $31 \pm 0.4\%$ at Post-implant; $p < 0.05$) vs. saline controls which reported a decrease in ejection fraction ($27 \pm 0.3\%$ at Pre-implant to $24 \pm 1.3\%$ at Post implant; $p < 0.05$)[110]. A further similar study suggested the use of a non-degrading acellular PEG hydrogel delivered into myocardium to inhibit suboptimal auto-remodelling of cardiac tissues post infarct and maintain more efficient cardiac geometry by retarding post infarct left ventricular dilation. Of note this study reported 33.3% and 43.3% reductions in end-diastolic diameter increase at 2 and 4 weeks respectively[111]. Hydrogels which release growth factors may also support cardiac remodelling post infarct, particularly VEGF due to its role in angiogenesis and subsequent revascularisation of compromised tissue[112-113]. One such study reported particularly interesting findings by intracardiac injection of a temperature-sensitive, aliphatic polyester hydrogel mixed with / or conjugated to VEGF (40ng/rat). The researchers showed that the material conjugated to VEGF supported improvements in several physiological features of cardiac functional preservation above that of the mixed equivalent, most noteworthy being a statistically significant increase in ejection fraction in the hydrogel without VEGF after 35 days. The group also report increases in scar thickness with accompanying decreases in scar size in the conjugated variant, suggesting this process and material as an ideal for maintaining cardiac tissue stability post infarct[112]. Other biologic factors have been investigated in conjunction

with hydrogels for their ability to improve cardiac function, engraftment of exogenous cells or angiogenesis including bFGF/gelatine, thymosin ß4/PEG and ephrin-B2/fibrin gels[114-116]. In the bFGF gelatine study, the researchers showed that inclusion of bFGF loaded gel particles into a porcine infact model significantly enhanced myocardial perfusion (89.7 +/- 5.9% vs. 26.3 +/- 0.6%) and left ventricular ejection fraction s (37.1 +/- 4.2% vs. 31.8 +/- 4.7%) after 4 weeks vs. control injections of DMEM alone. This research also included the delivery of cells; both cardiosphere derived cells and mesenchymal stem cells. They fascinatingly reported that although the gels improved retention of cells at the infarct site, the delivery of stem cells had no additive impact on cardiac recovery over delivery of the gel alone. However delivery of the cardiosphere derived cells resulted in further increases in left ventricular ejection fraction and decreases in infarct size[114].

A study performed by Mooney *et al*. considered the use of adult stem cells to repair infarct tissue in a rat model of myocardial ischemia. This study utilised a range of organic hydrogels and posed the question of deducing if differences in carrier vehicle influence the ability of the cells to be retained at the site of injury using in tissue monitoring of fluorescence in conjunction with DiD/GFP labelled stem cells. The study presented the cells to the infarct area in 2 formats, either as an onlay patch (collagen or alginate), or as a hydrogel injection (alginate or chitosan/β-glycerophosphate). The group demonstrated that over 24 hours all materials supported a significantly higher amount of cell retention compared to a saline injection, although there was no significant difference between the retention observed across the materials either hydrogels or patches, with all stem cell interventions retaining 50-62% percent of cells, compared to 9% for the saline control injection[210].

It has also been suggested that outcomes of hydrogel based cardiac tissue engineering may vary as a function of delivery time post insult, with early/immediate intervention potentially not being the best solution. This study utilised a PEG hydrogel and showed that delivering the gel either immediately or 1 week post infarct had radically different outcomes, with only the 1 week group demonstrating improvements in cardiac functionality using echocardiographic analyses. Interestingly, the study also showed that the distribution of the biomaterial in the cardiac tissue differed as a function of delivery time post infarct, with the 1 week injection forming a fibrilar structure which degraded rapidly and could not be recovered after 4 weeks. On the other hand, when implanted 1 week post infarct, the material formed a more bulky structure which was easily recoverable after the same length of time[117].

Hydrogels are also under investigation for the purpose of cardiomyocyte delivery into infarct tissue. One such study has suggested that conjugation of a peptide fragment derived from angioprotein-1 (QHREDGS) into a bio-organic chitosan-collagen hydrogel supports survival of cardiomyocyte cells both *in vitro* and *in vivo.* When the concentration of peptide modified chitosan was increased from 0-7.5mg/ml a concurrent increase in cardiomyocyte metabolic activity was observed *in vitro* using the XTT method. The highest concentration of QHREDGS also supported the greatest degree of cardiac functionality by generating the largest number of beating constructs *in vitro*[118]. A further study proposes the use of chitosan for an alternative reason; it's ability to scavenge reactive oxygen species, an incredibly destructive family of molecules present in ischemic tissue. This study showed that when treated with 30μm H_2O_2 adipose stem cell adhesion *in vitro* was reduced to 63.6 +/- 1.37%, however when treated concurrently with chitosan this could be recovered to ~90%. The study elegantly demonstrated that this restoration in adhesion was a concequence of chitosan reversing the down regulations to integrins β1 and αV and their intercellular signalling moieties FAK and Src induced by H_2O_2[119].

A study using adult stem cells for cardiac tissue engineering in combination with a synthetic HEMA-PTMC hydrogel elegantly suggested the 'modulus specific' differentiation of adult stem cells. This research used the gel as a 3D culture matrix to differentiate adult stem cells into cardiomyocytes based on engineering the material to present similar mechanical properties to endogenous myocardial tissue[120]. The study considered gels of three moduli; 16kPa, 45kPa and 65kPa and demonstrated that although modifying the modulus did not alter stem cell survival (quantified by numeration of total dsDNA), which the authors suggested was as a result of the cells becoming non-proliferative in favour of entering a differentiation state when in the gels. The research showed that cardiac differentiation was highly modulus specific, considering a number of cardiac genes using real time PCR. Most noteworthy was CACNA1c, a marker of calcium channels, which was hugely upregulated in the 65kPa gels whilst considerably less remarkable slight upregulations where observed in the lower modulus materials.

Embryonic stem cells have also been suggested as candidate cells for cardiac tissue generation. Embryonic stem cell derived cardiomyocytes have been used in conjunction with a micropatterned fibrin gel to generate organised, functional cardiac patches. This study also elegantly demonstrates the supportive role of non-cardiac cells in myocardial development by generating patches derived from embryonic stem cell derived cardiovascular progenitor cells

and fibroblasts. The authors demonstrated that these cells formed a synergistic role in tissue organisation and functionalisation and suggested that this was due to the fibroblast remodelling of the fibrin matrix which then allowed the cardiomyocytes to spread and functionlaise[121]. Outside of specific cardiac tissue engineering, hydrogels have been suggested as drug eluting vehicles to combat antiarrhythmias demonstrated using amiodarone in a PEG hydrogel administered in an epicardiac location[122].

Although for the purpose of this review integrated within the broad heading of cardiovascular applications, the propensity of a hydrogel to support the in growth and function of endothelial cells towards an effective mass transport network is over-arching in its importance in any engineered tissue. Despite their prevalence throughout the body endothelial cells have demonstrated specific preferences in hydrogel substrates based on chemical and physiomechanical properties. In one particularly elegant study Werner and co-workers compared the effectiveness of relatively complex hydrogels in which heparin was linked to star PEG via an MMP cleavage peptide using either an amide or ester linkage. The study showed that the ester variant was more rapidly degraded and this subsequently supported faster and deeper endothelial cell penetration. Even when VEGF was included in the gels, the amide linked material did not record any significant change in cell in growth after 24 hours (~12μm +/-VEGF), in comparison the ester linked gel showed deeper cell penetration during this time to a depth of ~20μm, which was accentuated further (~30μm) by inclusion of VEGF. From this the authors suggested that the early degradation was critical in permitting cell migration. Furthermore this study also showed that modifying gel stiffness from 3.5 to 7.5kPa modulated cell infiltration, with the more stiff gel supporting less cell ingrowth in the ester linked gel. Modifying the stiffness of the amide gel did not appear to alter its cellular behaviour over the initial 24 hours presented. These *in vitro* results mirrored data obtained from the materials in a CAM assay, with the ester gel demonstrating substantially greater vascular attraction than the amide equivalent which in both instances was enhanced by the inclusion of VEGF[123].

4.2 Intervertebral Disc

Engineering of intervertebral discs (IVD) presents a further soft tissue niche which the tuneable physical properties of hydrogels make them an ideal candidate material. The most pressing requirement for a disc regeneration strategy is in response to degenerative disc disease; the intrinsic degeneration of intervertebral discs as we age which may severely

compromise quality of life through compromised biomechanics. A hydrogel disc prosthesis would also prove invaluable in acute, traumatic injuries in which collateral damage to a native disc has impaired its function. Typical hydrogel strategies involve replacement or supplementation/reinforcement of nucleus pulposus tissue, the shock absorbent material in the core of the IVD. In addition to the mechanical properties of hydrogels which are easily tuneable to mimic soft tissues, their in situ swelling to fill a void space also presents them as ideal IVD prosthetics with the ability to be implanted either in a dry state or small volume and subsequently swell to fill any available space within the degenerated nucleus pulposus [124].

Many hydrogels are under investigation in this area using both *in vivo*[125] and *in vitro* strategies including poly-N-acetyl glucosamine which has demonstrated *in vitro* to possess similar mechanical properties to human nucleus pulposus tissue whist maintaining the phenotype of primary human disc cells[126]. The study considered two variants of poly-N-acetyl glucosamine in which poly-N-acetyl glucosamine nanofibres used to synthesise the hydrogels were shortened to 5-7μm or their native length of 80μm. Using MTT the group demonstrated that the native nanofibre structure was superior at maintaining cellular metabolic activity, whilst alcian blue staining for proteoglycans showed that this gel was also more appropriate for supporting the phenotype of the human disc cells. An interesting peptide derived hydrogel KLD-12 (AcN-KLDLKLDLKLDL-CNH_2) has also demonstrated an ability to support the viability and phenotype of rabbit nucleus pulposus cells[127]. The study confirmed using AFM that this peptide has the ability to self assemble into a hydrogel forming nanobibres 10-30μm in diameter. The group demonstrated statistically significant increases in disk cell proliferation when cultured with hydrogels containing this peptide with 93% cellular survival after 7 and 14 days culture and excellent expression of type II collagen. Chitosan hydrogels have shown promise in IVD tissue engineering due to their capacity to support the growth of appropriate cell types and possess disc mimetic biomechanics[128-129]. One of these studies considered the variable of chitosan molecular weight in addition to degree of hydroxybutyl substitution within the chitosan molecule. They considered 3 variables; (expressed as molecular weight and degree of substitution) 530,000+/-122,000 and 1.33+/-0.04, 417,000+/-11,000 and 1.75+/-0.02 and lastly 931,000+/-20,000 and 1.96+/-0.01. The research showed that chitosan variables within this range did not influence the viability of annulus fibrosis, stem or nucleus pulposus cells and claimed that the relative biological inertness of these gels would make them ideal candidates to add further

biomacromolecules to influence the phenotype of the cells which they contain[128]. One study suggested that whilst bovine derived nucleus pulposus cells generally survived in chitosan gels the survival of annulus fibrosus cells, derived from the outer peripheral tissue of the intervertebral disc was much more variable as a function of hydrogel fabrication and mechanical parameters[130]. This study showed that under 1% or 1.5% chitosan nucleus pulposus cells survived and produced proteoglycan to around 8-12% of that found in normal IVD tissue which was not reflective of chitosan concentration. The researchers reported that contrary to this under the same conditions annulus fibrosus cells did not survive. Interestingly the group then modified the mechanical properties of the gel by adding hydroxyethyl cellulose to make the material more malleable. Initially this yielded data similar to their first experiments in which nucleus pulposus cells flourished and annulus fibrosus cells died. However they remarked that when a second batch of hydroxyethyl cellulose was trialled, the annulus fibrosus cells also appeared to survive, and when stimulated with TGFβ, also produced proteoglycan. The group noted that this highlights the temperamental nature of annulus fibrosus cells and suggested that with appropriate stimulation to they have the propensity to behave in a similar manner to nucleus pulposus cells.

The influence of fabrication variables on cell phenotype has also been investigated using nucleus pulposus cells and alginate hydrogels which showed that cell phenotype was insensitive to rising concentrations of alginate and the associated modifications to gel physiomechanical properties[131]. This research considered 2%, 4% and 6% concentrations of alginate and reported that cells derived from nucleus pulposus, annulus fibrosus or articular cartilage did not modify their expression of aggrecan or collagens type IIa1 or type Ia1 as a function of alginate concentration. Ionic versus photo cross-linking of alginate (methylacrylated alginate in this instance) gels could also modify their suitability as a nucleus pulposus replacement[132]. The group reported that photo cross-linked alginate was more resistant to degradation and supported greater viability and matrix deposition from nucleus pulposus cells than their ionic counterparts.

Gels derived from type II collagen/hyaluronic acid blends have demonstrated potential as nucleus pulposus replacement prosthetics with mechanical and physical properties to support viability and gene expression of nucleus pulposus cells[133]. This study used a 4S-StarPEG linker with type II collagen and hyaluronic acid. The research considered 4 different ratios of type II collagen:hyaluronic acid; 9:0, 9:1, 9:4.5 and 9:9 and demonstrated no significant difference in their ability to support the viability of adipose derived stem cells over 1,3,7 or

14 days, ranging from 89.33%+/-5.84 to 98.08%+/-0.99 independent of gel composition. The authors also used microscopy to quantify cell distribution throughout the gels and also reported no significant difference in distribution as a function of gel type. This study also reported no change in the expression of type II or I collagen or aggrecan throughout the materials and noted that cell proliferation in these materials was rare, and only observed in the material without hyaluronic acid.

Addition of adipic acid dihydrazide or polyglycolic acid may also enhance hyaluronic acid gels for nucleus pulposus tissue engineering[134-135]. One study considered a 6% solution of hyaluronic acid and varied the adipic acid dihydrazide using 2%, 4% and 8% to elucidate the influence on nucleus pulposus cells. After 3 days the study demonstrated that there were no notable differences in the numbers of live or dead cells determined by fluorescence live/dead staining between the 4% and 8% materials, however a notable number of dead cells where apparent in the 2% material. This was also supported by an LDH assay which demonstrated a significant increase in cytotoxicity of the 2% material vs. the higher concentrations. The authors suggested that the increase in cytotoxicity in the lowest concentration material may in part be due to the presence of unreacted aldehyde groups which may interact unfavourably with the nucleus pulposus cells[134].

Synthetic and composite materials too have been evaluated as nucleus pulposus replacement prostheses including PVA/PVP, tween/NVP/cellulose and poly(N-isopropyl acrylamide) hydrogels which have been reported to have ideal rheometric properties to replace nucleus pulposus tissue[136-138]. Also particularly beneficial in this area are radiopaque hydrogels which allow real-time follow up of the material using x-ray without the further inclusion of x-ray markers[139]. The study used IEMA co-polymerised with either N-vinyl-2-pyrrolidinone or 2-hydroxyethyl methacrylate which was rendered radiopaque based on the presence of iodine in IEMA. The group considered co-polymers of IEMA: NVP or HEMA at concentrations of 95:5, 90:10, 85:15 and 80:20 and concluded that modulating the concentration of NVP did not influence cell viability, however decreasing the concentration of HEMA, decreased cell viability.

Further to cell delivery and biomechanical support, hydrogels are also being investigated as drug delivery vehicles to promote autologous repair and retard disc degeneration including a chitosan/gelatine/glycerol phosphate hydrogel to deliver the anti-oxidant ferulic acid to quench reactive oxygen species within the damaged disc which cause apoptosis of native

nucleus pulposus cells[140]. The research considered ferulic acid concentrations between 0.5-500μM and showed that all of these concentrations were capable of successfully scavenging the ROS produced by 100μM H_2O_2 using luminol as a measure of available reactive oxygen species to a similar extent, and later showed that the presence of ferulic acid in the hydrogel improved the phenotype retention of nucleus pulposus cells in the presence of H_2O_2 based on gene expression analysis, particularly type II collagen.

Outside of nucleus pulposus regeneration hydrogels such as crosslinked collagen are also explored to augment the annulus fibrosus as 'plugs' to prevent seepage of nucleus pulposus tissue or escape of implanted cells through the injection port post cell therapy[141]. This study included a particularly elegant experiment in which *ex vivo* rabbit IVDs where injected with fluorescently labelled collagen microspheres which were also loaded with mesenchymal stem cells and sealed with the photochemically cross-linked collagen annulus plug. The discs where subject to 7 days of continuous mechanical loading and after 7 days the authors reported that in the plugged disks 39.3% of the collagen microspheres where retained in the nucleus pulposus area vs. 23.4% in the unplugged control.

4.3 Neural

Tissue engineering and cell therapy approaches for neural repair is an incredibly prolific area of research. Damage, either acute (e.g. traumatic injuries, stroke) or chronic (e.g. Parkinsonism, multiple sclerosis) to central nervous tissues can have dire, debilitating consequences in a group of tissues with a very limited endogenous repair capacity[142]. Therefore the ability to supplement and assist this regeneration with an exogenous material and/or cells would present a seminal breakthrough healthcare technology.

Much like the other soft tissues discussed, hydrogels present themselves as ideal candidates for neural regeneration. This in part is due to their ability to be fabricated with rheometric properties similar to endogenous tissue and assembly criteria which permit precise and controlled localisation by injection in a liquid phase. Indeed it has been evidenced using alginate gels with mechanical properties akin to native brain tissue to stimulate appropriate neuronal gene expression from neural stem cells[143]. This study considered alginate gels of 4 different elastic moduli; 183Pa, 1028Pa, 1735Pa and 19700Pa, generated by manipulating the ratio of alginate and calcium chloride in the gelling mixture. The authors reported that the 183Pa gels supported greater cell proliferation, and neuronal gene expression evidenced by immunohistochemical and PCR characterisation of nestin and β-tubulin III. The study reports

that this was predicted as the lower modulus gel is in the same range as native brain tissue. Simiarly a further study used agarose/chitosan/methylcellulose/dextran blended gels and varied the polymer composition across a number of experimental materials to demsontrate that small changes in physical properties brought about by changing gel composition (polysaccharide ratio) has the ability to modify neural cell interaction with the material[144]. The group considered this by measuring cortical neuron attachment to the materials. They remaked that the more positively charged the surface of the material became, the more supportive it was of cell attachment.

On this theme, gelatine/hydroxylphenylpropionic acid is a promising hydrogel for use in brain tissue engineering as its mechanical properties can be easily tailored by modifying the reaction conditions of an enzyme mediated cross-linking reaction, allowing tuning to a rheometry similar to brain or other CNS tissue. In addition to supporting neuronal differentiation these materials also endogenously protect encapsulated cells from the influence of oxidative stress, which is often considerable in damaged, diseased or healing tissues[145]. In this study the researchers included HRP and H_2O_2 into the hydrogels to control both the gellation rate and degree of cross-linking respectively, enabling them creation of hydrogels with higher levels of cross linking by increasing the H_2O_2 concentration in the gellation reaction. The group considered 4 concentrations of H_2O_2; 0.85, 1.0, 1.2 or 1.7mM and remarked that including this oxidant in the gel did not significantly reduce the viability of neural stem cells, with live cells remaining above 90% in all incidences. The publication explores this further by challenging the encapsulated cells with exogenous H_2O_2 to model oxidative stress and reports that cells were considerably more resistant to the cytotixic effects of H_2O_2 in the gelatine/hydroxylphenylpropionic acid hydrogels than two control gels; collagen and alginate. This is best evidenced considering the highest concentration of H_2O_2 trialled by the researchers; 500μM in which viability was ~84% in the gelatine/hydroxylphenylpropionic acid hydrogels as opposed to ~8% and ~15% in collagen and alginate hydrogels respectively.

From the point of view of brain tissue regeneration, a particularly elegant study has explored the use of a self-assembling peptide gel $RADA_{16}$ (AcN-RADARADARADARADA-$CONH_2$) combined with a short IKVAV motif (AcN-RADARADARADARADAIKVAV-$CONH_2$) derived from laminin to regenerate brain tissue in a rat model of brain injury in which a 2mm wide/2mm deep defect was created with a punch at a distance of 2 mm to the right of the bregma. This hydrogel, particularly when seeded with neural stem cells caused significant

regeneration of brain tissue after 6 weeks vs. a saline control by supporting the survival and localisation of neural stem cells. Histopathology these cell loaded gels resulted in beautiful tissue engineering, with biomaterial resorbtion alongside simultaneous cell proliferation and matrix secretion to fill the defect cavity after 6 weeks *in vivo*[146]. Chitosan has also been explored for its ability to facilitate CNS tissue engineering; the combination of glycerophosphate with chitosan confers a temperature dependant gellation at 37^{o}C. These gels were shown to maintain murine fetal cortical cells, and that cell survival could be increased by covalent attachment of polylysine to the chitosan via azidoaniline photocoupling[147].

Drug or growth factor containing/eluting hydrogels have shown promise in the area of CNS regeneration. Epidermal growth factor (EGF) is a mitogen for neural stem cells. One study modified this growth factor to incorporate a collagen binding domain, allowing the molecule to be bound within a collagen hydrogel. The group utilised an interesting approach to localising these two molecules using recombinant DNA expression in *E.coli* to produce a fusion protein of EGF and a collagen binding polypeptide, when mixed with a collagen gel this protein therefore intimately associates with the hydrogel matrix. It was clear that this molecule provided advantageous cellular adhesion, spreading and proliferation over collagen alone, demonstrating double the amount of live cells on surfaces decorated with the fusion protein and collagen vs. either of the molecules in isolation which increased in a concentration dependant manner. The authors also report that after this time 98% of neuropshere derived cells cultured on this material were positive for the neuronal stem cell marker nestin, which was also largely absent when either collagen or EGF were used in isolation[148].

Growth factor containing hydrogels are also under investigation for acute spinal cord repair. One such study explored the use of recombinant PDGF containing hyaluronic acid/methyl cellulose (HA/MC) blended hydrogels to support oligodendrocyte differentiation of neural stem/progenitor cells. The study reported that the percentage of live cells after 7 days of culture was significantly higher in HA/MC alone (60+/-26%) or HA/MC/PDGF (79+/-22%) than without the HAMC hydrogel (7+/-2%). *In vivo* the group created a surgical spinal defect in a rat model which was then revisited after 9 days and repaired using combinations of cell loaded gels and control injections. After 9 weeks *in vivo* the study reported that implantation of neural stem cells with HA/MC/PDGF gel significantly decreased the size of the defect

cavity vs. delivery of cells solely with media. Neuron number marginal to these explants was also quanitified based on the $NeuN^+$ marker and similarly, the gel implants demonstrated a significantly greater neuron number compared to a media equivalent. Interestingly the group also reported, using the macrophage marker ED1, that the control group and the gel implants where both equally as immunoreactive. This study also considered functional indicators of CNS improvement one of which scored locomotor improvement based on the number of footfalls required to cross a ladder, with greater footfalls indicating a dragging limb and subsequently being considered as a marker of poor CNS regeneration. Using this technique it was shown that the cell loaded HA/MC/PDGF sinnificantly improved CNS locomotor activity after 7 weeks *in vivo*[149]. Brain derived neurotrophic factor (BDNF) has also been investigated for its ability to support neural cell phenotype and viability when bound to a hyaluronic acid hydrogel using an approach which genetically modified BNDF to contain a hexahistidine tag which then allowed binding to the hydrogel via metal ion (Zn^{2+}) chellation improving viability and neural phenotype above an unmodified hyaluronic acid hydrogel. This study showed significant increases in neural cell viability on the BDNF containing hydrogel compared to a control substrate and also noted that BNDF could be used to increase the βIII-tubulin expression of these cells in a dose dependant manner (50-100ng/ml range)[150]. Another potential means of conjugating cytokines to growth factors involves biotinylating the factor and then binding it into the hydrogel matrix via streptavidin which is associated with the gel base polymer. This has been tested using photo crosslinkable thiolated methacrylamide/chitosan hydrogels to which were tethered both streptavidin and a short RGD sequence via malemide conjugation. This allowed recombinant biotinylated interferon gamma to bind, yielding significant upregulation of neuronal gene expression compared to a control surface and in some instances soluble vs. immobilised IFN. In the instance of both RIP and nestin, significant increases at protein level where reported in the soluble IFN samples (RIP26.6%+/Nestin30.8%+) compared to the control surface (RIP6%+/Nestin19.6%+) and immobilised IFN (RIP13.7%+/Nestin17.8%+). Interestingly this was not the case for βIII-tubulin which reported statistically similar increases in expression vs. the control (9.4%+) for both the immobilised (72.1%+) and (71.8%+) soluble IFN[151].

The ability of hydrogels to elute bio-molecules may also be exploited in the absence of cells. One such study utilised a hyaluronic acid hydrogel to couple an antibody against the Nogo-66 receptor, an important receptor in inhibition of nervous system repair. The antibody was

coupled to the hyaluronic acid backbone via a hydrazone linkage and subsequently eluted and delivered focally throughout the degradation profile of the hydrogel in a pH dependant manner. This study reported interestingly, that the release kinetics of the antibody where not specifically related to the biodegradation rate of the hydrogel but more associated with the acid stability of the linker. At physiological pH (7.4) the release kinetics of the antibody where considerably slower (400 hours) compared to more acidic pH values; 5 and 6 in which the antibody was released over a much shorter time, 8 and 70 hours respectively[152].

Acellular gels have also been suggested as scaffolds to support autologous neural tissue regeneration. As evidenced in a study which used HPMA hydrogels (NeuroGel^{TM}) covalently grafted with the cell adhesive motif RGD, which facilitated tissue regrowth and some regain of CNS functionality in a rat spinal cord segment excision model. Although this study did not specifically quantify return no normalcy after spinal cord excision the authors confirmed that axons did migrate into the material from the spinal stumps at either side of the defect and also commented that the rats whom had undergone the reparative procedure did regain some functional motility of the hind limbs throughout the course of the study period which was absence in animals which had received the transect without any hydrogel based repair[153].

Further to regenerative medicine, hydrogels are also being explored as coatings for permanently implanted central nervous system electro stimulatory devices. Currently, implanted electrodes succumb to wear which is in part is attributed to adsorption of serum proteins onto their surface which triggers an inflammatory reaction against the device. Hydrogel coatings to these devices may prevent surface protein fouling, thus minimising host vs. implant type responses whilst also providing a more stable tissue/implant interface by presenting resident cells with a layer of material into which they can infiltrate. A number of hydrogels are being explored for this purpose including PEG/PU, alginate, PVA/PAA and PEDGA[154-169].

PEG/PU was considered as a coating for neurostimulatory electrodes with the hypothesis that it may retard or cease the degradation and tissue induced wear by interrogation of the device by inflammatory cells by virtue of a foreign body response and a result of physical recruitment by the tissue damage associated by the device placement process. The authors constructed model electrode systems based on PDMS rods which were implanted into a rat brain model. *In vitro*, the group demonstrated that PU supported attachment and growth of a

significantly higher population of neurite cells compared to PDMS alone. Furthermore the length of these neutrites was also statistically higher in PU as opposed to PDMS. Using immunohistochemistry the group reported *in vivo* that cells surrounding the PU coated implants displayed less GFAP staining and more NeuN staining than PDMS alone, demonstrating that the PU coating reduced glial scar formation and increased neuronal activity in the interfacial tissues surrounding the implants[154].

The inflammatory response towards an implanted electrode is clearly driven by protein adsorption onto its surface, therefore the ability of a coating to prevent this protein fouling of neurostimulatory electrodes would potentially increase their efficacy by increasing the clarity of their neurostimulatory potential whilst also minimising inflammatory cell recruitment to their surface. One study considered PDMS electrodes in this instance and reported that coating with blends of PVA and PVA/AA hydrogels had the ability to significantly reduce surface protein fouling. PDMS and PDMS with PVA and PVA/AA coatings where incubated for 3 hours with a 1mg/ml solution of I^{125} labelled fibrinogen, and the concentration of protein on their surface calculated using radioactivity measurements. The study reported that PDMS alone adsorbed 586ng/cm protein onto their surface whereas the PVA and PVA/AA coated materials adsorbed only 92ng/cm and 84ng/cm respectively. Based on image analysis of GFAP immunostaining, *in vivo* the PVA/AA coated electrodes also resulted in reduced glial scar formation compared to PDMS alone in a rat brain implantation model[156].

4.4 Bone

Although the physiomechanical properties of bone do not immediately appear to make engineering and reconstruction of this tissue favourable using hydrogels, given their pliability akin to much softer tissues, considerable research is being performed in the area of hydrogel mediated bone augmentation.

Calcium phosphate ceramics and their derivatives are perhaps the most exploited molecules in bone tissue engineering; therefore incorporation of calcium phosphate into hydrogels may present an ideal for engineering this tissue, providing cells with a calcified matrix whilst also conferring suitable mass transport and cell migration properties via the hydrogel component. One such study explored this using a hyaluronic acid-g-chitosan-g-poly (N-isopropylacrylamide) hydrogel containing irregularly shaped 0.5-1.0mm biphasic calcium phosphate microparticles (40 wt% hydroxyapatite and 60 wt% of β-tricalcium phosphate). The study reported that the calcium phosphate containing substrate supported better

osteogenic phenotype maintenance from human fetal osteoblast cells compared to the naked gel alone. This was qualified using fetal osteoblast cells and observing increased cell proliferation and osteoblastic phenotype maintenance considering ALP activity which in both instances was found to be significantly higher in BCP containing material vs. naked material at every culture period analysed (7, 14, 21 and 28 days). The group also report more appropriate expression of the osteoblastic genes, ALP, osteopontin and osteocalcin throughout the culture period. Cell loaded BCP gels also formed ectopic bone in a subcutaneous athymic mouse model, which after 3 months demonstrated visible osteoid formation with positive immunohistochemistry for the osteoblast lineage marker osteoclacin[160]. Regeneration may also occur in hydroxyapatite containing hydrogels implanted without a cellular component as has been shown in a rabbit calvarial defect using a hydrogel derived from PEG-PCL-PEG copolymer, collagen and nano-hydroxyapatite which reported excellent bone remodelling after 20 weeks. In this study the researchers created 2 10x5x2mm defects in the skull of New Zealand white rabbits one of which was filled with hydrogel, the other was left empty. The group analysed bone regrowth throughout the defect using μCT analysis and reported statistically higher bone volumes in the hydrogel treated vs. control groups at every time point analysed (4, 12 and 20 weeks). Within the treated defect the area of bone formed increased from 47.7+/- 8.6% to 82. 3+/- 4.7% between weeks 4 and 20 compared to 45.3 +/-12.5% to 71.6+/- 8.2% in the control group, allowing the authors to conclude that addition of the hydrogel improved bone remodelling beyond that achievable by the auto-regeneration process alone[161].

The manner that the calcium phosphate/hydroxyapatite is presented within the gel may also modify the osteogenic response even when co-administered with BMP-2. One such study considered 5 different ceramics; β-tricalcium phosphate (~45nm) and 4 hydroxyapatites; nanoHAP (~20nm), HAP (<50μm), clods of HAP (>100μm), and a biomimetic HAP called Ostim35® (~200nm). In a rat intramuscular model of ectopic bone formation the study reported that the group containing the nanoHAP with BMP-2 stimulated the formation of significantly greater density than the rest of the materials tested, although interestingly the materials all resulted in bone formation of a similar volume and mineral content[162].

The inclusion of phosphate alone into hydrogels has been investigated, in one such study oligo(polyethylene glycol) fumarate was phosphorylated using bis(2-

(methacryloyloxy)ethyl)phosphate (BP) resulting in materials which supported osteoblastic differentiation without addition of exogenous soluble factors. This study investigated the influence of increasing bis(2-(methacryloyloxy)ethyl)phosphate concentration (155, 310, 620 and 930μM) on osteoblast and MSC viability, proliferation and differentiation. Increasing BP concentration did not negatively impact cell viability, which remained comparable to a tissue culture plastic control throughout the culture period tested. Concurrently the general trend observed was that as BP concentration was increased, the proliferation and ALP activity of fetal osteoblast cells also increased. Proliferation of MSCs also was shown to increase as a function of BP concentration. The study interestingly reported that ALP activity of MSCs rose (almost doubled vs. control surfaces) when BP was present at the lowest concentration, and remained the same throughout the concentration gradient[163].

Further to calcium, an alternative cation, Zn^{2+} (0.01M), has been studied for its ability to maintain human osteoblast cells using chitosan/ß-glycerophosphate hydrogels as it is thought this ion plays a role in osteoblast mineralisation. In this study, culture media was 'conditioned' by incubating the gels in it for 24 or 72 hours, before removal and addition to cells to test the influence of degradation or elution products on cell viability. The authors considered various volumes of conditioned media (up to 100μl) and reported that no differences in cytotoxicity were quantifiable as a function of media volume or gel soaking time. The study interestingly reported that under normal media conditions the gel eluent did not stimulate osteogenic phenotype maintenance (qualified by alizarin red staining), however the eluent did act syntergistically with osteogenic media to improve osteoblast phenotype beyond that of the media alone[164].

Bone morphogenic protein-2 (BMP-2) is an incredibly prolific morphogenic factor in bone regenerative medicine. Bone formation has been demonstrated in a hyaluronic acid based hydrogel where the gel has been utilised as a carrier vehicle for BMP-2. In this study which used a rat calvarial defect model it was evidenced that the hydrogel alone supported limitted bone formation however inclusion of BMP-2 caused significant bone synthesis within the gel. This study also demonstrated the importance of endogenous environmental stimulation as gels implanted under the periosteum showed considerably enhanced bone deposition vs. an identical material implanted in a subcutaneous space. The study varied concentrations of BMP-2 in a 200μl injection; 5μg/ml and 150μg/ml alongside control gels in which no BMP-2

was included. The volume of bone formed increased as a function of BMP-2 concentration and anatomical location, with periostial implants forming significantly greater volumes of bone under the same hydrogel conditions as the subcutaneous equivilents[165].

A further study explored the inclusion of hMSCs in a hyaluronic acid hydrogel containing BMP-2, similarly this study reported neo-bone generation in the gel using a rat calvarial defect in acellular BMP-2 containing gels, which was increased further by the inclusion of MSCs[166]. This study reported poor viability for MSCs cultured for 3 days on the HA gel alone (72%) which could be increased to 81% by the addition of 500ng/construct BMP-2. The researchers placed the material into an 8mm rat clauvarial bone defect and demonstrated improvements in bone volume within the defect as a function of including both MSCs and BMP-2. Expressed as percentage of the defect occupied by bone, the HA gel alone supported 16%+/-2.3 bone remodelling, HA+BMP-2: 50.6%+/-9.5, HA+MSC: 60.8%+/-4.8 and HA+MSC+BMP-2: 84.3%+/-6.8, demonstrating beautiful synergy between material, growth factor and cells in supporting tissue regeneration.

A study utilising a hydrogel delivery vehicle for BMP-2 created a composite rat model of lower limb bone and vascular trauma in order to deduce the capacity of the growth factor laden gel to support bone regeneration in a realistic compound damage environment and to better understand the relationship between biomaterial induced bone remodelling and vascular biology. The group created an 8mm femoral bone defect in conjunction with ligation of the femoral artery and vein. The researchers repaired the bone defect using a perforated tubular electrospun nanofiber mesh with 150μL of rhBMP-2 (2μg or 0.5μg per defect).laden RGD functionalized alginate hydrogel injected into the defect. The group elegantly concluded that in the instances when concurrent limb ischemia was induced at the time of surgery, bone healing was superior to when no vascular insult was performed. In the instance of the ischemic group, they reported that bone regeneration both visually and mechanically were comparable between the two BMP-2 doses. Furthermore this study showed that in the loser dose BMP-2 groups, without concurrent vascular insult bone healing was inconsistent, with 50% of the animals demonstrating bony bridging of the defect, this however was upgraded to 100% when ischemia was present[211].

It has been suggested that enhancing the cell adhesion capacity of hyaluronic acid may improve its ability to support bone formation in the presence of BMP-2. This has been

investigated by covalent grafting of an integrin binding domain from fibronectin onto hyaluronic acid which showed excellent improvement of bone formation compared to unmodified hyaluronic acid. The group reported initially (after 4 hours) low adhesion of MSCs to the HA base material (56%) which became elevated to 79% on the peptide grafted HA, demonstrating an improvement in cell adhesion to HA as a result of including the FN fragment. *In vivo* the FN grafted gel formed twice the volume of ectopic bone as the base HA alone, quantified using μCT analysis after 7 weeks *in vivo*[167]. Several other hydrogels have also been investigated as BMP-2 carriers including PEGylated fibrinogen, silk fibroin and glycidyl methacrylated dextran/gelatin[168-170].

Particularly noteworthy was the study by the guldberg group which demonstrated a dose-response relationship of a silk fibroin hydrogel to support bone growth in a rat critical size defect with the addition of BMP-2. This was true of both a 1% and 2% silk fibroin gel, without the BMP-2 however neither gel was particularly successful at facilitating bone formation of a greater bone volume within the defect over a 1% equivalent. The study show a marginal increase in bone volume in the 2% vs. 1% fibroin gels with BMP-2 although the authors report that this increase was not found to be significant[169].

From the point of view of including exogenous molecules to enhance hydrogel mediated bone regeneration, one study reports the benefits of including a synthetic oxygen carrier; perfluorotributylamine, in a fibrin gel which may overcome the hypoxia often associated with the inner areas of tissue engineering scaffolds. In this study a modified MSC cell line was used which express recombinant BMP-2. Implant of these cells into a murine 2.5mm radial defect showed a PTFBA mediated improvement in non-union healing, average bone volume in no PFTBA implants was 0.7+/-0.2mm^3, in the 5% PFTBA group 0.98+/-0.26mm^3, and in the 10% PFTBA group 1.77+/-0.47mm^3, corresponding to a 2.5 fold increase in bone volume in the 10% PFTBA relative to the no PFTBA control. The study also reports similar PFTBA mediated increases in bone mineral density and trabecular thickness. Using a fascinating approach in which the host has a luciferase gene placed under the same promoter as its native osteocalcin gene, the researchers were able to identify upregulations in native osteocalcin expression after delivery of these implants using real-time *in vivo* fluorescence imaging. This data showed increases in native osteocalcin expression at 3 experimental time points (days 5,7 and 14) in animals which had received the PFTBA gels relative to the control materials, although only on day 5 was this difference found to be statistically significant[171].

Pre-committing MSCs towards an osteogenic lineage prior to bone repair has been demonstrated advantageous in a study which compared the ability of naive, and lineage committed MSCs to repair a murine critical size cranial defect using cell seeded gelatine hydrogels. Although gels containing naive MSCs did display some degree of tissue remodelling, gels containing osteogenic pre-committed MSCs showed considerably enhanced bone formation in comparison. In this study, MSCs were seeded onto gelatine hydrogels and cultured for 4 days prior to implant in either basal or osteogenic medium. During this time the group demonstrated using a 5mm murine clauvarial defect that after 8 weeks scaffolds seeded with osteogenic preconditioned cells supported formation of a significantly higher volume of bone than within the defect than those pre-cultured under basal conditions[172].

Hydrogels may also act synergistically to improve the integration and efficacy of existing metallic or ceramic implants. This has been evidenced using titanium screws in a canine mandible defect which showed that coating titanium screws (3.3mm diameter, 10mm length) with a bFGF containing gelatine hydrogel improved bone growth around the immediate vicinity of the implant. Furthermore, the study also considered modulating the gelatine content of the hydrogel between 98% and 95% to slow the degradation rate and showed that modifying the resorbtion rate of the gel influenced bone remodelling with gels with longer resorbtion profiles demonstrating superior bone formation. The slowest resorbing gel containing the greatest amount of bFGF (95% gelatine, 10µg bFGF) resulted in the most appropriate bone/material interface without fibrous tissue formation[173.]

Hydrogels with incredibly precise and defined 3D nano-structures have also been explored for bone tissue engineering. Elegantly, one such study fabricated rosette nanotube based hydrogels which are DNA inspired helical structures based around a nucleotide backbone. These gels were grafted with the peptide motif RGDSK and showed excellent osteoblast adhesion which increased as a function of increasing rosette nanotube number[174]. Other means of introducing 3D definition into hydrogels to promote osteogenesis have been investigated including electrospun PLA fibres[175].

The effect of RGD to promote osteogenesis in hydrogels has also been tested using poly(ethylene glycol) diacrylate gels, which showed that the inclusion of RGD increased the expression of certain osteogenic markers in a dose dependant manner. The authors considered 3 concentrations of RGD; 0.025mM, 1.25mM and 2.5mM and showed that increasing RGD

concentration resulted in a dose dependant increase in ALP production from goat mesenchymal stem cells of 77%, 357% and 1344% respectively relative to a no RDG control. This trend was also true for osteocalcin secretion, resulting in 143%, 211% and 277% respective increases in osteocalcin concentration in the surrounding media relative to a no RGD equivalent[176].

Drug elution may also be beneficial in bone regeneration. This has been explored using a simvastatin releasing gelatine hydrogel which improved bone formation vs. the hydrogel alone in a rabbit tooth model. In this study gelatine hydrogel micelles containing 1 and10μg/implant Simvastatin both resulted in the appearance of bone formation in the tooth defect using radiographic observation, with the authors commenting that in these specimens most of the defect was occupied by newly formed bone tissue after 5 weeks. Contrary to this, in gels without simvastatin insignificant bone formation was observed[177]. Gelatine has also been utilised to release growth factors including bFGF and TGF-ß1 focally to regenerate bone. One study using TGF-ß1 in a rabbit calvarial defect showed that although bone formation was supported by gelatine/TGF-ß1 it was heavily dependent on hydrogel degradation rate and subsequent TGF-ß1 release. The study considered gelatine hydrogels of varying water contents between 85 and 90% +/-0.1μg TGF-ß1. In a rabbit clauvarial defect the group demonstrated that this material was exceptional at supporting bone regeneration reporting that in the 90% and 95% materials the defect was completely occupied by bone, with an associated bone mineral density of 84.3+/-5.2 mg/cm^2 and 84.3+/-12mg/cm^2 respectively. Interestingly the gels tested either side of these; 85% and 98% did not support such a significant generation of neo-bone with associated 60.5+/-8 mg/cm^2 and 65.0 +/-6.1 mg/cm^2 bone mineral densities respectively. Those values being similar to the bone mineral densities observed using either control gels of identical water contents without TGF-ß1 or free TGF-ß1[178].

The synergistic role of insulin like growth factor (IGF) with TGF-ß1 has also been tested using a gelatine system, which reported enhanced bone formation in a rat mandibular defect model than when either of the 2 growth factors were used singly. This research considered gelatine gels impregnated with combinations of IGF (25ng) and TGF-ß1 (0.1μg) implanted into 3x4mm drilled defects in the rat mandible. The authors showed bone closure rates of 37%, 38%, 24%, 14%, after 3 weeks, and 94%, 91%, 84%, 72%, after 6 weeks, in TGF- ß +/IGF-1, TGF- ß1 alone, IGF alone and gelatine alone[179].

4.5 Cartilage

Cartilage damage in joints causes pain, swelling and impaired mobility. This damage can be a function of chronic auto-degeneration such as osteoarthritis or acute trauma[180]. Once articular cartilage is damaged its capacity to regenerate and repair is limited due to avascularity[181] and low proliferation rate of resident chondrocytes[180].

The goal of tissue engineering is to regenerate tissue and repair its function using scaffolds with or without biologically active components (e.g. cells and growth factors). Biomimetic scaffolds deliver and retain cells to a damaged tissue and possess appropriate rheometry to mimic the mechanical properties of healthy tissue, i.e. cartilage, providing a substitute for ECM[182, 214]. In cartilage, ECM is composed of primarily type II collagen and proteoglycans[183]. Ideally, a scaffold will be resorbable, providing a transitory ECM until native or clinically delivered chondrocytes deposit load bearing matrix. With appropriate tuning, the autologous cell matrix will replace the scaffold at a rate parallel to its degradation profile[184-185] (Fig.1). When designing a scaffold for cartilage regeneration the mechanical properties of the construct need to bear the load and sheer stress of the joint and protect cells and tissue within the scaffold[180]. Another important design property is the biocompatibility of the construct; the material should induce a balanced immunological response without excessive inflammation.

Although regeneration and reconstruction is our grand unifying objective as tissue engineers, hydrogels also present a tool which elegantly allows us to understand the intricacies of the biology by which tissue repairs, providing model systems to study cells which preserve their functionality under our microscopes the same as that *in vivo*. This has been beautifully demonstrated in a study by Jeon and co-workers, who demonstrated using alginate hydrogels that gene expression in chondrocytes was dependent on their mechanical loading, and this was further modified based on the depth (zone) within the joint from which the chondrocytes where isolated[186]. Thereby using a hydrogel and chondrocytes as a model system to demonstrate the over-arching paradigm that when engineering biomedically functional cells, thought must be given to the dynamics of their native tissue niche and not solely their soluble environment and focal contact points.

Similarly, hydrogels have enabled the study of cyclic tensile strain (CTS) in its ability modify the actin arrangement and cellular alignment of myoblasts. In this research a robust NCO-sP(EO-stat-PO) hydrogel was decorated with micropatterened lines of fibronectin 30μm wide at differing orientations (0^o [parallel to the strain direction], 45^o and 90^o [perpendicular to the strain direction]) relative to the direction of CTS. This hydrogel was ideal based on its ability to retain its mechanical properties throughout large numbers of cycles of mechanical loading. The study showed that 0^o resulted in irregular actin organisation whereas 90^o resulted in actin alignment following the fibronectin lines; perpendicular to the direction of the loading (average fibre angle 91^o)[187].

One of the main advantages of hydrogels for cartilage tissue engineering is that encapsulated chondrocytes maintain their rounded morphology similar to that in native cartilage[188-189]. The ideal concept would a hydrogel which is injectable (i.e. less invasive), biocompatible, non-cytotoxic with appropriate, tuneable mechanical and biodegradable properties. The literature suggests that a wide range of polymers, synthetic and natural, have been used to prepare hydrogels as scaffolds for cartilage TERM[182]. One class of polymers which is often used for hydrogels are polysaccharides. They are usually non-toxic and biocompatible[189], and some can be enzymatically degraded *in vivo*[190-191] or excreted renally[192]. Two polysaccharides which have been extensively studied in this area are chitosan[191, 193-194] and dextran[192, 195]. Polysaccharides are attractive as base material for cartilage engineering because cartilage ECM contains an abundant polysaccharide[182, 196].

Scaffold composition has been shown to influence the chondrogenic potential of mesenchymal stem cells even in the presence of chondrogenic growth factors, demonstrating the synergy between surface parameters and soluble factors. In one such study the group of Guldberg *et al*. showed pre-treatment with FGF-2 helped to predispose mesenchymal stem cells towards a chondrogenic lineage, and chondrogenic differentiation could be completed based on quantification of sGAG concentration when cells were seeded into gels containing dexamethasone and TGF-β1. However the group demonstrated using both alginate and agarose gels that the chondrogenic influences of these factors were enhanced in the alginate gel compared to agarose by observation of more rapid sGAG production after both 14 and 21 days. Under FGF-2+, TGF-β1+ and dexamethasone+ conditions the study also reported substantially higher viability in alginate (69%) compared to agarose (29%) gels[197].

An extensive review on chitosan based hydrogels highlighted the similarity in molecule structure of this polymer and glycosaminoglycan (GAG), which can be commonly found in cartilage. *In vitro* tests have shown that chitosan in combination with chondroitin sulphate or hyaluronic acid can be used as a carrier material for of autologous chondrocytes and/or as a scaffold for cartilage-like tissue[191].

Other cartilage TERM hydrogels are based on hyaluronic acid (HA) backbone modified with functional groups MeHA, MeLAHA, and MeCLHA can alter cell behaviour and functionality. A recent overview showed the influence of various network densities and ratios of inserted functional groups on distribution and connectivity of matrix deposited by mesenchymal stem cells (MSCs)[198]. The inclusion of GAG such as HA in chondrogenic gels appears logical, based on the abundance of native GAG in collagenous matrix and therefore the *ex vivo* similarity to the cells native niche. One study however interestingly showed a preference for HA as the GAG of choice for hydrogel incorporation when compared to chondroitin sulphate (CS). This research demonstrated more complete chondrogenesis in HA gels vs. CS equivalents. For instance, the HA gel stimulated a 9 fold increase in type II collagen gene expression of primary chondrocytes compared to 3 fold in the CS gel[199].

Over the years, composite materials have been under investigation for cartilage tissue engineering. Examples include electrospun poly(L-lactide-co-ε-caprolactone) scaffolds combined with heparin-based hydrogels to improve cell encapsulation, collagen deposition, mechanical properties[200] and a composite material of gelatine and methacrylamide to achieve photo polymerised hydrogels to encapsulate growth factors (e.g. TGF-β1) and cells[201]. This latter composite can also be combined with viscosity enhancing additives such as hyaluronic acid and/or reinforcing support structures as poly(caprolactone) to provide optimised constructs for engineering cartilage[202].

One of the primary functions of scaffolds is to protect cells within the construct however scaffolds have also been designed to instruct the cells inside, for example to enhance matrix deposition. As a result a higher rate of cartilage regeneration might be achieved, improving quicker restoration of joint function[180]. Known signal molecules (integrins), and ECM components of articular cartilage, are collagen, fibronectin, lamina and vitronection[203-205]. Specific binding of these signal molecules with cell-surface receptors induces complex intracellular signalling cascades, influencing cell behaviour[205, 206] therefore scaffold polymers may be linked with bioactive ligands to influence specific cell functions such as adhesion and

migration. Results show that hESC derived cells encapsulated within RGD-modified polyethylene glycol (PEG) hydrogels produced matrix high in type II collagen similar to articular cartilage[198].

Another method to influence the cell environment is by embedding enzyme sensitive molecules in the hydrogels to achieve control resorbtion. A study by Bahney *et al.* made use of an enzyme associated with cartilage development: matrix metalloproteinase 7 (MMP7). This study incorporated MMP7 peptide substrates in poly(ethylene glycol) diacrylate (PEGDA) and were therefore able to create an MMP7-sensitive hydrogel with distinctive degradation rates. When hMSCs were photoencapsulated, these constructs produced cartilage with more extensive collagenous matrices when compared with nondegradable scaffolds[207].

Materials which used one or several integrins showed regulation of chondrocyte activities[205], and stem cell chondrogenesis[204, 208]. When using integrins in scaffolds for cartilage tissue engineering, it would be beneficial to physically bind these integrins to avoid unrestricted elution. Peptides can be synthesised with similar structures to integrins (e.g. vitronectin, laminin) to introduce instructive signalling between cell and polymer, highlighting potential for smart biomaterials in cartilage regeneration[203].

When considering cartilage as a whole it is naive to contemplate this tissue as a cellularly homogenous entity, rather than the reality of multiple zones of cartilage containing chondrocytes with genomic and transcriptomic adaptations to habit that particular area. In part this is governed by their proximity and cross-talk with osteoblasts at the osteochondral junction. This interface has been modelled using a bilayered oligo(poly(ethylene glycol) fumarate) hydrogel containing gelatine micropatricles of 50-100μm. In these experiments the system contained a layer of gel containing stem cells which had been predifferentiated in chondrogenic media for either 7 or 14 days on top of a layer of predifferentiated osteoblasts. Using gene expression analysis the researchers reported that this bilayered approach was ideal for maintaining the phenotype of both cells quantified by increases in cartilage genes in the chondrogenic layers (aggrecan, type II collagen) in addition to repression of the fibroblast associated type I collagen. Interestingly however the study reported that enzyme activity of the bone biomarker alkaline phosphatise (ALP) was actually higher when the subchondral layer contained undifferentiated stem cells rather than linage committed osteoblasts. This was explained by the prediction that in the cells committed to an osteogenic lineage before embedding in the gel, their mineralization may have been previously completed rendering

this enzyme obsolete. Thereby the MSCs presenting this protein when embedded in the gel suggests endogenous cues either by the material or by locality to chondrocytes which is driving them towards an osteogenic fate[209].

5. Disease Models

The added value of ECM and microenvironmental mimesis provided by hydrogels proposes this class of materials as ideal candidates to augment *in vitro* disease model systems, to provide more rapidly translatable data by using cells which are functioning in a manner more akin to what would be expected of them *in vivo*.

Hydrogels have been used in this manner to support the study of a diverse range of diseased tissues including a PEDGA gel to study the behaviour of bone cells in Apert Syndrome. Using cells derived from Apert syndrome model mice the study demonstrated that cells from these mice cultured in hydrogel exhibited increased osteoblastic differentiation, decreased bone matrix remodelling and abnormal chondrogenesis which correlated well between *in vitro* and *in vivo* disease pathology[219].

An additional culture system incorporated several tissue culture technologies in order to create a model environment in which intestinal epithelial cells could be maintained. In this instance Sephadex beads where coated with a mixture of thiol-modified HA derivative CMHA-S and thiol-modified gelatin derivative Gelatin-DTPH which formed a sythethtic disulphide bonded ECM structure on the beads. Interestingly this gel could be disrupted by adding NAcCys which dissolves the gel by thiol sulphide exchange, meaning that cells can be released from the ECM structure in a minimally disruptive manner without enzymatic digestion. These tissue structures were used in combination with a rotating wall bioreactor to culture intestinal epithelial cells which demonstrated excellent maintenance of epithelial cell phenotype confirmed by characterisation of the proteins ESA, ZO-1 and CK19 using immunohistochemistry. The group comment concisely that such a real-time organotypic culture will provide an excellent platform from which to study infectious disease, environmental toxicology, cancer and drug discovery[220].

6. 3D Printing of Hydrogels

At this present time, no biomaterials based review article should be comcluded without mentioning the influence of 3D printing on scaffold design and fabrication. 3D printing is a technique which will undoubtedly influence the bioengineering and regenerative medical

field immensely over the coming years. This technology enables the intricately controlled deposition of sequential rounds of polymer 'inks' on top of one another to form a three dimensional matrix with defined external shape and topography as well as internal porosity. Most clearly such a technology concurs images of beautifully personalised medicine in which prosthetic cardiac valves, blood vessels or even larger more complex organs are generated bespoke to fit an individual patient. However the applications of the technique are much more far reaching than this as an important in vitro system for accurately generating a 3D microenvironment at a nanometer scale in which cellular behaviour can be studied singly or as organotypic cultures.

Hydrogels lend themselves well to deposition as a 3D printing ink based on their ability to be deposited as a liquid often without the use of cytotoxic solvents or high temperatures which may render cells unviable or growth factors and enzymes denatured. One such study utilised gelatin methacrylamide with the photoinitiators1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one or 2,20-Azobis[2-methyl-N-(2-hydroxyethyl)propionamide]. A bioplotter was used to produce 3D gel within interconnected porosity that was printed laden with HepG2 cells. Using the later photoinitiator the group demonstrated, >97% viability of cells inside the constructs in addition the cells constructs displaying a maintained expression of liver specific proteins albumin and HNF4α, characterised using immunohistochemistry[216].

Several other hydrogel materials have also been used to deposit cell laden constructs including alginate, atelocollagen, and decellularised ECM which all reported 95% viability after deposition of human adult stem cells[217].

3D printing also presents researchers with the ability to generate exquisite in vitro systems which provide cells with a more appropriate mechanical and topographic microenvironment to function in a manner more akin to that *in vivo*. One such study elegantly used 3D printing to deposit a collagen base layer, onto which a gelatine cylinder laden with endothelial cells (HUVECs) was printed. Sequential layers of collagen were printed around the gelatine before it was liquefied and removed to leave am endothelial cell lined collagen tube onto which a pump could be attached to provide fluidic sheer. Such a system would be ideal for testing the influence of substances on vascular integrity under the same mechanical and fluidic parameters that these cells are accustomed to *in vivo*, whilst also being able to monitor the diffusion of substances through the endothelial lumen into the surrounding collagen[218].

7. Conclusion

Hydrogels are a versatile class of materials with a plethora of favourable physical and chemical properties which make them unrivalled candidates as cell scaffolds or molecular delivery vehicles in biomedicine. Their high water content mimetic of native tissue, ease through which cells can migrate and intricately controllable resorbtion profiles have thrust hydrogels into the forefront of tissue engineering. Over the last 2 decades particularly, this group of materials have established a track record in the experimental reconstruction of a significant and diverse array of adult tissues demonstrating their ability to support appropriate cell and tissue dynamics to augment several chronic degenerate and debilitating conditions. Future research in this area will focus on translation of these promising pre-clinical studies and bioprocesses into life changing clinical interventions with the ability to increase quality of life and healthy ageing on a world wide scale.

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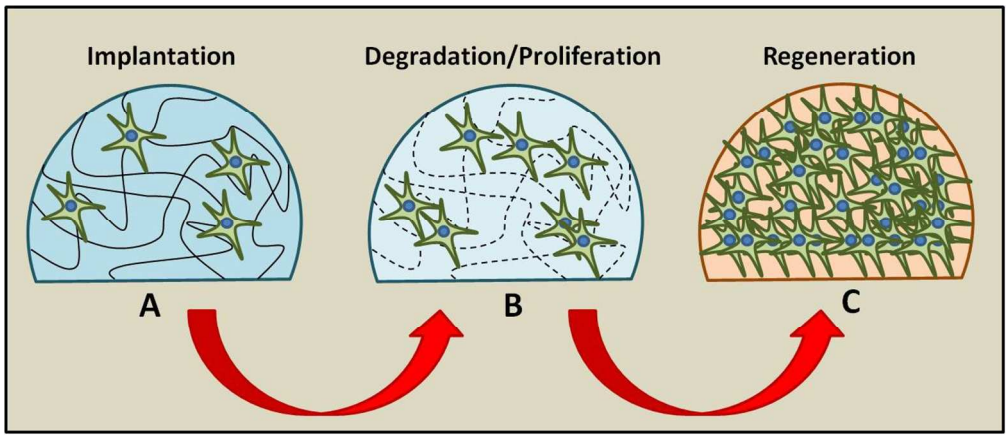
Figure/Table Legends

Fig.1 The ideal pathway to hydrogel mediated tissue regeneration. A. Hydrogel implanted at a defect site loaded with stem/progenitor cells. B. The material degrades whilst simultaneously cells within the implant proliferate and secrete their own ECM. C. The material is completely degraded resulting in a neo tissue composed entirely of autogenous cellular matrix, integrated seamlessly with the matrix of surrounding tissues.

Table 1. Overview of noteworthy synthetic and organic hydrogels used throughout experimental regenerative medicine grouped by tissue of interest.

Hydrogel	Regenerative Application	Notes	Reference
poly(N-isopropylacrylamide-co-propylacrylic acid-co-butyl acrylate)	Cardiac	Gellation Stimuli ofsupreesed pH	108
alpha-cyclodextrin/MPEG–PCL–MPEG	Cardiac	Delivery vehicle for EPO	109
alginate	Cardiac		110
PEG	Cardiac		111,117
gelatine	Cardiac	Delivery vehicle for bFGF	114
chitosan-collagen	Cardiac	contains peptide fragment derived from angioprotein-1	118
chitosan	Cardiac		119
HEMA-PTMC	Cardiac	displayed modulus specific cell differentiation	120
fibrin	Cardiac	micropatterend surface	121
star PEG	Vascular	heparin linked via an MMP cleavage peptide using either an amide or ester linkages	123
poly-N-acetyl glucosamine	IVD	5-7μm or 80μm poly-N-acetyl glucosamine nanofibres	126
chitosan	IVD	considered chitosan molecular weight and degree of hydroxybutyl substitution	128
alginate	IVD	ionic versus photo cross-linking of alginate	132
type II collagen/hyaluronic	IVD	4S-StarPEG linker	133
IEMA co-polymerised with N-vinyl-2-pyrrolidinone or 2-hydroxyethyl methacrylate	IVD	radiopaque	139
chitosan/gelatine/glycerol phosphate	IVD	delivery vehicle for anti-oxidant ferulic acid	140
crosslinked collagen	IVD		141
alginate	CNS		143
agarose/chitosan/methylcellulose/dextran	CNS		144
gelatine/hydroxylphenylpropionic	CNS	HRP and H_2O_2 controlled the gellation rate and degree of cross-linking	145
$RADA_{16}$ (AcN-RADARADARADARADA-$CONH_2$)	CNS	combined with laminin IKVAV motif	146
Chitosan	CNS	cell survival increased by attachment of polylysine via azidoaniline photocoupling	147
collagen	CNS	EGF eluting	148
hyaluronic acid/methyl cellulose	CNS	PDGF containing	149
hyaluronic acid	CNS	BNDF linked via hexahistidine tag	150
thiolated methacrylamide/chitosan	CNS	streptavidin and RGD tethered via malemide conjugation to bind biotinylated IFNgamma	151
HPMA	CNS	covalently grafted with RGD	153
PEG/PU	CNS	coating to PDMS rods	154
PVA/AA	CNS	coating to PDMS rods	156
hyaluronic acid-g-chitosan-g-poly (N-isopropylacrylamide)	Bone	containing irregularly shaped calcium phosphate microparticles	160
PEG-PCL-PEG	Bone	collagen and nano-hydroxyapatite	161
oligo(polyethylene glycol) fumarate	Bone	phosphorylated using bis(2-(methacryloyloxy)ethyl)phosphate	163
chitosan/ß-glycerophosphate	Bone		164
hyaluronic acid	Bone	carrier vehicle for BMP-2	165,166
hyaluronic acid	Bone	covalent grafting of an integrin binding domain	167
silk fibroin	Bone	carrier vehicle for BMP-2	169
fibrin	Bone	synthetic oxygen carrier; perfluorotributylamine	171
gelatine	Bone	coating titanium screws	173
poly(ethylene glycol) diacrylate	Bone	added RGD	176
gelatine	Bone	simvastatin releasing	177
alginate	Cartilage		186
NCO-sP(EO-stat-PO)	Cartilage	decorated with micropatterened lines of fibronectin	187
chitosan	Cartilage		192, 193-194
dextran	Cartilage		192, 195
alginate	Cartilage	gels contained dexamethasone and TGF-β1	197
heparin	Cartilage	combined with electrospun poly(L-lactide-co-ε-caprolactone)	200
gelatine and methacrylamide	Cartilage		202
polyethylene glycol	Cartilage	RGD-modified	198
PEGDA	Cartilage	incorporated MMP7 peptide substrates	207
oligo(poly(ethylene glycol) fumarate)	Cartilage	containing gelatine micropatricles	209



The ideal pathway to hydrogel mediated tissue regeneration. A. Hydrogel implanted at a defect site loaded with stem/progenitor cells. B. The material degrades whilst simultaneously cells within the implant proliferate and secrete their own ECM. C. The material is completely degraded resulting in a neo tissue composed entirely of autogenous cellular matrix, integrated seamlessly with the matrix of surrounding tissues.
231x99mm (150 x 150 DPI)