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Review

Hyaluronic acid based scaffolds for tissue engineering—A review

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ABSTRACT

This review focuses on hyaluronic acid (HA) tissue scaffolding materials. Scaffolds are defined in terms of formation mechanisms and mode of action. Solution properties are discussed as an understanding of the hydrodynamics of HA is fundamental in optimising the subsequent modification and the chemistries behind important tissue engineering applications that are emerging from recent research on this increasingly valuable carbohydrate polymer are described. Key scaffold characteristics such as mechanical, biological function and degradation are discussed. The latest technologies behind scaffold processing are assessed and the applications of HA based scaffolds are discussed.

Contents

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1. Introduction

Hyaluronic acid (HA) is a glycosaminoglycan that is found in extracellular tissue in many parts of the body. It is a material of increasing importance to biomaterials science and is finding applications in diverse areas ranging from tissue culture scaffolds to cosmetic materials. Its properties, both physical and biochemical, in solution or hydrogel form, are extremely attractive for various technologies concerned with body repair. This review describes the solution and hydrogel properties of HA and considers the chemistry behind some of the important tissue engineering applications that are emerging. Three general strategies have been utilised in the creation of new tissue using scaffolds; (1) the replacement of only those isolated cells or cell substitutes needed for function (Conductive approach); (2) production and delivery of tissue-induced substances such as growth factors and signal molecules (Inductive approach); (3) cells placed on or within a scaffold made from synthetic materials such as polyethylene glycol or natural materials such as hyaluronic acid (cell seeding approach) Fig. 1 illustrates these approaches. The latest technologies behind scaffold processing are also assessed and the applications of HA based scaffolds are discussed.

HA has been the subject of previous reviews focusing on chemical modifications (Prestwich, 2001, 2011; Prestwich & Vercruysse, 1998; Schante, Zuber, Herlin, & Vandamme, 2011), its biological functions (http://www.glycoforum.gr.jp/science/hyaluronan/hyaluronanE.html) and medical applications such as viscosupplementation (Moreland, 2003), wound healing (Jiang, Liang, & Noble, 2007) and drug delivery (Eenschooten et al., 2012; Oh et al., 2010). However, this review focuses on HA and HA derivatives prepared and used specifically for tissue engineering applications. The first section defines scaffold function, formation and mode of action. The second section describes HA in detail and its suitability as a scaffolding material. The third section summarises solution properties of HA which are important as an understanding of the hydrodynamics of HA is fundamental in optimising modification and crosslinking reactions used for scaffold construction. This is followed by examination of the latest developments relating to HA modification and crosslinking for tissue engineering. The following section describes the latest technologies behind scaffold processing and this leads to a section on scaffold properties including degradation, mechanical and biological functions. Finally, the latest applications of HA based scaffolds are discussed in detail.

1.1. Scaffolds

The scaffold, by definition, is a temporary supporting structure for growing cells and tissues (Murugan & Ramakrishna, 2007). It is also called synthetic extracellular matrix (ECM) and plays a critical role in supporting cells. These cells then undergo proliferation, migration, and differentiation in three dimensions, which leads to the formation of a specific tissue with appropriate functions as would be found in the human body. To facilitate these measures, the scaffold should possess a few basic characteristics. The following section highlights the general characteristics of a scaffold that are desirable for most tissue engineering applications.

According to Chen, Ushida, and Tateishi (2002) and O'Brien (2011) scaffolds for tissue engineering should meet several design criteria:

- The surface should permit cell adhesion, promote cell growth, and allow the retention of differentiated cell functions;
- The scaffolds should be biocompatible, neither the polymer nor its degradation by-products should provoke inflammation or toxicity *in vivo*;
- The scaffold should be biodegradable and eventually eliminated;
- The implanted scaffold must have sufficient mechanical integrity to function from the time of implantation to the completion of the remodelling process. A balance between mechanical properties and sufficient porous architecture to allow cell infiltration and vascularisation is key to the success of any scaffold;
- The porosity should be high enough to provide sufficient space for cell adhesion, extracellular matrix (ECM) regeneration and minimal diffusional constraints during culture, and the pore structure should be interconnected to allow even spatial homogeneous tissue formation. Cells primarily interact with scaffolds via chemical groups (ligands) on the material surface. Scaffolds synthesised from natural extracellular materials (e.g. collagen) naturally possess these ligands in the form of Arg-Gly-Asp (RGD) binding sequences, whereas scaffolds made from synthetic materials may require deliberate incorporation of these ligands through, for example, protein adsorption. The ligand density is influenced by the specific surface area of pores to which cells can adhere. Pores thus need to be large enough to allow cells to migrate into the structure, where they eventually become bound to the ligands within the scaffold, but small enough to establish a sufficiently high specific surface, leading to a minimal ligand density to allow efficient binding of a critical number of cells to the scaffold. Therefore, for any scaffold, a critical range of pore sizes exists which may vary depending on the cell type used and tissue being engineered (O'Brien, 2011);
- The material should be reproducible and processable into three-dimensional structures with properties or design variables tailored for the intended scaffold application and environment into which the scaffold will be placed.

Scaffolds can be derived from synthetic and natural materials. Common synthetic scaffolding materials include poly(lactide-co-glycolide) (PLG) which are FDA approved degradable polymers with good mechanical properties (Wong & Mooney, 1997). However they are hydrophobic which makes entrapment of viable cells a challenge. Alternatively, hydrogels are being employed as

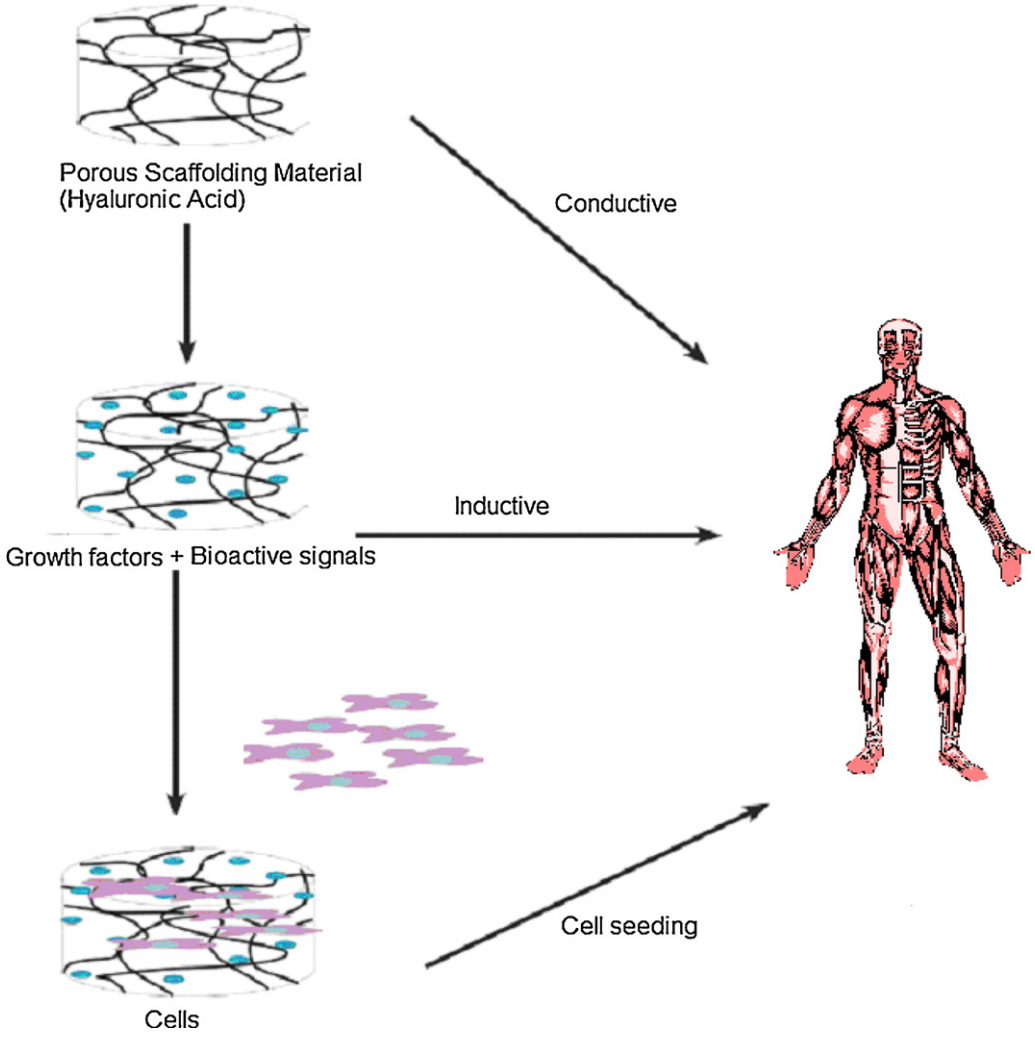

Fig. 1. Conductive: a biodegradable scaffold is implanted directly at the site of injury where it provides the mechanical support and space needed for tissue growth. Inductive: scaffold serves the same role as in the conductive approach but bioactive signals are introduced to the scaffold to better guide the cells through the tissue formation process. Cell seeding: cells are seeded *ex vivo* into the inductive approach scaffold and tissue is grown in a bioreactor adapted with permission from Segura (2004).

scaffolding materials. Hydrogels offer many advantages as they are composed of hydrophilic polymer chains which can be synthetic or natural in origin. They are biodegradable, easily processed, delivered in a minimally invasive manner and have structural properties similar to tissues and the ECM which are controllable by chemical crosslink densities. This review focuses on hydrogels as scaffolds for tissue engineering. The hydrogel scaffold mode of action depends on physical properties (e.g. mechanics, degradation, gel formation), mass transport properties (diffusion) and biological properties (cell adhesion and signalling) which can all be carefully engineered.

As shown in Fig. 1 scaffolds can be used as space filling agents (conductive), as bioactive molecule delivery systems (inductive) and as cell/tissue delivery systems (cell seeding). In space filling applications they provide a framework in which tissue may be regenerated. The scaffold or construct system can be opened or closed. Open cell scaffold systems are implanted in the body and become completely integrated with the host tissue. In a closed construct system, cells are isolated from the body by a membrane that permits nutrient and gas exchange while acting as a barrier for large entities such as antibodies and immune cells. In this capacity, the physical properties of the material are inherent to the success of the scaffold. Specific physical properties include gel formation mechanisms and dynamics, mechanical characteristics, and degradation behaviour (Slaughter, Khurshid, Fisher, Khademhosseini, & Peppas, 2009). In hydrogels, these properties are governed by the intrinsic properties of the main chain polymer and the crosslinking characteristics, as well as environmental conditions.

1.2. Scaffold properties

For tissue scaffold applications the following key properties have been analysed, see Table 1:

- Physical properties (compressive stress and modulus, storage and loss modulus, porosity, density and swelling ratios)
- Degradation properties (enzymatic degradation, swelling studies)
- Biological properties (*in vitro* and *in vivo* studies, cell culture, histology, immunology)

The physical and degradation properties of HA based scaffolds largely depend on the molecular weight of HA, whether HA is composited with another polymer, degree of grafting, crosslinker type and crosslink densities. Biological properties are largely influenced by interactions with cell surface receptors.

Table 1
Commonly used techniques for HA scaffold assessment.

Key scaffold property assessed	Techniques used
1. Mechanical behaviour	
Storage/Loss moduli	Dynamic mechanical thermal analysis (DMTA), rheology
Compressive strength/modulus	Instron
Crosslink density, mesh size	Swelling tests in distilled water and phosphate buffer solution (PBS)
2. Chemical analysis	
Material composition	Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR)
3. Porosity	
Pore size and morphology	Scanning electron microscopy (SEM)
Pore density	Immersion technique
4. Thermal behaviour	
Degradation temperature	Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA)
Bound and unbound water content analysis	Differential Scanning Calorimetry (DSC)
5. Diffusion	
Molecular diffusion	Fluorescence recovery after photobleaching (FRAP)
6. Biological	
In vitro and in vivo cell attachment, growth and proliferation	Confocal microscopy, cell culture, histology, immunology
Protein incorporation	Fluorescence imaging
7. Degradation	
Enzymatic	Exposure to hyaluronidase
Hydrolysis	Swelling tests in distilled water and Phosphate buffer solution (PBS)
	Size exclusion chromatography–gel permeation chromatography (SEC–GPC)

1.2.1. Physical properties

The suitability of hydrogels as scaffolding material depends largely on their bulk structure. For example important parameters used to characterise the network structure of hydrogels include: (1) the molecular weight of the polymer chains between two neighbouring crosslinks (Mc), (2) the corresponding mesh size (ξ), and (3) the effective network density. These parameters are inter-related and can be determined by applying the equilibrium-swelling theory (Collins & Birkinshaw, 2008) and the rubber-elasticity theory (Van Vlierberghe, Dubruel, & Schacht, 2011).

Hydrogel formation mechanisms and crosslink densities also dictate how molecules and cells are incorporated into a scaffold and how that scaffold is then delivered. Formation of tissues with desirable properties relies on scaffold material mechanical properties on both the macroscopic and the microscopic level. Macroscopically, the scaffold must bear loads to provide stability to the tissues as it forms and to fulfil its volume maintenance function. On the microscopic level, evidence suggests that cell growth and differentiation and ultimate tissue formation are dependent on mechanical input to the cells (Sikavitsas, Temenoff, & Mikos, 2001; Wang, Jin He, Wang, & Cui, 2012). As a consequence, the scaffold must be able to both withstand specific loads and transmit them in an appropriate manner to the surrounding cells and tissues.

Methods used to measure mechanical properties of HA based hydrogels include dynamic mechanical thermal analysis (Collins & Birkinshaw, 2008) and rheology (Ananthanarayanan, Kim, & Kumar, 2011; Bhattacharyya, Guillot, Dabboue, Tranchant, & Salvetat, 2008) where storage and loss moduli have been obtained. Various researchers have detailed compressive tests used to obtain compressive moduli and strengths of potential HA based scaffolding materials (Collins & Birkinshaw, 2011; Ibrahim, Kang, & Ramamurthi, 2010; Kim et al., 2012a; Oldinski, Ruckh, Staiger, Popat, & James, 2011; Tan, Chu, Payne, & Marra, 2009; Zhang et al., 2010; Zhang, Xiao, Jiang, Fan, & Zhang, 2010). Results agree with swelling ratios, showing that increasing the crosslink densities improves the overall mechanical performance of the scaffolding material (Collins & Birkinshaw, 2008; Ibrahim et al., 2010). The porosity of scaffolds is measured by imaging using scanning electron microscopy (SEM). Collins et al. (Collins & Birkinshaw, 2011) showed using SEM images of scaffolds obtained before and after crosslinking, that porous structures were retained after crosslinking. Scaffolds displayed interconnected pores with mean diameters of 40, 90 or 230 μm and porosity of 58–66%, depending on the freezing temperature, as shown in Fig. 2.

1.2.2. Degradation properties

The desired kinetics for scaffold degradation depends on the tissue engineering application. Degradation is essential in many small and large molecule release applications and in functional tissue regeneration applications. The rate of scaffold degradation should mirror the rate of new tissue formation or be adequate for the controlled release of bioactive molecules. For hydrogels, there are three basic degradation mechanisms: hydrolysis, enzymatic cleavage, and dissolution. Most of the synthetic hydrogels are degraded through hydrolysis of ester linkages (Metters, Anseth, & Bowman, 2000). Carbohydrate polymer based scaffolding materials such as hyaluronic acid and chitosan are degraded by enzymatic action. The rate of enzymatic degradation will depend both on the number of cleavage sites in the polymer and the amount of available enzymes in the scaffold environment.

Enzymatic degradation of HA based scaffolds in biological environments is catalysed by hyaluronidase. Recently, Schante et al. have published work on improved enzymatic stability of hyaluronic acid by grafting with amino acids (Schante, Zuber, Herlin, & Vandamme, 2011; Schante, Zuber, Herlin, & Vandamme, 2012).

1.2.3. Diffusion

Diffusion is an important parameter in tissue scaffold design as enhancing the supply of oxygen and nutrients and the removal of waste products is essential to the survival of implanted cells. Diffusion rates through scaffolds are influenced by the molecular weight and size of the diffusion species (defined by Stokes radii) compared to the size of the scaffold pores. Fluorescence recovery after photobleaching (FRAP) experiments has been performed to measure the diffusive properties of dextrans through scaffolds (Leddy, Awad, & Guilak, 2004). Dextrans were studied because they are uncharged and are available in a range of sizes, which encompass a wide range of physiologically relevant molecules from small growth factors to large matrix macromolecules.

1.2.4. Biological properties

HA based scaffolding materials can bind to proteins and cells through cell surface receptors such as CD44 (Knudson, Chow, & Knudson, 2002), RHAMM (Lapcik, Lapcik, Smedt, Demeester, & Chabrecek, 1998) and ICAM-1 (Laurent, Hellstrom, & Stenfors, 1986). HA scaffolds can bind to chondrocytes via CD44 and chondrogenic gene expressions were analysed for adipose tissue mesenchymal stem cells (AT-MSC) using a HA based scaffold (Jakobsen, Shahdadfar, Reinholt, & Brinchmann, 2010) and a poly(ethylene glycol) diglycidyl ether crosslinked scaffolds (Yoon et al., 2010). The expression of RHAMM in both the extracellular and intracellular space is needed for HA scaffold mediated cell locomotion (Lei, Rahim, Ng, & Segura, 2011; Masters, Shah, Leinwand, & Anseth, 2005; Solis et al., 2012), and it has been identified in a wide variety of mobile cells. In the wound healing response RHAMM bound to HA scaffolds is up regulated in keratinocytes, macrophages, and migrating fibroblasts to enhance wound contraction and re-epithialization (Teh, Shen, Friedland, Atlas, & Marano,

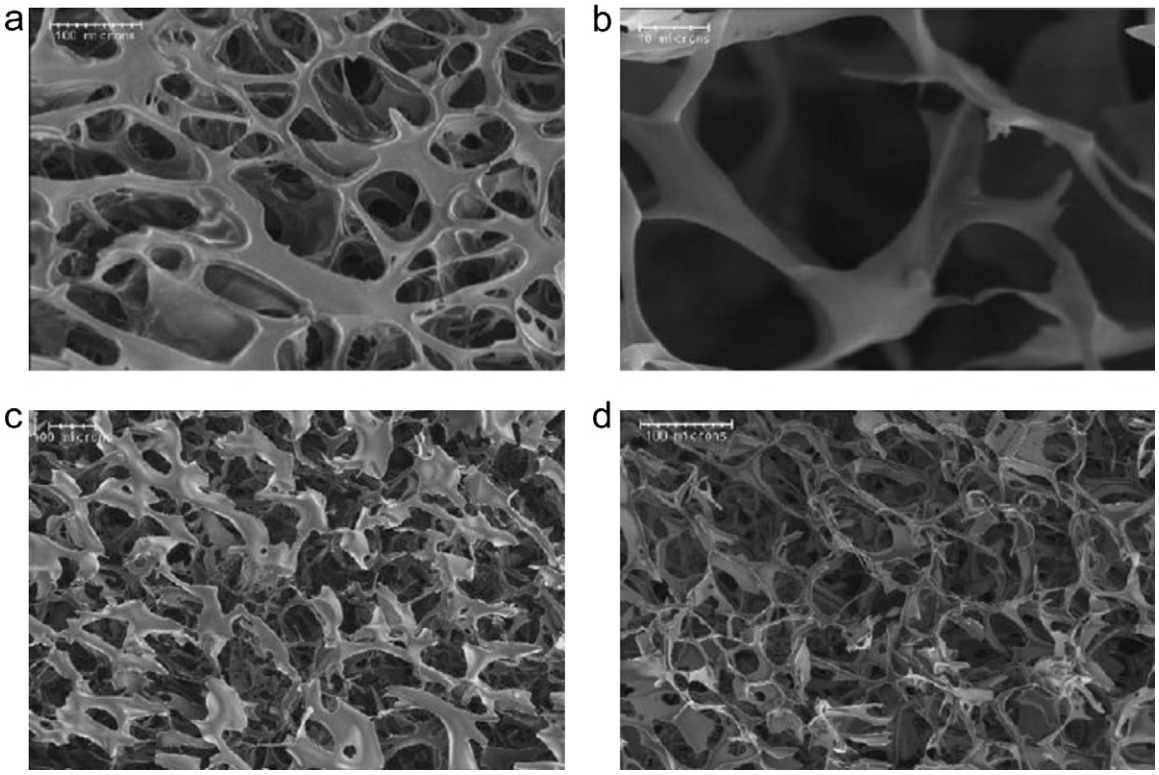

Fig. 2. Homogenous hydrogel matrices (a) solution crosslinked scaffold by GTA (200× magnification), (b) solution crosslinked scaffold by GTA (1500× magnification), (c) HA EDC/LME crosslinked scaffold (100× magnification), and (d) HA EDC/LME crosslinked scaffold (200× magnification) (Collins & Birkinshaw, 2011).

2012; Turley, 1999). The Intercellular Adhesion Molecule (ICAM) is a cell adhesion molecule widely distributed in endothelial cells, and macrophages. ICAM-1 binding to HA scaffolds may contribute to the control of ICAM-1-mediated inflammation activation (Chen & Abatangelo, 1999).

Many of the biological processes mediated by HA scaffolds are essential to the wound healing process; this coupled with the ability of HA based scaffolds to provide an open, hydrated structure for the passage of nutrients make them ideal candidates for tissue regeneration and repair.

2. Carbohydrate polymers in tissue engineering

2.1. Hyaluronic acid

In 1934, Karl Meyer and John Palmer, described a procedure for isolating a novel glycosaminoglycan from the vitreous humour of bovine eyes (Meyer & Palmer, 1934). They showed that this substance contained a uronic acid and an aminosugar, they proposed, the name "hyaluronic acid" (HA), and it is sometimes referred to as 'Hyaluronan', reflecting the fact that it exists *in vivo* as a polyanion and not in the protonated acid form. It is a glycosaminoglycan since each glucuronate unit carries an anionic charge at physiological pH, and there are negative charges associated with its carboxylate group which are balanced by mobile cations such as Na^+.

2.1.1. Structure and sources

HA is present in all vertebrates. It is a major constituent of the ECM, for example, in the vitreous humour of the human eye (0.1–0.4 mg/g wet weight), in synovial joint fluid (3–4 mg/ml), and in the matrix produced by the cumulus cells around the oocyte prior to ovulation (~0.5 mg/ml) (Salustri & Fulop, 1998). Rooster comb has high amounts of hyaluronan (up to 7.5 mg/ml). More recently HA has been extracted from bacteria-streptococci through fermentation, thereby eliminating the possibility of inter-species disease transfer (Manna et al., 1999).

HA offers many advantages as a tissue scaffold which include:

a) Biodegradability, biocompatibity and bioresorbability;
b) HA is a major intracellular component of connective tissues where it plays an important role in lubrication, cell differentiation and cell growth. These functions can be transferred to the scaffold;
c) HA contains functional groups (carboxylic acids and alcohols) along its backbone that can be used to introduce functional domains or to form a hydrogel by crosslinking;
d) Since HA is part of every step in the wound healing process exogenous HA has the potential to provide faster healing;
e) Due to its ability to maintain a hydrated environment conducive for cell infiltration, HA based hydrogels are ideal as wound grafts to treat chronic wounds or wounds in patients with impaired healing such as diabetic patients;
f) HA can be part of a new kind of tissue engineering scaffold that is bioactive both in its full length and in the degraded form. It exhibits low non-specific adsorption of proteins and specific interactions between the scaffold and growing cells can be tailored using cell receptors (CD44, RHAMM, ICAM-1) to enhance tissue growth and repair.

Researchers have developed scaffolds based on hyaluronic acid in the form of hydrogels (Leach, Bivens, Patrick, & Schmidt, 2003; Lei et al., 2011; Seidlits et al., 2011), sponges (Collins, 2007; Kim et al., 2012b; Perng, Wang, Tsi, & Ma, 2011) and meshes (Park et al., 2011).

2.1.2. Solution properties

The application of HA as a tissue scaffold material is hindered by its short residence time and lack of mechanical integrity in an

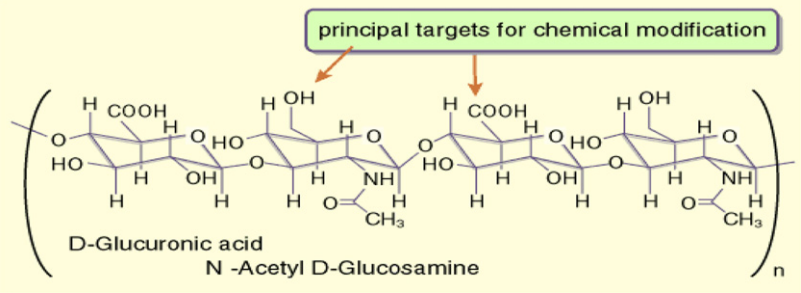

Fig. 3. Hyaluronan showing the disaccharide repeat units and primary sites for chemical modification. Reproduced with permission from Luo, Kirker, and Prestwich (2002).

aqueous environment and these drawbacks are addressed by chemical modification and crosslinking (Collins & Birkinshaw, 2007). However, to ensure repeatability of reported HA modifications and crosslinking procedures and ultimately scaffold properties, it is essential to have an understanding of the solution properties of HA.

Control of HA dissolution is important because dissolution and solution degradation are concurrent processes. Complete dissolution is also important to maximise intermolecular crosslinking and reduce wasteful intramolecular reactions. Viscosity time profiles of HA solutions during various stages of dissolution have been obtained (Collins, 2007) to optimise the crosslinking efficiencies. Hydrodynamic parameters have been measured using viscometric techniques such as Ubbelohde and Size Exclusion Chromatography (SEC) for pre-crosslinked (Mendichi & Schieroni, 2002) and degraded HA solutions (Collins & Birkinshaw, 2008). Mark–Houwink parameters, molecular weights, and radii of gyration and hydration have been measured as a function of HA dissolution time to determine optimum dissolution conditions. α parameters derived from the Mark–Houwink equation increase, depending on molecular weight, until 24 h dissolution indicating that the HA chains are in a fully solvated extended state which is desirable for optimised subsequent chemical modifications and wasteful intramolecular reactions mentioned above are minimised (Collins, 2007).

2.2. Other carbohydrates

Several carbohydrate polymers have been used in tissue engineering applications because they are components of the ECM, or have macromolecular properties similar to the ECM. They are all hydrophilic linear polysaccharides which interact well *in vivo* so they have been utilised as hydrogel scaffolding materials (Piskin, 2002).

Alginate has been used in cell encapsulation and drug delivery because it gels easily, has low toxicity and is readily available. The crosslinking density, mechanical properties and pore size of the ionically crosslinked gels can be manipulated by varying the molecular weight of the chains. Gels can also be formed by covalently crosslinking alginate with the adipic hydrazide and polyethylene glycol (PEG) using standard carbodiimide chemistry (Eiselt, Lee, & Mooney, 1999) and via the Schiff base reaction (Chen et al., 2011).

Chitosan is structurally similar to glycosaminoglycans (GAGs) and is degradable by enzymes in humans. It is a linear polysaccharide of (1–4) linked D-glucosamine and *N*-acetyl-D-glucosamine residues derived from chitin. Once dissolved, chitosan can be gelled by increasing the pH or extruding the solution into a non solvent (Suh & Matthew, 2000), by glutaraldehyde crosslinking, UV radiation and thermal variations as well as complexation with hyaluronic acid (Ma et al., 2012). Much interest has been shown in the development of chitosan based products for burn treatment (Dai, Tanaka, Huang, & Hamblin, 2011).

3. Chemical modifications and crosslinking of hyaluronic acid

The fabrication of new HA based scaffolding materials has been achieved by a variety of chemical modifications to provide mechanically and chemically robust materials. The resulting derivatives have physicochemical properties that may significantly differ from the native polymer, but most derivatives retain the biocompatibility and biodegradability, of native HA. The following part of this review is not intended as an exhaustive examination of HA modifications but details the more relevant chemistries that produce HA derivatives and hydrogels for tissue engineering applications. For a more detailed review the reader is referred to (Prestwich, 2001, 2011; Prestwich & Vercruysse, 1998; Schante et al., 2011b).

The most common modification of hyaluronan is crosslinking to form a hydrogel crosslinking has been accomplished under acidic, neutral and alkaline conditions, by various workers and will be described later. Also, several methods have been devised for functionalisation of hyaluronan with side groups that permit attachment of reporter groups to produce new drugs for targeted and controlled release (Xu, Jha, Duncan, & Jia, 2011). The preparation of composite materials has also been accomplished (Seidlits et al., 2011), with hyaluronan being grafted onto natural and synthetic polymers to provide improved biomechanical and physiological properties.

The chemical structure of hyaluronan with the two most commonly used sites of covalent modification: the carboxylic acid and hydroxyl functionalities are shown below in Fig. 3. The Hydroxyl groups on HA may be crosslinked via an ether linkage and carboxyl groups via an ester linkage. If desired, the HA may be chemically modified prior to crosslinking to form other chemically reactive groups. For example, HA may be treated with acid or base such that it will undergo at least partial deacetalization, resulting in the presence of free amino groups. These amino groups may be crosslinked via an amide, imino, or secondary amine bond. The imino linkage can be further converted into an amine linkage in the presence of a reducing agent. Crosslinking reactions have been accomplished under acidic, neutral, and alkaline conditions using carbodimides, aldehydes, sulphides and polyfunctional epoxides. Autocrosslinking (defined in Section 3.3) and photocrosslinking have also been reported.

Reactions for scaffold productions may be carried out using heterogeneous or homogeneous methods (Collins & Birkinshaw, 2011). Heterogeneous reactions are carried out on preformed HA scaffolds, in which case diffusion rates may be at least as important as chemical kinetics, whereas homogeneous reactions are carried out using HA solutions. The former method has the advantage of

allowing shaping of a product before crosslinking, whereas the latter method offers the advantage of better control of the chemistry with greater product homogeneity.

3.1. Esterification of HA

Esterified HA biomaterials have been prepared by alkylation of the ammonium salt of hyaluronan with an alkyl halide in dimethylformamide (DMF) solution. At higher percentages of esterification, the resulting HYAFF® materials (Fidia Advanced Biopolymers) became insoluble in water. These hyaluronan esters can be extruded to produce membranes and fibres, lyophilized to obtain sponges, or processed by spray-drying, extraction, and evaporation to produce microspheres. The polymers show good mechanical strength when dry, but the hydrated materials are less robust. The degree of esterification influences the size of hydrophobic patches, which produces a polymer chain network that is more rigid and stable, and less susceptible to enzymatic degradation.

These materials have been used as meshes and sponges for growth of cultured human fibroblasts and for culture of chondrocytes and bone marrow derived mesenchymal cells for repair of cartilage and bone defects (Caravaggi et al., 2003; Lisignoli et al., 2003). Information on the chemico-physical properties of non-woven hyaluronan benzyl ester has been obtained by Milella et al. (2002). The non-woven Hyaff®11 showed thermal stability up to 220 °C allowing thermal processing, and mechanical tests showed that when submitted to a press treatment, the samples have best mechanical properties. More recently HYAFF has been used to produce scaffolds using a combination of particulate leaching and Gas Antisolvent (GAS). These scaffolds displayed compressive moduli of up to 10 MPa with vitality tests and histological analysis confirming their suitability for cell culture (Flaibani & Elvassore, 2012). Also the properties of the biomaterial in terms of tissue meniscus regeneration are reported to be promising: the implants remained in position, retained their shape, and showed adequate mechanical properties (Chiari et al., 2006).

3.2. Hydrazide modifications

HA has been modified with adipic dihydrazide, mono and polydihydrazides. HA-ADH forms by carbodiimide mediated coupling of ADH with the carboxyl group of HA and this allows further crosslinking and addition of polypeptides (Prestwich, Marecak, Marecek, Vercruysse, & Ziebell, 1998).

Researchers have produced hydrogels from these materials which are suitable for tissue engineering applications by further crosslinking using a Michael type reaction with peptide polyethylene glycol tetra thiol crosslinker (Park et al., 2010), poly(ethylene glycol) propiondialdehyde (Kirker, Luo, Nielson, Shelby, & Prestwich, 2002), genipin (Zhang et al., 2010a), 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) (Lei et al., 2011; Prestwich et al., 1998; Yeo et al., 2006) and conjugation with polyethylene glycol (Park et al., 2011). All these materials offer the advantage of intrinsic biodegradation pathways and recognition by biological systems.

3.3. Autocrosslinking (non-covalent reactions)

Hyaluronan aggregates with itself, partly associated with bonding between its hydrophobic patches. Electrostatic repulsion between the negative charges is countered not only by hydrophobic interactions but also by H-bonding between acetamido and carboxylate groups. These short-range interactions are favoured when the hyaluronan interactants are antiparallel to each other. The interactions that hold the meshworks together are fairly weak, so that aggregates form and dissociate, depending on conditions and temperatures.

The autocross-linked polymer (ACP™, Fidia) is an internally esterified derivative of hyaluronan, with both inter- and intramolecular bonds between the hydroxyl and carboxyl groups of hyaluronan. ACP™ can be lyophilized to a white powder and hydrated to a transparent gel (Acunzo et al., 2003; Guida et al., 2004). This biomaterial has been used as a barrier to reduce post-operative adhesions and as a scaffolding for cell growth repair of tissue defects. After 20 weeks implantation ACP scaffolds showed more bone growth in osteochondral defects than HYAFF 11 (Allison & Grande-Allen, 2006).

Other forms of autocrosslinked HA involve the synthesis of a HA-benzoyl cystein derivative which can self assemble in physiological buffer by π–π stacking interactions and after oxidation to produce a chemical hydrogel by means of S–S bridges (Palumbo, Pitarresi, Albanese, Calascibetta, & Giammona, 2009). These gels offer the advantage of being injectable and in situ cross-linkable without the use of toxic crosslinkers.

3.4. Crosslinking with polyfunctional epoxides

The epoxy group reacts with –COOH and the –OH functional groups, therefore forming ester and ether bonds, respectively.

Zhang et al., have recently produced HA–Agarose composite materials by using epichlorohydrin as a crosslinking agent. The crosslinked materials were subsequently crosslinked using Freeze drying to produce scaffolding materials which offer the advantage of controlled degradation rates (Zhang et al., 2012).

In another recent study, HA–Collagen scaffold materials for cartilage regeneration applications have been produced using ethyleneglycoldiglycidyl ether. Mechanical, degradation and biological response of these scaffolds were assessed with favourable results being reported (Kim et al., 2012a).

HA has been conjugated with photopolymerisable methacryloyl groups as described by Zawko, Suri, Truong, and Schmidt (2009) to form glycidyl methacrylate modified hyaluronic acid (GMHA). GMHA and HA were crosslinked in basic solution with 1,4-butanediol diglycidyl ether. The GMHA materials were subsequently photocrosslinked to produce shape changing gels suitable for tissue scaffolding and molecular delivery applications.

3.5. Glutaraldehyde (GTA) crosslinking

Dialdehydes are believed to crosslink through formation of acetal or hemiacetal groups on neighbouring chains, with kinetic and spectroscopic evidence indicating a prevalence of the hemiacetal (Tomihata & Ikada, 1997). Glutaraldehyde (GTA) is believed to form either a hemiacetal or an ether link with HA under acidic conditions. Scaffolding materials produced from GTA crosslinked HA are shown in Fig. 2.

Tomihata and his co-worker (Tomihata & Ikada, 1997) crosslinked HA (Denkikagaku, Japan) with glutaraldehyde (GTA) to produce water insoluble gels. The crosslinking reaction was performed using HA films in acetone–water mixtures. This method was further refined by Collins et al. (Collins & Birkinshaw, 2007) to compare the crosslinking efficiency of GTA with EDC, divinyl sulfone (DVS) and diglycidyl ether (EX 810). This immersion crosslinking technique has been further modified recently to produce HA based tissue scaffolds using GTA as a crosslinker (Antunes et al., 2011; Collins & Birkinshaw, 2011). Freymann also used GTA crosslinked HA-PGA scaffolds to study gene expression for meniscus cells (Freymann et al., 2011).

3.6. Reaction with divinyl sulphone (Hylans)

With divinyl sulfone (DVS), the crosslinking occurs via the hydroxyl groups forming an ether bond. Hylans are hyaluronic acids which are chemically crosslinked with divinyl sulfone (Balazs, Leshchiner, Leshchiner, & Band, 1987). The hydroxyl groups of the HA chains react to form an infinite network of sulfonyl *bis*-ethyl crosslinks. A general method to crosslink HA chains is based on the reaction of formaldehyde and HA at a neutral pH, which involves polysaccharide hydroxyl groups and protein amino or imino groups. DVS reacts readily with HA in aqueous alkaline solutions at room temperature to provide crosslinked HA gels. The reaction is very fast, and strong gels are obtained within minutes (Collins & Birkinshaw, 2008).

HA gels, based on DVS chemistries, were assessed using swelling, degradation and cell culture studies (Ibrahim et al., 2010). HA coated scaffolds using template leaching (Arnal-Pastor, Valles-Lluch, Keicher, & Pradas, 2011) and carbon nanotube reinforced scaffolds (Bhattacharyya et al., 2008) have been produced using DVS crosslinked materials. DVS crosslinked HA that has been oxidised by periodate to produce aldehyde functionalities has been combined with HA-ADH to form a double crosslinked network (Jha et al., 2009).

3.7. Crosslinking with carbodiimides

With carbodimides, the crosslinking occurs through the initial formation of anhydride on the polysaccharide, through reaction with neighbouring carboxyl groups, and this anhydride then reacts with nearby hydroxyls to give both inter- and intramolecular crosslinks. L-lysine methyl ester offers the opportunity to form higher stability amide crosslinks with carbodiimides as shown in Fig. 2c and d (Collins & Birkinshaw, 2008).

HA–Collagen based scaffold materials were fabricated by freeze drying and crosslinked using EDC for enhanced mechanical stability (Davidenko, Campbell, Thian, Watson, & Cameron, 2010; Wang & Spector, 2009).

In a further study by Perng et al. (2011) HA–collagen scaffolds crosslinked with EDC were assessed using histological studies in mice. These scaffolds resulted in enhanced angiogenesis. Tuneable porous structures were produced using HA–gelatin based scaffolds crosslinked with EDC (Zhang et al., 2011). These scaffolds were assessed with L929 fibroblasts and showed no cytotoxicity effect. As discussed in Section 3.2 EDC is widely used in the modification of HA to produce HA-ADH based systems which can be further modified by chemical and photocrosslinking.

3.8. Other relevant reaction mechanisms

HA has been oxidised using periodate to form hyaluronic dialdehyde (HDA), the HDA is subsequently attached to Chitosan by Schiff base links between the amino groups in the Chitosan and aldehyde groups in the HDA (Nair, Remya, Remya, & Nair, 2011; Tan et al., 2009). These chemistries offer the advantage of gel and scaffold formation without the use of crosslinking agents. Oxidised HA has also been successfully grafted to hydroxyethylmethacrylate (HEMA) for application in pulmonary tissue regeneration (Radhakumary, Nandkumar, & Nair, 2011).

HA has also been methacrylated and photocrosslinked using Irgacure 2959 in N-vinyl pyrrolidone as an initiator to form hydrogels and scaffold materials (Leach et al., 2003; Park, Tirelli, & Hubbell, 2003; Seidlits et al., 2011; Skardal et al., 2010). Shoichet's group have used Diels–Alder click chemistry to produce HA based hydrogels. These gels were cytocompatible and results indicate that they could potentially be used as soft tissue construct materials (Nimmo, Owen, & Shoichet, 2011). Park et al. have recently reported on the use of cucurbit[6]uril-conjugated hyaluronic acid (CB[6]-HA) hydrogel based on a thiol-ene click reaction. The in situ supramolecular assembly is intended to be exploited as a 3D artificial extracellular matrix for various tissue engineering applications (Park et al., 2012).

4. Scaffold processing technologies

Several approaches to the fabrication of porous degradable polymer scaffolds have been developed and are summarised in Table 2. However, only the commonly used techniques are discussed here.

4.1. Phase separation

Phase separation of a polymer solution can be induced in several ways including non solvent induced phase separation, chemically induced phase separation and thermally induced phase separation (TIPS). In the TIPS process a relatively new approach for preparing porous membranes, the temperature of a polymer solution is decreased to induce phase separation. After the solvent is removed by extraction, evaporation, or sublimation, the polymer in the polymer-rich phase solidifies into the skeleton, the space occupied by the solvent becomes porous (Baker, 2000). The pore morphology varies depending on the polymer, solvent, concentration of the polymer solution and the phase separation temperature (Chen, Wang, & Chen, 2009). Nanofibrous scaffolds consisting of high surface area-to-volume ratio which enhances cell adhesion, migration, proliferation, and differentiation have also been produced using phase separation (Venugopal, Low, Choon, & Ramakrishna, 2008).

4.2. Rapid prototyping – solid freeform fabrication (SFF) and bioprinting

The development of 3D porous matrices for cell support in tissue engineering has been the focus of intensive research in recent years (Barbetta, Carrino, Costantini, & Dentini, 2010; Brenner, Schiffman, Thompson, Toth, & Schauer, 2012; Cai et al., 2012; Dehghani & Annabi, 2011; Dijkman, Driessen-Mol, Frese, Hoerstrup, & Baaijens, 2012; Eenschooten et al., 2012; Flaibani & Elvassore, 2012; Kelleher & Vacanti, 2010; Madurantakam, Cost, Simpson, & Bowlin, 2009; Skardal et al., 2010; Zhang & Chen, 2011). Three-dimensional (3D) printing produces components by ink-jet printing a binder into sequential powder layers (Hutmacher, 2000). The binder is delivered to the powder producing the first layer, the bed is then lowered to a fixed distance, powder is deposited and spread evenly across the bed, and a second layer is built. This is repeated until the entire part, e.g. a porous scaffold is fabricated. The speed, flow rate and drop position can be controlled to produce the complex 3D product. Patterned scaffolds were created using SFF manufacturing and UV crosslinking. The scaffolds exhibited various geometries including hexagonl as shown in Fig. 4a and b (Suri et al., 2011).

Other rapid prototyping techniques such as fused deposition modelling (FDM) and stereolithography are also being explored for scaffold fabrication (Ma, 2004). A Hyaluronic acid dextran hydroxyethylmethacrylate (HA-dex HEMA) material has been produced by bioprinting scaffolds as shown in Fig. 4c using a layer by layer deposition technique, where scaffolds are formed by deposition of cell laden material (Pescosolido et al., 2011). HA–gelatin hydrogels have been printed in the form of tubular constructs using a Fab@Home printing system (Skardal et al., 2010). This technique offers the possibility of priniting customised scaffolds with cells incorporated.

Park et al. (2011) have used SFF to produce scaffold materials of HA grafted a Poly(lactic-co-glycolic acid) with incorporated bone morphogenetic protein-2 (BMP-2) while maintaining biological activity. Hyaluronic acid scaffolds were also fabricated using

Table 2
Currently applied 3D scaffolding technologies.

Fabrication technology	Processing	Pore size (μm)	Porosity (%)	Pore architecture	Advantage	Disadv.	Reference
Phase separation	Casting	<200	<97	Interconnected micropore structure	3D scaffold	Limited control of pore size and shape	Chen et al. (2009), Hutmacher (2000), Venugopal et al. (2008)
Rapid prototyping	Solid free form fab. Layer by layer fab. Bioprinting	100–300		Interconnected macro (various shapes) incorporation of biological molecules	Control of pore geometry and size	Difficult processing	Kang et al. (2011), Pescosolido et al. (2011), Skardal et al. (2010)
Supercritical fluid technology	Casting	<50 micro <450 macro	<95	Non-interconnected micro structure with interconnected macro structure			Dehghani and Annabi (2011), Hutmacher (2000), Sakai et al. (2012)
Porogen leaching	Casting	30–300	20–50	Spherical pores, salt particles remain	3D scaffold	Limited control of pore size and shape	Antunes et al. (2010), Flaibani and Elvassore (2012), Hutmacher (2000)
Electrospinning	Needling	20–100	<95	Poor mechanical properties	3D scaffold	Limited control of pore size and shape	Ekaputra et al. (2011), Hutmacher (2000), Nesti et al. (2008)
Freeze drying	Casting	<200	<97	Interconnected macro structure	Easy processing, 3D scaffold	Limited control of porosity	Ananthanarayanan et al. (2011), Collins (2007), Perng et al. (2011), Zhang et al. (2010a)
Centrifugal casting	Casting and spinning	>50	Low	Circular	Tubular sections	Pores need to be machined	Kasyanov et al. (2009)
Templating	Solid free form fabrication Layer by layer fabrication	30–200	<80	Interconnected macropores	Cell incorporation	Slow processing time	Barbetta et al. (2010), Ko et al. (2011)
Micro patterning	Casting and femtosecond laser induced two photon polymerisation Lithography Nanoimprinting	50–250	High	Spherical	Geometry control chemical cues nanoscale topography Controlled degradation	Slow processing	Kelleher and Vacanti (2010), Suri et al. (2011), Takahashi et al. (2009), Zhang and Chen (2011)

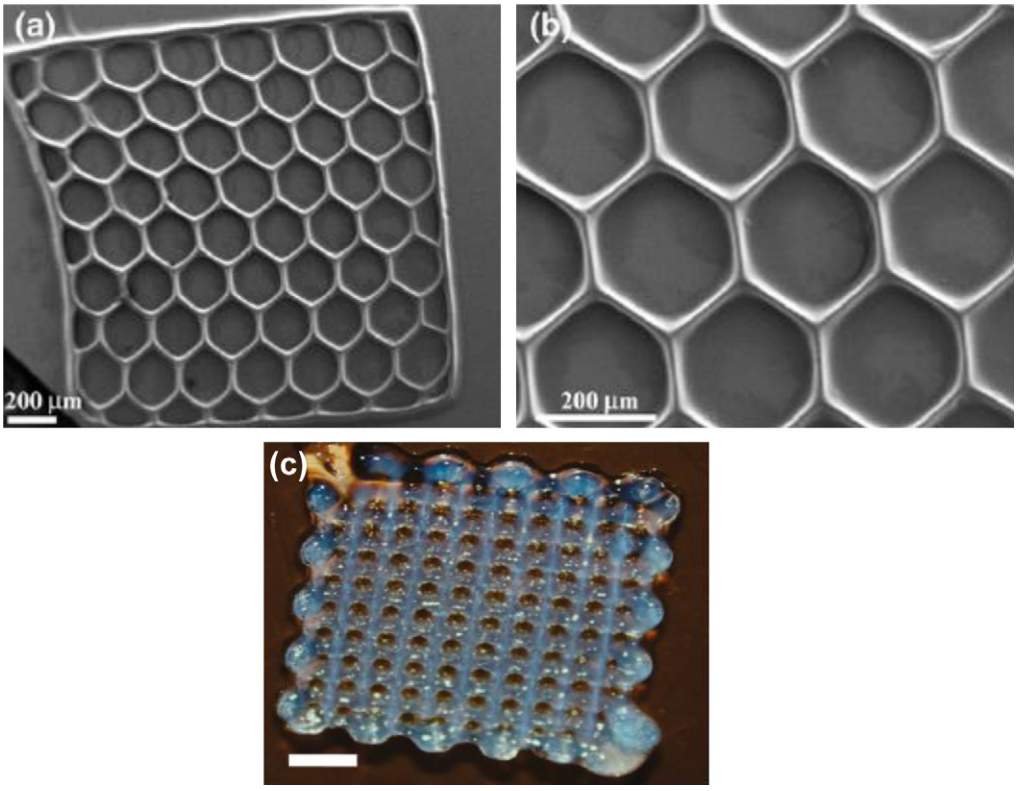

Fig. 4. a) and b) SEM micrograph of single layered HA based scaffolds with hexagonal patterns reproduced with permission from (Suri et al., 2011) and c) HA based scaffold produced from bioprinting.
Reproduced with permission from Pescosolido et al. (2011).

a digital micromirror-array device (DMD) microfabrication system that consists of a computer controlled servo-stage, see Fig. 5a and b.

4.3. Supercritical fluid technology

Porogens are used to create pores or increase porosity for example NaCl. In this process gas is dissolved in a polymer with or without a porogen, at high pressures; homogeneous nucleation occurs when gas molecules combine in response to the thermodynamic driving force to produce small gas bubbles homogeneously throughout the polymer. Saito recently reported on the use of a batch foaming process using supercritical carbon dioxide (20 MPa) for 5 h at 80 °C in an autoclave(Saito, Liu, Migneco, & Hollister, 2012). For further information on engineering scaffold materials from gas based techniques the reader is referred to a recent review by Dehghani and Annabi (Dehghani & Annabi, 2011).

4.4. Porogen leaching

Porogen leaching allows control over pore size and porosity leading to scaffolds with a much more homogeneous pore morphology. Porous structures from several relevant polymers in the biomedical field, such as: poly(D,L-lactide) (Zhang, Wu, Jing, & Ding, 2005) and poly(ε-caprolactone) (Lebourg et al., 2008) have been produced using this method.

Antunes et al. (Antunes et al., 2011) and Flaibani and coworkers (Flaibani & Elvassore, 2012) have developed HA based scaffolds using salt leaching. Porosities of the scaffolds are varied by adjusting the polymer to salt ratio and the pore size is controlled independently by varying the leachable particle size.

4.5. Electrospinning

The applications of electrospinning in tissue engineering has been reviewed (Agarwal, Wendorff, & Greiner, 2009). Three-dimensional fibrous (Ji et al., 2006; Nesti et al., 2008) and nanofibrous (Brenner et al., 2012; Ma et al., 2012) HA based scaffolds have been successfully produced by electrospinning. Micron-sized fibres of thiolated HA-heparin were electrospun in combination with Heprasil to successfully address the problem of cellular infiltration into dense electrospun nanofibrous structures (Ekaputra, Prestwich, Cool, & Hutmacher, 2011). However, it is difficult to control porosity using this technique.

4.6. Freeze drying

The material in hydrogel form, is frozen and the water content of the solution forms dense pockets, or nuclei of ice, throughout the polymer. These nuclei act as the porogen and are removed by sublimation under vacuum (<100 mTorr) leaving behind a porous sponge network. The size of these nuclei can be controlled by exposing the hydrogel to different freezing conditions e.g. −20 and −80 °C (O'Brien, Harley, Yannas, & Gibson, 2005) thereby producing sponges with varying pore diameter and internal structure, due to varying heat transfer coefficients.

4.7. Centrifugal casting

Centrifugal casting of thiolated HA materials with encapsulated endothelial cells has been employed to create a variety of tubular constructs, with crosslinking occurring during axial spinning (Burdick & Prestwich, 2011). The abundance of tubular tissues in the human body—from capillaries to bones, kidney tubules,

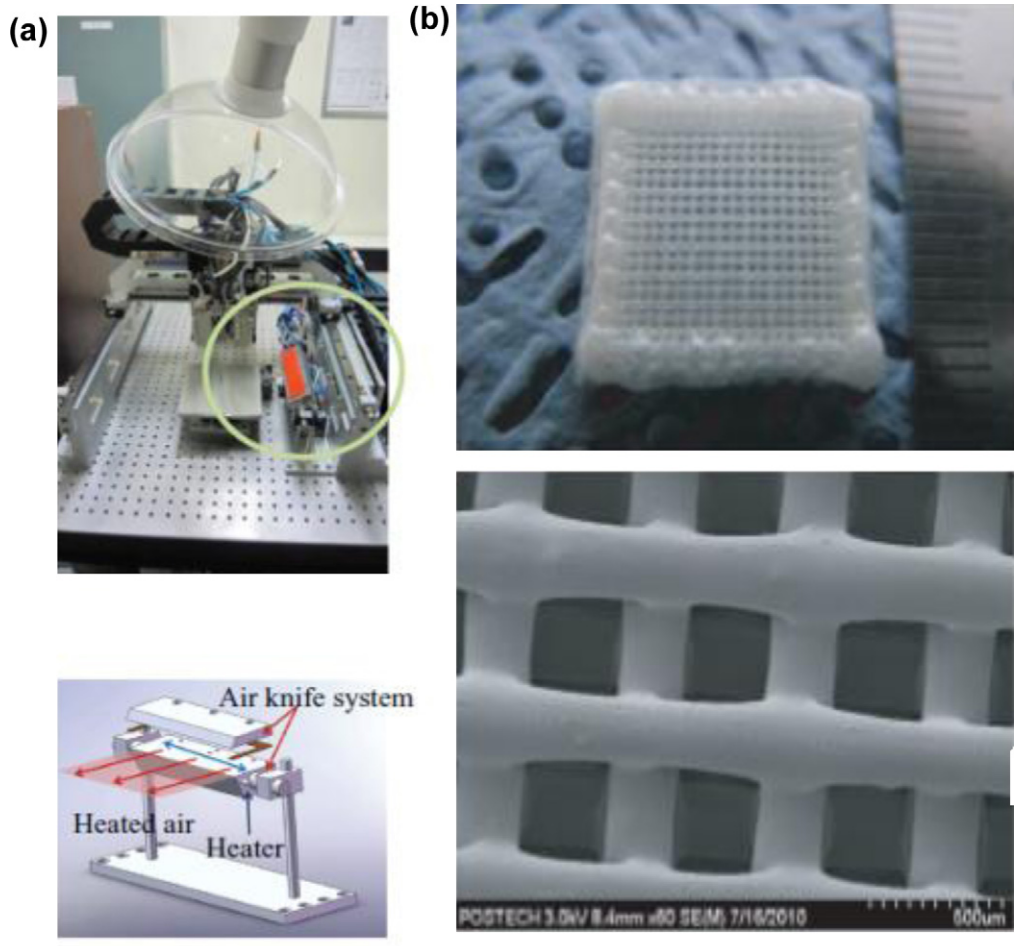

Fig. 5. a) Photograph and schematic representation of the SFF system with heated air blower/air knife (green circle) and suction systems, b) photograph and SEM image of SFF produced HA-PLGA/PEG/BMP-2 scaffold at a magnification of 60×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Adapted with permission from Park et al. (2011).

genitourinary structures—suggests that centrifugal casting has the potential to have an important impact on tissue engineering.

Rapid biofabrication of tubular tissue constructs by centrifugal casting combined with laser-machined micropores to facilitate cell seeding without compromising the biomechanical properties of the scaffold have been described by Kasyanov et al. (2009), see Fig. 6.

4.8. Scaffold templating techniques

HA based hydrogels may also be injected into moulds to fabricate hydrogels with variations in shape and size and a microsphere templating process can be used to fabricate a macroporous scaffold, in this process the HA is crosslinked around beads with the beads subsequently being dissolved leaving adequate porosity for cell and tissue invasion. Open porous HA scaffolds have been produced using an ice particulate template method by Ko, Oh, Kawazoe, Tateishi, and Chen (2011). Barbetta et al. (2010) describe a gas-in-liquid templating technique followed by foam freezing in liquid nitrogen which can be used to produce HA based scaffolds. These scaffolds display an interconnected morphology consisting of pores a few hundreds of μm in dimension, which should be beneficial for cell attachment.

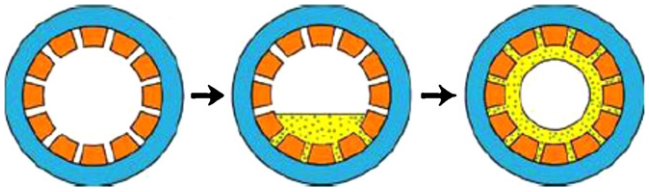

Fig. 6. Centrifugal casting of laser-perforated tubular SIS scaffold with in situ cross-linkable hyaluronan-based hydrogel and living cells.

Reproduced with permission from Kasyanov et al. (2009).

4.9. Micro patterning techniques

3D patterning of HA based scaffolding materials is possible using spatially controlled light exposure (Suri & Schmidt, 2009) and using capillary force lithography on HA films with deposited cells, as shown in Fig. 7 (Takahashi, Yamazoe, Sassa, Suzuki, & Fukuda, 2009). The micro pattering is used to influence and tailor scaffold properties for example protein microstructures can be patterned within 3D HA hydrogels with sub-micrometer resolution to enhance the complexity of scaffolds for use in regenerative medicine, including for neural applications (Burdick & Prestwich, 2011).

Also, Zhang et al. (Zhang & Chen, 2011) have described a femtosecond laser induced two photon polymerisation (TPP) technique that is capable of producing 3D submicron patterns on hydrogel scaffolds with precise control of geometry. PEG diacrylate scaffolds were successfully produced with incorporated chemical cues and

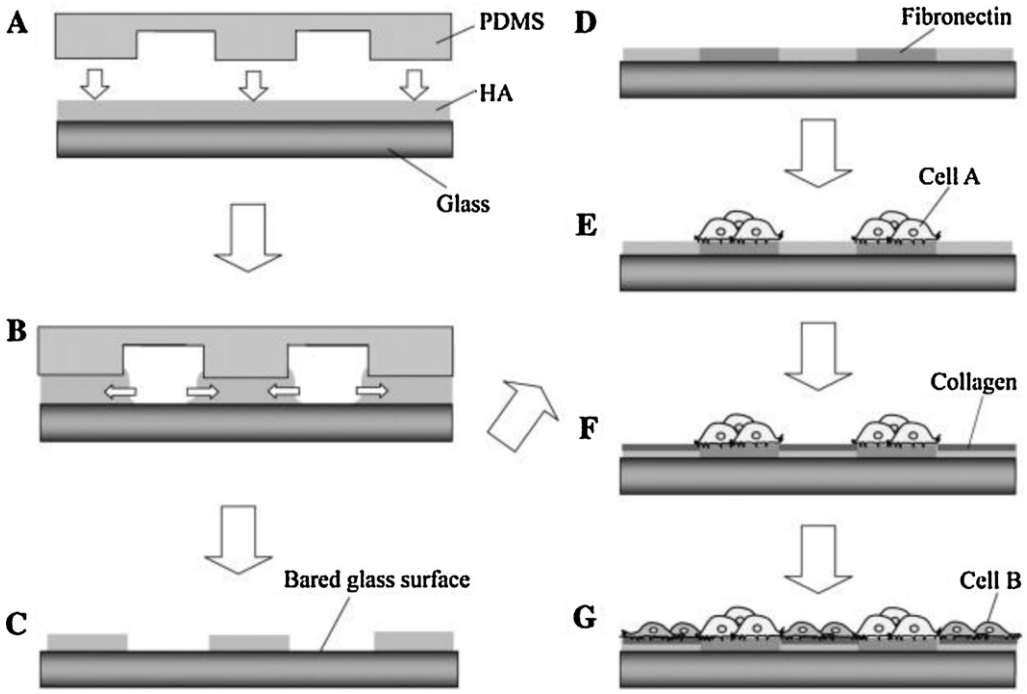

Fig. 7. Schematic for the fabrication of a coculture system by using capillary force lithography and layer by layer deposition a) HA solution is spin-coated onto a glass slide, and a PDMS mold is immediately placed on the thin HA layer, b) HA under the void space of the PDMS mold recedes until the glass surface is exposed, c) the mould is peeled off, d) the exposed region of the glass substrate is coated with fibronectin, e) cells adhere to this region, f) HA surface forms a complex with collagen, g) enabling the subsequent adhesion of secondary cells.

Reproduced with permission from Takahashi et al. (2009).

nanoscale topographical patterning, used to mimic biological cues associated with the ECM.

For further information on the engineering of potential scaffolding materials by nanotechnology techniques the reader is referred to a review by Kelleher and Vacanti (2010).

5. Applications of HA based scaffolding materials

Refer to Table 3 of the current review for a detailed overview of the latest chemistries and applications of HA based scaffolds.

5.1. Space filling and wound healing

Space filling scaffolds provide bulking, prevent adhesions, or function as bioadhesives. In this capacity, the most basic design requirements for a hydrogel based scaffolds are the abilities to maintain a desired volume and structural integrity for a specific length of time. As a filling material, these implants are used to treat conditions such as urinary incontinence (Bent et al., 2001), to maintain alveolar space (Chang, Huang, Yang, Kuo, & Lee, 2012), for cosmetics (Jones, 2011), otolaryngology (Kogan, Šoltés, Stern, & Gemeiner, 2007) and reconstructive surgery (Fagien & Cassuto, 2012; Mossaad & Frame, 2012).

HA (Hyaff) scaffolds have been widely used since 2001 in burn care, for difficult-to-heal wounds, post-traumatic and complicated surgical wounds (Faga et al., in press; Galassi et al., 2000). Recently, HA/Argarose (Zhang et al., 2012) and photocrosslinked HA/fibronectin (Seidlits et al., 2011) based scaffolds have shown great potential in wound healing applications.

5.2. Bone and cartilage tissue repair and regeneration

The encapsulation of cells such as auricular chondrocytes for cartilage regeneration has been investigated with photopolymerisable HA based scaffolds (Erickson et al., 2012; Kim et al., 2012b), with freeze dried HA/Chitosan (Tan et al., 2009) and freeze dried HA/Collagen based scaffolds (Kim et al., 2012a; Zhang et al., 2010b). Human meniscus cells were successfully seeded on freeze dried glutaraldehye crosslinked HA/polyglycolic acid (PGA) scaffolds (Freymann et al., 2011), for further details see Table 3. The presence of HA is sufficient to increase both chondrocyte proliferation and protein secretion. In general, initial proliferation is important to ensure an adequate cell population, but ultimate cartilage formation depends on synthesis of GAGs and type II collagen.

An injectable and biodegradable hydrogel system comprising hyaluronic acid-tyramine (HA-Tyr) conjugates which undergo covalent crosslinking *in vivo* by the addition of small amounts of peroxidase and hydrogen peroxide (H_2O_2) have been investigated by Toh, Lim, Kurisawa, and Spector (2012). Findings suggest that these tunable three-dimensional microenvironments are capable of modulating cellular condensation during chondrogenesis and improve on overall cartilage tissue histogenesis.

Polyethylene glycol diacrylate (PEGDA) crosslinked thiolated HA has been used to deliver mesenchymal stem cells to defects in the patellar groove of rabbit femoral articular cartilage. After 12 weeks, defects were completely repaired (Liu, Shu, & Prestwich, 2006). More recently, porous hyaluronic acid scaffolds have been used for bone morphagic protein (BMP-2) delivery for bone growth and *in vitro* results suggested that the BMP-2 were continuously released for controlled times in an active form from the scaffolds (Kim & Valentini, 2002; Park et al., 2011). Solid free form fabrication of polylactic–co–glycolic acid grafted HA/polyethylene glycol (PEG) scaffolds has successfully delivered BMP-2 *in vivo* with controllable release from the scaffold for up to a month. Histological analyses and staining after implantation in rats revealed active bone regeneration, the BMP-2 released from the scaffold was thought to contribute to enhanced bone regeneration (Park et al., 2011).

Table 3
Recent HA based scaffold chemistries, mode of manufacture, property and biological assessments.

Scaffold Material	Crosslinking reagent	Chemistry	Scaffold manufacture	Scaffold Application	Properties assessed	Biological Assessment	Advantages	Disadv	Reference
HA	Butanediol diglycidyl ether with NaOH pH adjustment	Photochemistry Methacryloyl modification and crosslinking	Gel	General TE applications	Swelling, crosslink densities	–	Shape changing/Anisotropic swelling	Requires prior mod	Zawko et al. (2009)
HA	DVS		Gel	Tissue grafts	Crosslink density, swelling, degradation	Cell culture (rat aortic cells)		Excess crosslinker must be removed	Ibrahim et al. (2010)
HA	EDC		Gas in liquid templating	General TE applications	–	–	–	–	Barbetta et al. (2010)
HA	DL-dithiothrelitol with RGD peptide functionalisation	Methacrylate functionalisation with cystein thiol groups on peptide (Michael type reaction)	Freeze drying	Brain tissue	Swelling, porosity	Human Glioblatoma cells Rat C6 glioma cells	ECM tunable ligand density	–	Ananthanarayanan et al. (2011)
HA	EDC	Immersion	Ice particulate templating	General TE applications	Pore size	Cell culture/seeding	Controlled porous structures		Ko et al. (2011)
HA based	Adipic dihydrazide (ADH) mod EDC PEG tetra thiol Peptides	1. MMP peptide PEG thiol crosslinker 2. HA-ADH mod. Crosslinker lamine peptide PEG thiol crosslinker (Michael type reaction)	–	Spinal cord	–	*In vitro* and *in vivo* testing hMSCs proliferation Cytoxcity	Biomemmetic scaffold Positive influence on regeneration of motor function		Park et al. (2010)
HA	GTA, EDC, DVS	Immersion	Freeze drying	General soft tissue applications	Swelling, thermal, porosity compression	–	Macroporus, interconnected, adequate mechanical properties, easily processed	Excess crosslinker removal required	Collins and Birkinshaw (2011)
GMHA	Photocrosslinking Irgacure 2959 N-vinyl pyrrolidone	Photochemistry	–	Tissue applications and protein delivery	Porosity	Protein release	Controlled degradation		Leach et al. (2003)
Polylactic-co-glycolic acid g HA/PEG	Adipic acid Dihydrazide	HA-ADH mod. HA-ADH conjugated with PEG	Solid free form fabrication Multi head deposition system	Bone regeneration	–	Bone morphagic protein (BMP) implanted in rats	New scaffold prep approach BMP attached	Phase separation may occur between HA and PEG	Park et al. (2011)
HA based	–	CDCHR receptor	–	Periodontal tissue		Cell culture Cell adhesion PCR Histology		Inadequate scaffold details	Takeda et al. (2011)
HA based	Sodium periodate (oxidation)	Oxidation EDC coupling of ADH	Gel	Anti cancer drug delivery		Cell culture C42B cells	Performs better than 2D systems in anticancer drug delivery		Gurski et al. (2009)
HA/PGA	Glutaraldehyde	–	Freeze drying	Meniscal repair	Porosity	*In vitro* cell viability (human meniscus cells)	Gene expression for meniscus cells		Freymann et al. (2011)
HA/Collagen	EDC	–	Freeze drying	Brain tissue	Compression	Neural stem cells	Good mech properties		Wang and Spector (2009)
HA/Collagen	EDC	–	Freeze drying	Adipose tissue	Swelling	3T3–C1 Preadipocytes	Degradation resistant	Highly swollen	Davidenko et al. (2010)

Table 3 (*Continued*)

Scaffold Material	Crosslinking reagent	Chemistry	Scaffold manufacture	Scaffold Application	Properties assessed	Biological Assessment	Advantages	Disadv	Reference
HA/Gelatin	EDC	–	Freeze drying	Soft tissue	Swelling	L929 fibroblasts	Good mech properties Degradation resistant No cytotoxcity effect		Zhang et al. (2011)
HA/Collagen	EDC	–	Freeze drying	Angiogenesis	Swelling, porosity	Histological studies with mice	Good angiogenesis		Perng et al. (2011)
Poly ε caprolactone/Collagen/HA	Hepracil™	Hepracil™	Electrospinning	Vascular		Growth factor incorporation	Supports cellular attachment and vascularisation		Ekaputra et al. (2011)
HA/Chitosan		Schiff base links	Freeze drying	Encapsulant/tissue scaffold		Fibroblasts	Non toxic chemistries	Pretreatment required	Nair et al. (2011)
HA/Collagen	Ethyleneglycol diglycidyl ether		Freeze drying	Cartilage	Mechanical, degradation	*In vitro* cell adhesion and proliferation/chondrocyte cells implantation in rabbit ears	Easily processed		Kim et al. (2012b)
HA/Argarose	Epichlorohydrin		Freeze drying	Wound healing	Porosity, swelling, degradation, thermal	*In vitro* cytoxicity/ Fibroblasts mice implanation	Controlled degradation		Zhang et al. (2012)
HA/PLLA	–	–	Electrospinning	Spinal	Porosity	PCR Immunohistology Human bone marrow cells	–		Nesti et al. (2008)
HYAFF 11		Benzyl ester chemistry	Salt leaching		Mechanical, porosity	C2C12 cell line, cell seeding and viability	Solvent free manufacture Porosity control		Flaibani and Elvassore (2012)
HA/Chitosan		Schiff base reaction	Freeze drying	Cartilage tissue	Swelling, compression, gelation, morphology	Bovine articular chondrocytes	Non toxic reagents, injectable	Oxidation of HA required	Tan et al. (2009)
HA/Fibronectin	Irgacure 2959 N-vinyl pyrrolidone	Photocrosslinking HA acrylation	Gel	Wound healing	Swelling	Endothelial cells	Wound healing enhancement	HA acrylation required	Seidlits et al. (2011)
HA/Fibrin	ADH and EDC		Gel	Cancer therapy		DNA delivery	Prevents polyplex aggregation		Lei et al. (2011)
HA/Collagen/ Chondroitin sulphate	ADH, EDC, genipin	ADH mod EDC and genipin crosslinking	Freeze drying	Cartilage tissue	Compression-swelling, degradation	Histolgical staining in rabbits	Crosslinking in mild conditions	Fast degradation	Zhang et al. (2010b)
HA g HEMA	Ceric ammonia nitric	Oxidation/grafting	–	Pulmonary tissue	Thermal, swelling, mechanical	Mammalian cells (alveolar) L929 fibroblasts cytotoxicity studies	Uncrosslinked		Radhakumary et al. (2011)
HA/PEGDA	EDC, Cysteamine	Michael type addition of thiolated HA RGD functionalisation	Gel	General tissue engineering applications	Degradation (Enzymatic) Swelling, mechanical, rheology	L929 fibroblasts (MTS Assay)	Tuneable cell material interaction	Disulfide bonds form by wasteful intramolecular reactions, phase seperation	Ouasti et al. (2011)
HA	DVS	Carbon nanotube reinforcement	Freeze dried	General tissue engineering applications	Swelling, porosity, rheology – mechanical	–	Increased mechanical performance		Bhattacharyya et al. (2008)

5.3. Nerve and brain tissue repair

For applications of hyaluronic acid based scaffolding materials in central neural tissue engineering please refer to a recent comprehensive review by Wang et al. (2012). A crosslinked thiol-modified heparin, HA/gelatin (HyStem-HP) has been investigated as a possible vehicle for cell delivery to provide a translational therapy for stroke recovery. Results show that cell survival was improved, glial scar formation was reduced, and local inflammation was minimised for the scaffold delivered cells in comparison to neural progenitor cells (NPCs) delivered in buffer only (Zhong et al., 2010). Freeze dried HA/Collagen scaffolds with enhanced compression strengths were studied by Wang and Spector (2009). An electrospun HA/poly lactic acid (PLLA) scaffold was used to deliver human bone marrow cells by Nesti et al. (2008). Human glioblatoma cells have been seeded on a freeze dried methacrylate functionalised HA scaffolds for brain tissue applications (Ananthanarayanan et al., 2011). Biomimetic HA based scaffolds used to deliver human mesenchymal stem cells have shown a positive influence on the regeneration of motor function (Park et al., 2010).

5.4. Scaffolds for bioactive molecule and cell delivery

HA based hydrogels that are both hydrolytically (via ester group hydrolysis) and enzymatically degradable were synthesized by introducing hydrolytically degrading esters (e.g., lactic acid or caprolactone) between the HA backbone and the photoreactive groups (Sahoo, Chung, Khetan, & Burdick, 2008). These materials allow for enhanced control over the degradation of the HA hydrogels and therefore towards their application as delivery vehicles (Ekaputra et al., 2011; Gurski, Jha, Zhang, Jia, & Farach-Carson, 2009; Lei et al., 2011; Nair et al., 2011; Perng et al., 2011).

5.5. Soft tissue repair and smooth muscle engineering

The engineering of heart valves is prevalent due to diseases and damage that inflict natural heart valves. Valvular interstitial cells (VICs) have been difficult to culture in a range of common culture environments, including peptide- and protein-modified surfaces; however, these cells adhered to and proliferated on HA based hydrogels (Burdick & Prestwich, 2011).

A hybrid scaffold of poly (ε-caprolactone)-collagen blend (PCL/Col) and HA hydrogel, was created with incorporated growth factors. These scaffolds were shown to support cellular attachment and the recapitulation of primitive capillary network in the scaffold's architecture (Ekaputra et al., 2011). Ibrahim, Joddar, Craps, and Ramamurthi (2007) describe the immobilisation of HA oligomers, utilizing the carboxyl functional group, and it is envisaged that this technique could be used to tailor the design of HA scaffolds suited to vascular tissue engineering applications. Perng and co-workers have utilised freeze dried HA/collagen scaffolds to promote angiogenesis (Perng et al., 2011) while Davidenko et al., have seeded similar scaffolds with 3T3-C1 preadipocyctes for adipose tissue regeneration (Davidenko et al., 2010). HA/gelatin scaffolds with slow degradation times have been produced for soft tissue applications by Zhang and co-workers (Zhang et al., 2011). Embryonic endothelial progenitor cells (eEPC, murine) were encapsulated in HA-hydrogels (HyStem-C/Extracel) to create a stem cell niche for renal regeneration (Ratliff et al., 2010).

A recent excellent paper by Espander and his group describes the growth *in vivo* of human adipose stem cells on various HA based scaffolds which have successfully expressed human cornea-specific proteins opening up future possibilities for a bioengineered cornea (Espandar et al., 2012).

Table 4
Assessment of scaffolds coated with HA.

Scaffold	Material	Crosslinking reagent	Scaffold manufacture	Scaffold application	Properties assessed	Biological assessment	Advantages	Disadv	Reference
Elastomeric scaffold/HA	DVS	Template leaching	General tissue engineering applications	Water sorption, porosity	–				Arnal-Pastor et al. (2011)
Poly-L-lactic acid/HA	GTA	Salt leaching	Bone tissue	Mechanical, Thermal	L929 culturing and seeding	Mechanical performance is enhanced	Reduced cell viability in some instances		Antunes et al. (2011)
PCL-PLGA/HA-Fibrin		Solid free form (multi head deposition system – MHDS)	Bone tissue	Porosity	Stromal cells	Bone formed well	MHDS not suitable for proteins		Kang et al. (2011)

5.6. HA coated scaffolds

Recently, HA hydrogels have been used to coat scaffolds produced from poly-L-lactic acid to enhance bone tissue regeneration (Antunes et al., 2010), poly(caprolactone) poly(lactic-co-glycolic acid) to enable protein incorporation (Kang et al., 2011) and elastomeric materials to influence swelling (Arnal-Pastor et al., 2011), see Table 4.

6. Conclusion and future trends

Hyaluronic acid offers great practical potential as a scaffolding material, a range of crosslinking techniques are available, both to enhance the material residence time as well as to control its mechanical properties. The resulting materials offer advantageous properties such as bioresorbability, inhibition of scar formation and promotion of angiogenesis (Pan, Ren, Cui, & Xu, 2009; Shoichet, 2009). Cell adhesion ligands and growth factors can also be incorporated in the HA based scaffold to enhance the rate of tissue regeneration. Future tissue engineering applications will be based on the emergence of technologies such as micropatterning of hydrogels and hydrogel processing using lithography and microfluidics to produce micro-bioreactor systems allowing micrometer level spatial control over cell growth and differentiation. Much fundamental work has now been published and it is expected that the next few years will see the practical realisation of some of these ideas in clinical trials.

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