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ALBUMIN-BASED HYDROGELS FOR REGENERATIVE ENGINEERING AND CELL TRANSPLANTATION

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ABSTRACT

Albumin, the most abundant plasma protein in mammals, is a versatile and easily obtainable biomaterial. It is pH and temperature responsive, dissolvable in high concentrations and gels readily in defined conditions. This versatility, together with its inexpensiveness and biocompatibility, makes albumin an attractive biomaterial for biomedical research and therapeutics. So far, clinical research in albumin has centred mainly on its use as a carrier molecule or nanoparticle to improve drug pharmacokinetics and delivery to target sites. In contrast, research in albumin-based hydrogels is less established albeit growing in interest over recent years. In this mini-review, we report current literature and critically discuss the synthesis, mechanical properties, biological effects and uses, biodegradability and cost of albumin hydrogels as a xeno-free, customisable and transplantable construct for tissue engineering and regenerative medicine.

Keywords: serum albumin, hydrogel, crosslinking, stem cells, tissue engineering, regenerative medicine

INTRODUCTION

Albumin, an endogenous, non-glycosylated protein, is produced predominantly in the liver by hepatocytes and secreted into blood as a major constituent of plasma. It is comprised of 585 amino acids, has a molecular weight of 66.4 kDa, and an iso-electric point of pH 4.7 (Vlasova & Saletsky, 2009). *In vivo*, albumin is a stable molecule because it is poorly metabolised, poorly immunogenic and poorly filtered in the renal glomerulus (Lee & Youn, 2016). As a result, albumin has a physiological half-life of approximately 19 days, during which it maintains oncotic pressure in the circulatory system, acts as a weak buffer, and stabilises other important proteins, hormones, metal ions, nanoparticles and drugs *in vitro* and *in vivo.* Albumin has two significant non-covalent binding sites that exogenous substances attach to; Binding Site 1 and Binding Site 2. In so doing, the half-life and treatment efficacy of drugs such as antibiotics, anti-inflammatories and synthetic insulin preparations are increased (Kratz, 2008; Lee & Youn, 2016). Other important biological characteristics of albumin include its accumulation at sites of inflammation from leaky capillaries and its active uptake by cancer cells, making it useful for targeting disease in molecular cancer therapeutics (Elsadek & Kratz, 2012; Lee & Youn, 2016).

Despite extensive research in albumin as a molecule for drug therapy, its use as a hydrogel in biomedical research is comparatively under-studied. However, interest is steadily growing because albumin hydrogels offer a non-synthetic, xeno-free and biocompatible biomaterial for the fields of tissue engineering and regenerative medicine which increasingly employ three-dimensional (3D) cell cultures, tissue scaffolds and constructs for disease modelling and transplantation. In addition, its inertness, stability, ability to gel at low concentrations and the possibility of deriving patient specific albumin, make albumin hydrogels an attractive option. This review summarises work on albumin hydrogels over the past decade and

specifically discusses the (i) synthesis, (ii) mechanical properties, (iii) biological effects and uses, (iv) biodegradability and (v) cost.

For this review, the adopted definition of a hydrogel is: a two- or multi-component system, consisting of a 3D network of polymeric chains, where water occupies the spaces between those polymeric chains (Ahmed, 2015). Articles reporting hydrogels formed by other polymers but functionalised with albumin have been excluded in this review. A brief overview of the properties of albumin-based hydrogels is provided in Table 1.

SYNTHESIS OF ALBUMIN HYDROGELS

pH-induced albumin hydrogels.

Albumin exists either as monomers or oligomers depending on its environment (Barone et al., 1995; Molodenskiy et al., 2017). By manipulating pH, albumin in solution polymerises and forms a clear hydrogel. Baler, Michael, Szleifer, and Ameer (2014) reported that by lowering the solution pH to 3.5 followed by 37°C incubation, bovine serum albumin (BSA) changes structure from the "N-form" to the "F-form" isomer which then self-assembles into a hydrogel network by hydrophobic interactions and counter ion binding (Figure 1). Crucially, neutralisation of the acid-induced hydrogels by leaching in Dulbecco's Modified Eagle Medium (DMEM) was required before acellular hydrogels could be transplanted into murine models. This implies it is not feasible to encapsulate pH-sensitive cells in the bulk of the gel using this method of gelation. However, it does not preclude acid-induced albumin hydrogels from being functionalised and used as a scaffold after pH neutralisation is achieved. Also noteworthy is that BSA is only 76% similar in amino acid sequence compared to human serum albumin (HSA) (Carter & Ho, 1994; X. M. He & Carter, 1992) therefore gelation behaviour and properties of HSA hydrogels may differ even if gelation methods and conditions are standardised.

Recently, both pH and temperature-dependent gelation behaviour in BSA and HSA have been extensively studied by Arabi et al., 2018. This led to the physical characterisation of both BSA and HSA hydrogels through several phase diagrams (Figure 2). Interestingly, the authors established that gelation of BSA and HSA can occur over a wide pH range and temperatures (pH 1.0 - 4.3 and pH > 10.6 at 37 ºC or pH 7.0 - 7.2 at 50 - 65 ºC). However, the gelling mechanism of BSA and HSA, or the biocompatibility of alkali-induced albumin hydrogels were not investigated. It is highly likely molecular and structural differences in albumin isomers exist across the different gelling conditions, and this will in turn affect the properties of the albumin hydrogel such as available binding sites. Further research in this area can help in the conjugation or functionalisation of albumin hydrogels with target proteins in the future.

In contrast to acidic pH, albumin transitions from the N-form isomer to the B-form (basic form) around pH 8 then to the A-form (aged form) around pH 10 and above (Amiri, Jankeje, & Albani, 2010; J. Chen et al., 2019; Leggio, Galantini, & Pavel, 2008). The A form isomers then form aggregates and the exact mechanism of gelation remains poorly understood (J. Chen et al., 2019). Recently, J. Chen et al., 2019 reported that alkali-induced BSA hydrogels formed at pH 12 and 37 ºC incubation were mechanically stable, and exhibited self-healing and auto-fluorescence properties. However, similar to acid-induced albumin hydrogels, alkali-induced hydrogels required neutralisation with DMEM to pH 7.4. Biocompatibility of the neutralised hydrogel was subsequently demonstrated by cell culture of human lung carcinoma cells (A549 cell line) over a 48-hour period. Unfortunately, no quantitative data on cell experiments were provided and the long term (weeks to months) *in vivo* stability of these alkali-induced hydrogels remains unknown.

Thermally-induced albumin hydrogels

Heat induced gelation is a more commonly reported method to obtain stable albumin hydrogels (Amdursky et al., 2018; Arabi et al., 2018; Baler et al., 2014; Nandlall et al., 2010; Peng et al., 2016). However, one important consideration is that applying high heat will cause the denaturation of albumin since its structure starts to unfold at temperatures above 65 ºC (Borzova et al., 2016). The higher the temperature above 65 ºC, the greater the degree of unfolding and aggregation. This denaturation temperature of albumin can also be lowered by changes in pH and the addition of ions or redox reagents e.g. magnesium (Haque & Aryana, 2002) and urea (Gonzalez-Jimenez & Cortijo, 2002) respectively. Another important consideration is that with the denaturation and aggregation of albumin, its binding sites for ions, drugs and proteins can change, together with other physical properties of the albumin hydrogels such as turbidity.

pH-neutral, thermally-induced albumin hydrogels increase in turbidity as temperature, albumin concentration and ionic content increase due to extensive denaturation of albumin molecules (Amdursky et al., 2018; Murata, Tani, Higasa, Kitabatake, & Doi, 1993). This is in stark contrast to pH-induced albumin hydrogels which still have a clear to translucent appearance at higher albumin concentrations when incubated at room temperature to 37 ºC, even though denaturation still occurs. Baler et al., 2014 have demonstrated that thermally induced BSA hydrogels have larger pore sizes, a higher Young's modulus and lower degradability compared to pH-induced BSA hydrogels, however these properties vary with albumin concentration and more extensive characterisation over a wider range of gelation conditions is needed, particularly in HSA. The tuneable characteristics of thermally-induced albumin hydrogels indeed make it seem appealing, but opaque or turbid hydrogels have limited usefulness in biological studies since it precludes normal brightfield microscopy. However, this may be overcome by the addition of sodium chloride. To reduce the turbidity of thermally-induced albumin hydrogel, Murata et al., 1993 reported that the addition of

sodium chloride into BSA solution resulted in transparent gels within a specific concentration range. Studies to determine if this effect is reproducible in HSA are still pending.

Interestingly, apart from applying high heat, it has been recently demonstrated that albumin hydrogels could be formed by salt induced cold gelation (Ribeiro et al., 2016). With the addition of calcium chloride and DL-Dithiothreitol to a BSA/HSA mix and heating at 60 ºC for 30 minutes, followed by cooling and freezing at -20 ºC for two days, an albumin hydrogel can be obtained. The resulting hydrogel was freeze-dried to create a porous scaffold which was later shown to be biocompatible.

In summary, current methods to derive pH-induced or thermally induced hydrogels have shown that the (i) albumin concentration, (ii) the presence of ions or redox reagents, (iii) the range of pH, (iv) heating temperature, and (iv) duration of heating, are all crucial factors for gelation. These in turn affect the final properties of the albumin hydrogel.

Chemically crosslinked albumin hydrogels

Chemical crosslinking is the most reported method to derive albumin hydrogels (Abbate, Kong, & Bansal, 2012; Bai et al., 2019; Feldman & McCauley, 2018; Gallego, Junquera, Meana, Alvarez-Viejo, & Fresno, 2010; Gallego, Junquera, Meana, Garcia, & Garcia, 2010; P. He, Jean-Francois, & Fortier, 2012; Hirose, Tachibana, & Tanabe, 2010; Kim et al., 2015; Li et al., 2014; Lisman, Butruk, Wasiak, & Ciach, 2014; Ma et al., 2016; Manokruang & Lee, 2013; Noteborn, Gao, Jesse, Kros, & Kieltyka, 2017; Oss-Ronen & Seliktar, 2011; Overby & Feldman, 2018; Raja, Thiruselvi, Mandal, & Gnanamani, 2015; Scholz et al., 2010; Upadhyay & Rao, 2019; Zhao et al., 2019; Zhou et al., 2018). Synthetic polymers such as Polyethylene Glycol (PEG) are activated to form PEG-albumin complexes (e.g. with 4-nitrophenyl-chloroformate), or alternatively functional groups may be added to the ends of the PEG molecule to target specific chemical compositions or binding sites of other target

proteins for conjugation. For example, the methoxy-polyethylene glycol group at the ends of each PEG- succinimidyl propionate (PEG-SPA), PEG-succinimidyl succinate (SS) and PEG-succinimidyl glutarate (PEG-SG) molecule, are able to exchange a hydroxyl group with a N-hydroxysuccinimide (NHS) group. These functionalised PEG-NHS molecules can then form amide linkages with amino acids such as lysine from target proteins.

The configuration of the PEG backbone and the number of hydrolytically cleaved functional groups determine the overall stability of PEG-NHS molecules. As such, PEG-SS and PEG-SG molecules are more easily degraded compared to PEG-SPA since these molecules contain esters in their backbone which are affected by hydrolysis. *In vivo* once hydrolysed, PEG chains are cleared mainly through the kidneys, and to a lesser extent, the liver and gut (Baumann et al., 2019). This can be taken advantage of to suit the rate of biodegradability desired. Apart from functionalisation with NHS groups, PEG can also be functionalised with maleimide (PEG-MAL) or diacrylate (PEG-DA). The -MAL end group crosslinks thiol groups that are present in amino acids such as cysteine and thiolated target proteins such as thiolated albumin. PEG-DA is activated by exposure to ultraviolet light and photo-crosslinking ensues. However, intracellular damage from reactive oxygen species (ROS) and cytotoxicity may result from prolonged or high intensity UV exposure (de Jager, Cockrell, & Du Plessis, 2017) if cells were encapsulated in bulk during gelation. This can be circumvented by creating a porous scaffold through sacrificial moulding then seeding cells within it (Shirahama et al., 2016). Other less commonly used agents to crosslink albumin include glutaraldehyde (Gallego, Junquera, Meana, Alvarez-Viejo, et al., 2010; Gallego, Junquera, Meana, Garcia, et al., 2010; Ma et al., 2016; Upadhyay & Rao, 2019; Zhao et al., 2019), glutathione (Bai et al., 2019; Raja et al., 2015), dithiothreitol (Hirose et al., 2010), transglutaminase (Li et al., 2014), polyaminourethane (Manokruang & Lee, 2013), oxidised dextran (Lisman et al., 2014) and N,N-methylenebisacrylamide (MBA) (Abbate et al., 2012).

With PEG being the most common polymer used in the crosslinking of albumin, perhaps the greatest concern in the clinical application of PEG-albumin hydrogels is immunogenicity. Although it is well established that albumin itself is poorly immunogenic, there is growing evidence that PEG is not bio-inert. Clinical trials involving PEGylated drugs have demonstrated that the occurrence of PEG-specific IgM and IgG antibodies in patients is not infrequent and it can result in reduced drug efficacy, mild to moderate immune reactions and adverse outcomes (Zhang, Sun, Liu, & Jiang, 2016). This considered, other methods of albumin gelation and conjugation should be explored.

MECHANICAL PROPERTIES

Uniaxial compression and tension, and indentation have been employed to measure the Young's modulus of albumin hydrogels (Amdursky et al., 2018; Baler et al., 2014; Fleischer et al., 2014). The main concern when testing compliant materials such as hydrogels is separating inelastic (time-dependent) and elastic characteristics since the Young's modulus should be independent of time. In this context, the load and displacement measuring systems and the inertia of the testing setup are important. Often hydrogels require a customised set-up suitable for low load testing rather than conventional mechanical testing set-ups. Nano- and micro-indenters on the other hand have accurate load and displacement measuring systems, but inevitably indentation is likely to generate regions of high local stress, which make inelastic deformation even more likely. Furthermore, unless a relatively large indenter tip is used, indentation may not be suitable for property measurement since it cannot sample volumes large enough to be representative.

Baler et al., 2014 measured the Young's modulus of pH- and thermally-induced BSA hydrogels using a custom built flat-ended cylindrical indenter with a radius of 0.44 mm. For a 17 wt% pH-induced hydrogel, the values were found in the range of 3 to 35 kPa for pH

between 3 and 4, with the highest value obtained at pH 3.5. No solid gels were formed for pH below 3 and above 4. The 20 wt% pH- and thermally-induced hydrogels gave values of about 46 and 67 kPa respectively. Fleischer et al., 2014 measured the Young's modulus of electrospun 10% (w/v) BSA scaffolds under uniaxial tension. Scaffolds were submerged in PBS for 15 min prior to testing. The Young's modulus values were 1.22 ± 0.07 and 0.43 ± 0.07 MPa respectively for uniaxially-aligned and randomly-oriented albumin scaffolds, with ribbon-like fibres. No information was provided on the loading direction. It is assumed that they measured the through-thickness Young's modulus of the scaffolds and that the albumin fibres in both scaffolds were lying in-plane. The randomly-oriented fibrous scaffolds were reported to have a larger pore size and slightly wider albumin fibres. No information was provided on the scaffold porosity and fibre density so it is difficult to make any comparisons. Amdursky et al., 2018 measured the Young's modulus of 3-9 wt% BSA hydrogels under tension. The hydrogels were not submerged in solution during testing. The Young's modulus was found to increase from ~5 to 17 kPa with increasing albumin concentration. Under confined compression, the values varied from ~0.2 to 4.4 MPa for 3-9 wt% BSA hydrogels. In all of the above studies, it would have been useful if the authors provided an expanded view of the low strain region used to measure the Young's modulus.

Rheology tests have also been used to characterise the viscoelastic behaviour of BSA hydrogels. Baler et al., 2014 investigated the gelation kinetics of both pH- and thermally-induced BSA hydrogels at 37 and 80 °C respectively, with a 0.5% oscillatory strain. pH-induced BSA hydrogels (pH 3.5) formed slowly (~330-2300 s) compared to thermally-induced BSA hydrogels (~20-65 s). They exhibited a lower storage modulus (G') compared to thermally-induced hydrogels with the same BSA concentration. The G' and loss modulus (G'') values for both 16 and 20 wt% thermally-induced BSA hydrogels reached a plateau at around 120 and 60 kPa respectively after 30-50 s. Amdursky et al., 2018 obtained G' ~ 13 kPa

and G''~ 4 kPa for a 9 wt% thermally-induced BSA hydrogel (10 Hz, 80°C and 0.5% oscillatory strain).

There is limited data on the strength and failure strains of albumin hydrogels (Amdursky et al., 2018; Zhou et al., 2018). Zhou et al., 2018 reported tensile strengths of about 40 kPa for both a 20 wt% HSA hydrogel and a 0.5% Bioglass-activated/HSA composite hydrogel. Amdursky et al., 2018 measured tensile failure strains between 35-100% for 3-9 wt% BSA hydrogels. The stress-strain curves suggest that increasing BSA concentration does not have a strong effect on the measured fracture strengths, which are a few kPa, whereas failure strains tend to decrease markedly reducing the toughness of the hydrogels.

BIOLOGICAL EFFECTS AND USES

Albumin is confined mainly to the vascular and interstitial space within the human body. It binds to nine different cell surface receptors and is relatively inert to many cell types (Merlot, Kalinowski, & Richardson, 2014). Several binding sites on albumin allow the attachment of important molecules, proteins and ions which in turn provides stability in solution. It is therefore used commonly in cell culture media as a carrier protein, however albumin alone in its normal form (N-form) has rarely been used as a culture matrix because of limited cell attachment in two dimensional cultures (Hirose et al., 2010). Several groups have overcome this problem successfully by functionalising albumin hydrogels with fibronectin (Amdursky et al., 2018), laminin (Fleischer et al., 2014) or culturing cells in 3D hydrogels with crosslinked or denatured albumin. Below, studies grouped by experimental cell or tissue types are discussed.

Bone and Cartilage Regeneration

Two separate studies (Gallego, Junquera, Meana, Alvarez-Viejo, et al., 2010; Gallego, Junquera, Meana, Garcia, et al., 2010) isolated human osteoblasts from teeth (third

mandibular molars) and cultured these cells in HSA enriched media. An albumin-rich gel was then created from patient derived serum by first crosslinking with glutaraldehyde. Further freezing at -70 ºC overnight and dehydration with ethanol created a porous scaffold which was later seeded with osteoblasts. These constructs were transplanted beneath the skin of immuno-deficient mice. Osteoblast proliferation was reported both *in vitro* and *in vivo*. After 75-150 days, analysis of transplanted constructs demonstrated the deposition of human vimentin, osteocalcin, calcium and phosphate matrix along with bone within the pores of the scaffold. Here the significance of vimentin positivity is unclear. Vimentin inhibits osteoblastic differentiation, but the deposition of bone matrix by mature osteoblasts was reported. It is also important to note that the albumin-rich scaffolds were derived from human serum which may contain other proteins and growth factors that were not removed by the gelation and drying process. Therefore, it is not possible to attribute any biological effects observed in this study solely to albumin although there is evidence in literature that albumin itself encourages osteoblast proliferation (Ishida & Yamaguchi, 2004).

Apart from bone-forming cells (osteoblasts), chondrocytes derived from human articular cartilage were able to proliferate in a PEGylated albumin hydrogel supplemented with hyaluronic acid (Scholz et al., 2010). It was reported that cells cultured within this hydrogel had a characteristic gene signature for aggrecan, collagen type I and type II. Unfortunately with the presence of three polymers in the hydrogel, it is not discernible what the actual effects of albumin are. Nonetheless it serves its function as a biocompatible scaffold.

Interestingly, Li et al., 2014 reported that freeze-drying an albumin gel crosslinked with transglutaminase produced a scaffold with physical and mechanical properties similar to collagen scaffolds. More significantly, the authors were able to successfully differentiate human mesenchymal stem cells (MSCs) seeded in these scaffolds into osteoblasts,

demonstrating the potential of such a scaffold for bone tissue engineering and regenerative medicine. However, one important limitation is that these scaffolds were made with BSA and further research is needed to determine if this is reproducible with HSA hydrogels for future clinical applications.

Skin Regeneration and Wound Healing

Feldman & McCauley, 2018 reported that a species-specific, albumin hydrogel scaffold could accelerate the epithelialisation rate of full thickness wounds after two weeks. This effect was further augmented with the introduction of MSCs expressing $TGF\beta_3$ in the bulk of the scaffold. These experiments were conducted using albumin derived from rabbits and transplanted into immuno-competent rabbits, however no significant differences were noticed when comparing the overall rate of wound healing with control groups. Admittedly, the study was also underpowered to detect an effect. Zhou et al., 2018 created a composite albumin hydrogel by crosslinking HSA with PEG-SS_2. Bioglass was added to increase the gelation time of the composite gel and to allow the delivery of calcium and silicon ions at the site of injury after injection of the acellular hydrogel. The authors demonstrated wound healing, measured by epidermal thickness, dermal thickness, and angiogenesis, were significantly increased by acellular HSA-PEG-SS_2 hydrogels, but the greatest effect was observed in the composite hydrogel with Bioglass (HSA-PEG-SS_2-Bioglass) at 14 days. It is noteworthy that an immuno-deficient mouse model was used (BALB/c nude). As such, the effect of the HSA-PEG-SH_2-Bioglass hydrogel in the presence of a competent immune system is not known and this may conceal immune-mediated reactions or detrimental effects on wound healing in normal test subjects.

Apart from human MSCs, human skin fibroblasts (BJ-5ta) (Ribeiro et al., 2016) and mouse adipose fibroblasts (L929) (Hirose et al., 2010; Lisman et al., 2014) have also been cultured

successfully on albumin hydrogels but these were used only in the context of cytotoxicity testing.

Lung & Breast

Cell survival studies have been performed successfully with lung cancer cell lines A549 (Bodenberger, Kubiczek, & Rosenau, 2017; Jun Chen et al., 2016; Ma et al., 2016), and breast cancer cell lines MCF7 (Bodenberger et al., 2017) and ZR75-1 (Nandlall et al., 2010). However, the use of albumin hydrogels in lung and breast tissue engineering as well as regenerative medicine is limited.

Heart

Albumin hydrogels have been reported to enhance the functionality of neonatal rat ventricular cardiomyocytes (NVRM) and cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) (Humphrey et al., 2017). Humphrey et al., 2017 reported albumin hydrogels had a positive effect on calcium handling (time to peak and rate of decay) in NVRM and hiPSC-CMs. The authors used glass as a negative control, however a positive control with an alternative matrix was missing. It is therefore not possible to discern what the effects of albumin hydrogels are in comparison to physiological standards. Amdursky et al., 2018 reported that NVRM cultured on a pH-induced albumin hydrogel and functionalised with fibronectin, produced NVRM with gene profiles (Myh7, Myh6, Myl2, Actn2, Tnnt2, Acta2, SERCA2, Atp2a2, Slc8a1, Pln and Ryr2) closely resembling that of freshly isolated cardiomyocytes, whilst NVRMs cultured on glass alone began to de-differentiate. Furthermore, co-culture of NVRM with rat endothelial cells, smooth muscle cells and fibroblasts on the surface of the hydrogel resulted in contractile cardiac tissue which could be paced by external electrical stimulation (Figure 3). Interestingly, neutralisation of the acidic

albumin hydrogel by leaching with neutral-pH media was not performed and the effect of the albumin hydrogel acidity (pH 2) on NVRM is not known.

Fleischer et al., 2014 created an electrospun scaffold from albumin hydrogels crosslinked by trifluoroethanol (TFE) and β-mercaptoethanol (BME). Interestingly, the authors reported that NVRM proliferated, self-organised and formed cardiac tissue in these albumin scaffolds when functionalised with laminin. Furthermore, indices of cardiac function; rate of contractility and amplitude, were significantly enhanced compared to scaffolds made from polycaprolactone (PCL). However, it is important to note that the laminin was applied by coating the albumin scaffolds with fetal bovine serum (FBS) instead of pure laminin alone. This suggests other soluble proteins and growth factors in FBS could also be present on the fibres and not just serum laminin alone. Also, the control group with PCL scaffolds were coated with fibronectin instead of FBS so a fair comparison cannot be made.

Liver

There is limited research on albumin hydrogels in liver tissue engineering and regenerative medicine. Zhao et al., 2019 created a ruthenium-albumin hydrogel crosslinked by glutaraldehyde and reported cell survival of both liver cancer cell line HepG2 and normal human fetal hepatocyte cell line L02. The survival rates of HepG2 cells decreased with increasing concentrations of ruthenium but this was an intended effect.

Nerves

Albumin scaffolds promoting the proliferation, differentiation and branching of human iPSC derived neural stem cells (hiPSC-NSC) was reported by (Hsu, Serio, Amdursky, Besnard, & Stevens, 2018). An electrospun scaffold was created from albumin hydrogels crosslinked by TFE and BME, then coated with hemin, laminin and basic fibroblast growth factor (FGF2).

hiPSC-NSCs seeded on uncoated albumin scaffolds were observed to have significantly high death rates. Oddly, the cell death rates on both coated and uncoated albumin scaffolds were similar. In contrast, cell death rates on uncoated glass (negative control) were significantly lower. More Ki67 positive cells were also observed on uncoated glass than on coated scaffolds although there were more β3-tubulin positive cells in coated scaffolds. Neurite branching was only observed to be more significant than the negative controls when an electrical stimulus was applied. Given the mixed results, further investigation is needed in this area.

Drug delivery

The role of albumin molecules in drug delivery is well established, however research in albumin hydrogels for controlled drug release and delivery is still growing. Kim et al., 2015 utilised a PEG-HSA hydrogel loaded with an apoptotic TRAIL protein to successfully induce cancer cell death and reduce tumour size in a murine model injected with a pancreatic cancer cell line (Mia Paca-2). Successful controlled drug release was also demonstrated using a composite hydrogel (Dextran-HSA-PEG) loaded with anti-cancer drug doxorubicin to eliminate breast cancer cells (MCF-7) *in vitro* (Noteborn et al., 2017). More recently, Zhao et al., 2019 demonstrated the ability of albumin hydrogels to selectively deliver metal ions to liver cancer cells (HepG2) for anti-cancer therapy or imaging.

BIODEGRADABILITY

The biodegradability of albumin hydrogels depends on the way the albumin hydrogel is synthesised. Baler et al., 2014 demonstrated that albumin hydrogels formed by electrostatic self-assembly in acidic pH were easily degradable *in vitro* and *in vivo*, whereas thermally-induced albumin hydrogels were resistant to degradation. *In vitro*, an 8M solution of urea degraded acid-induced albumin hydrogels within 17 hours, whereas *in vivo* degradation

occurred in an immuno-competent rat model (Sprague-Dawley) over four weeks with little evidence of inflammation and the site of transplantation. In contrast, thermally-induced albumin hydrogels were resistant to chemical and physiological degradation. Thermally-induced BSA hydrogels were still intact four weeks post-transplantation and a fibrous capsule around the scaffold was noted. Interestingly, local inflammation was noted when untreated BSA was injected but this resolved with time.

Albumin hydrogels derived by glutaraldehyde-induced crosslinking seem to exhibit poor biodegradability and local immunogenicity. (Gallego, Junquera, Meana, Alvarez-Viejo, et al., 2010) reported that glutaraldehyde-crosslinked HSA hydrogels, when transplanted in an immuno-deficient mouse model, remained partially degraded at 150 days. Calcification of the scaffolds and injury to overlying skin were also noted. Ma et al., 2016 reported hyperkeratosis in all mice after the injection of glutaraldehyde crosslinked BSA hydrogels but complete degradation after two months. In one out of two test subjects, inflammation was noted in the surrounding skin and a fibrous capsule around the BSA hydrogel was developing. The strain of mice used were immuno-deficient. The crosslinking process could account for the skin reactions since other methods of gelation e.g. electrostatic self-assembly, did not produce the same effect. Difference in degradation times could also be explained by the inherent differences between HSA and BSA although further studies are needed to confirm this.

BSA hydrogels synthesised by glutathione-mediated oxidative refolding (Raja et al., 2015) produce less of an immunogenic response compared to BSA hydrogels synthesised by glutaraldehyde-induced crosslinking. When transplanted into an immuno-competent rat model (Wistar), these hydrogels did not precipitate skin reactions. No obvious signs of inflammation were noted but the formation of a fibrous capsule around the hydrogel persisted

in a dose dependent manner i.e. higher albumin concentrations were associated with thicker fibrous capsules. Degradation times also increased with increasing albumin concentrations e.g. 15-20 days for 300 μM gels and 30-40 days for 600 μM gels. It would be both important and useful for future studies to determine if immuno-competency in animal models accelerates the rate of hydrogel degradation.

PEG-albumin hydrogels generally have a more predictable degradation period of approximately 2 to 4 weeks with no local side effects. A HSA-SH/PEG-MAL hydrogel was reduced to 28% of its initial weight after 21 days in an immuno-deficient mouse model (Figure 4) (Kim et al., 2015). Interestingly, only one study to date has created albumin hydrogels derived from species-specific serum for *in vivo* experimentation. Feldman & McCauley, 2018 created an albumin-($PEGSG_2$)-$TGF\beta_3$, hydrogel scaffold from rabbit albumin and transplanted these into immuno-competent rabbits. The degradation time was reported to be 2 weeks with no immunogenic complications observed. In contrast to the above, hydrogel created from the conjugation of BSA with PEG-derived poly-amino-urethane showed poor degradability after 3 weeks in immuno-competent rats (Manokruang & Lee, 2013). Unfortunately, local effects in the surrounding skin were not assessed.

COST

Animal-derived albumin is inexpensive; however all hydrogels should ideally be xeno-free for clinical utility. Human albumin is considerably more expensive than animal-derived albumin but relatively cheaper compared to other substrates used in regenerative medicine. For example, Matrigel costs approximately £1933.33/g (Sigma) and rat tail collagen-1 costs £4810.00/g (Sigma); whereas human albumin costs £20.30/g (Sigma). Other preparations of albumin such as 20% human albumin solution may be procured at cheaper and larger volumes, e.g. 20 g for £54 (Octapharma Ltd).

Apart from albumin, additional costs may also be incurred by the reagents used to induce gelation of albumin and chemically crosslink target proteins. For example, hydrogel synthesised by glutaraldehyde (£3.60/mL for 70% glutaraldehyde, Sigma) is considerably cheaper compared to functionalised PEG (£108.12/g for 4-arm 10K PEG-SG, Creative PEGWorks). However as discussed above, albumin hydrogels crosslinked by glutaraldehyde or glutathione have a propensity to be immunogenic. As cell attachment to N-form albumin is generally poor and conjugation of target proteins with PEG costly and laborious, better methods of functionalising HSA hydrogels are needed.

CONCLUSION

There is a broad scope for further exploitation of albumin hydrogels in regenerative medicine. In the study of the lung, the creation of macroporous albumin scaffolds from hydrogels could be useful in engineering lung parenchyma. Pore size and thickness could be tuned to recapitulate the alveolar space onto which lung stem cells and auxiliary cell types could be seeded. This offers an alternative to decellularised animal scaffolds and lung organoid biology could be studied in xeno-free conditions. In regenerative cardiology, the growth of contractile heart tissue on HSA has not yet been demonstrated but remains an attractive area of research to pursue. Xeno-free, injectable HSA hydrogels could then be a viable method of delivering cardiac stem cells or cardiomyocytes directly into injured myocardium. In regenerative hepatology, a similar approach to cell or tissue delivery could be adopted to transplant hepatic stem cells, hepatocytes or organoids in liver failure. However albumin, an important marker of synthetic liver function, is released by the degradation of HSA hydrogels which may make albumin ELISAs (enzyme-linked immunosorbent assays) difficult to interpret, particularly in animal models. In regenerative neurology, studies to determine if HSA hydrogels enhance proliferation, differentiation and branching of hiPSC-NSC are needed since these were previously reported in BSA.

In summary, apart from drug delivery, albumin hydrogels hold great potential as a biomaterial for 3D cell culture, platform for cell delivery and scaffold for tissue transplantation. The inertness, poor immunogenicity, biodegradability, cost and possibility to derive patient specific albumin make albumin hydrogels useful in regenerative medicine and tissue engineering. However, these have not been fully exploited and better methods of synthesising and functionalising albumin hydrogels are needed.

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List of Figures

Figure 1 - (A) Ribbon diagrams showing the partial denaturation of N-form to F-form albumin, protein aggregation and hydrogel formation. Inverted vial shows a transparent pH-induced BSA hydrogel (PBSA) next to a tubular PBSA cylinder made in mold at 37 °C. Cryo-SEM images of freeze-fractured hydrogels formed at pH 3.5 at 37 °C (B) and by thermally-induced gelation at 80 °C (C) illustrating differences in porosity. (D) Hydrogel turbidity of thermally-induced BSA hydrogels increases with BSA concentration. Images (A),

(B) and (C) were reproduced with permission from Baler et al. (2014); https://pubs.acs.org/doi/abs/10.1021%2Facs.accounts.5b00438. Further permissions related to the material excerpted should be directed to the ACS. Image (D) was reproduced with permission from Amdursky et al. (2018).

Figure 2 - Phase diagram of (A) human serum albumin (HSA) and (B) bovine serum albumin (BSA) after 48 hours of heating, at different concentrations and at neutral pH. (C) Phase diagram for 20% w/v HSA solution at varying pH values and heating times. Gels at high pH values (pH > 10.6) form in less than 2 hours at room temperature. (D) Phase diagram for 20% w/v BSA solution at varying pH values and heating times. Image reproduced with permission from Arabi et al. (2018).

Figure 3 - (A) Isolation of neonatal rat ventricular cardiomyoscites. (B) BSA-hydrogel construct had folded spontaneously at Day 14 to create a 3D environment. (C) No differences in cardiomyocytes function (beats/minute) were noticed at Day 7 and Day 14 ($p > 0.05$). Good cell survival was demonstrated over 2 weeks by (D) Live/Dead staining and (E) Picogreen double stranded DNA quantification. Image reproduced with permission from Amdursky et al. (2018).

Figure 4 - Cross-sections of epidermal tissue at the site of transplantation of HSA-PEG hydrogels demonstrated no evidence of inflammation or apoptosis; TUNEL negative. (TUNEL = Terminal deoxynucleotidyl transferase dUTP Nick-End Labelling, an assay for apoptosis). Image reproduced with permission from Kim et al. (2015).

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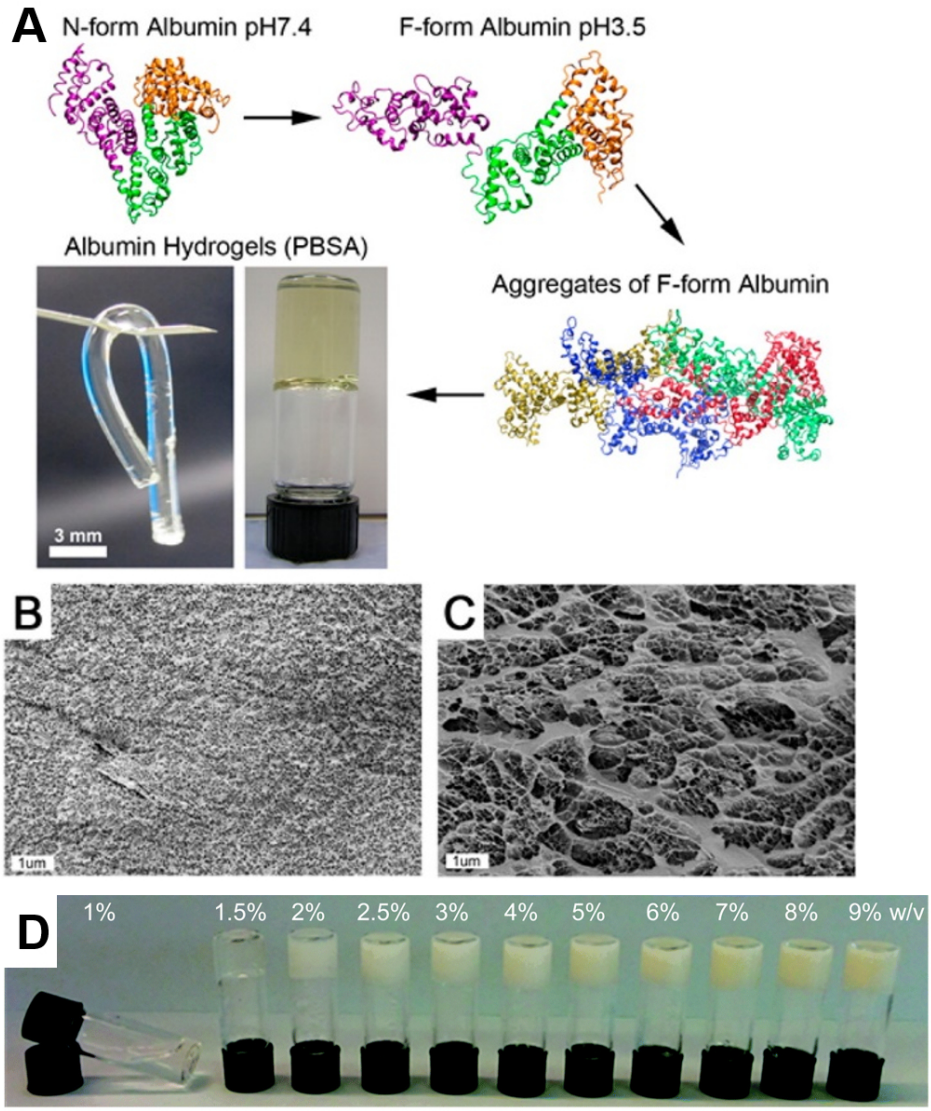

Figure 1

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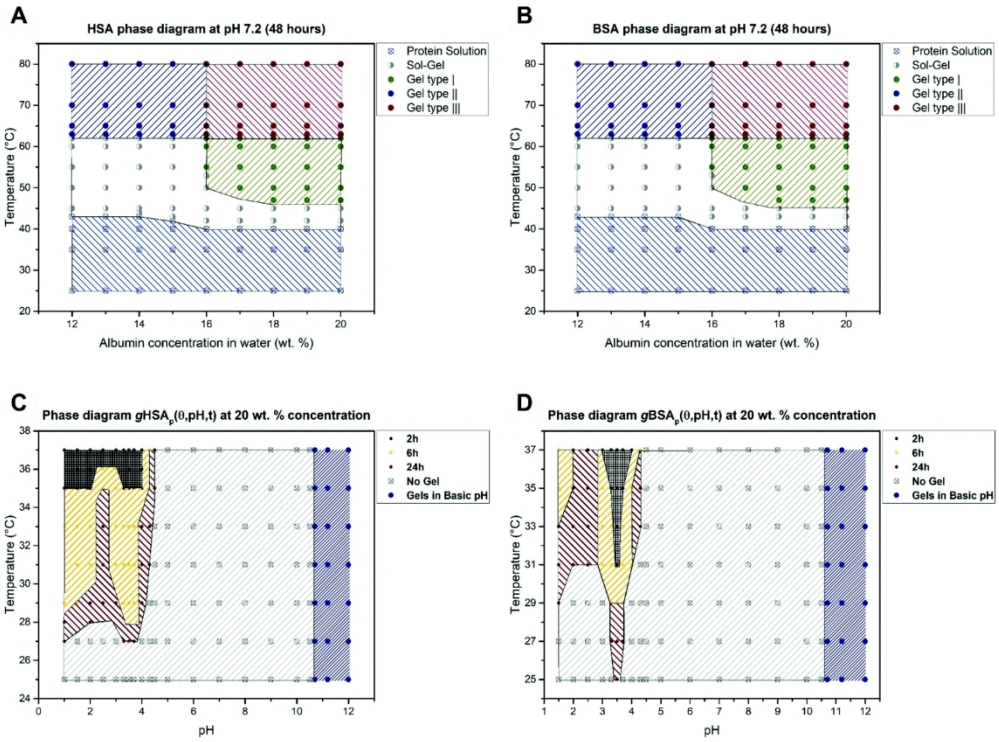
A
HSA phase diagram at pH 7.2 (48 hours)
Protein Solution
Sol-Gel
Gel type I
Gel type II
Gel type III
Temperature (°C)
Albumin concentration in water (wt. %)
B
BSA phase diagram at pH 7.2 (48 hours)
Protein Solution
Sol-Gel
Gel type I
Gel type II
Gel type III
Temperature (°C)
Albumin concentration in water (wt. %)
C
Phase diagram gHSA$_p$(0,pH,t) at 20 wt. % concentration
2h
6h
24h
No Gel
Gels in Basic pH
Temperature (°C)
pH
D
Phase diagram gBSA$_p$(0,pH,t) at 20 wt. % concentration
2h
6h
24h
No Gel
Gels in Basic pH
Temperature (°C)
pH

Figure 2

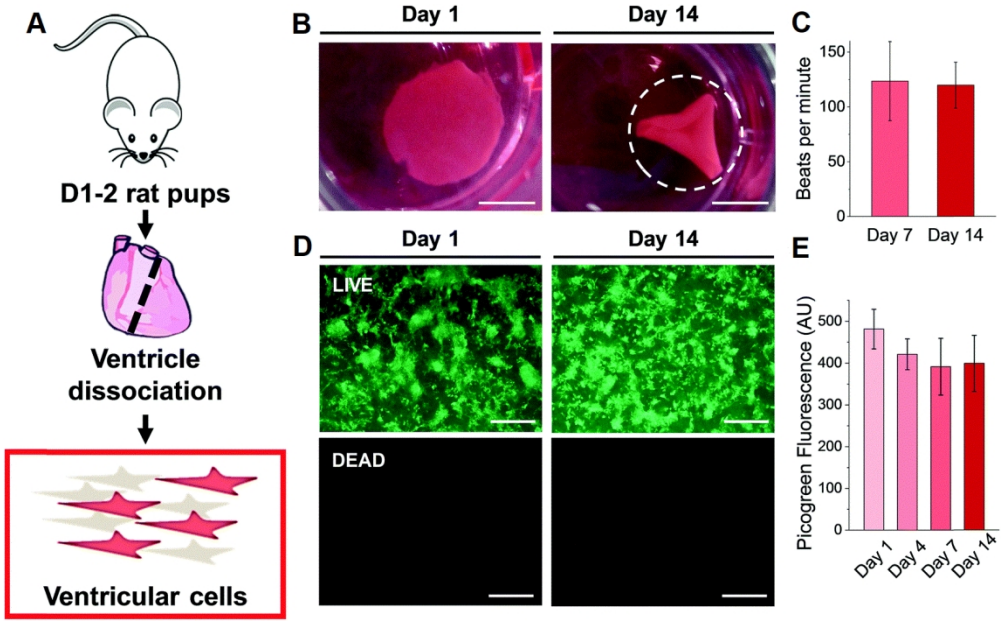
A
D1-2 rat pups
Ventricle dissociation
Ventricular cells
B
Day 1
Day 14
C
Beats per minute
150
100
50
0
Day 7
Day 14
D
Day 1
Day 14
LIVE
DEAD
E
Picogreen Fluorescence (AU)
500
400
300
200
100
0
Day 1
Day 4
Day 7
Day 14

Figure 3

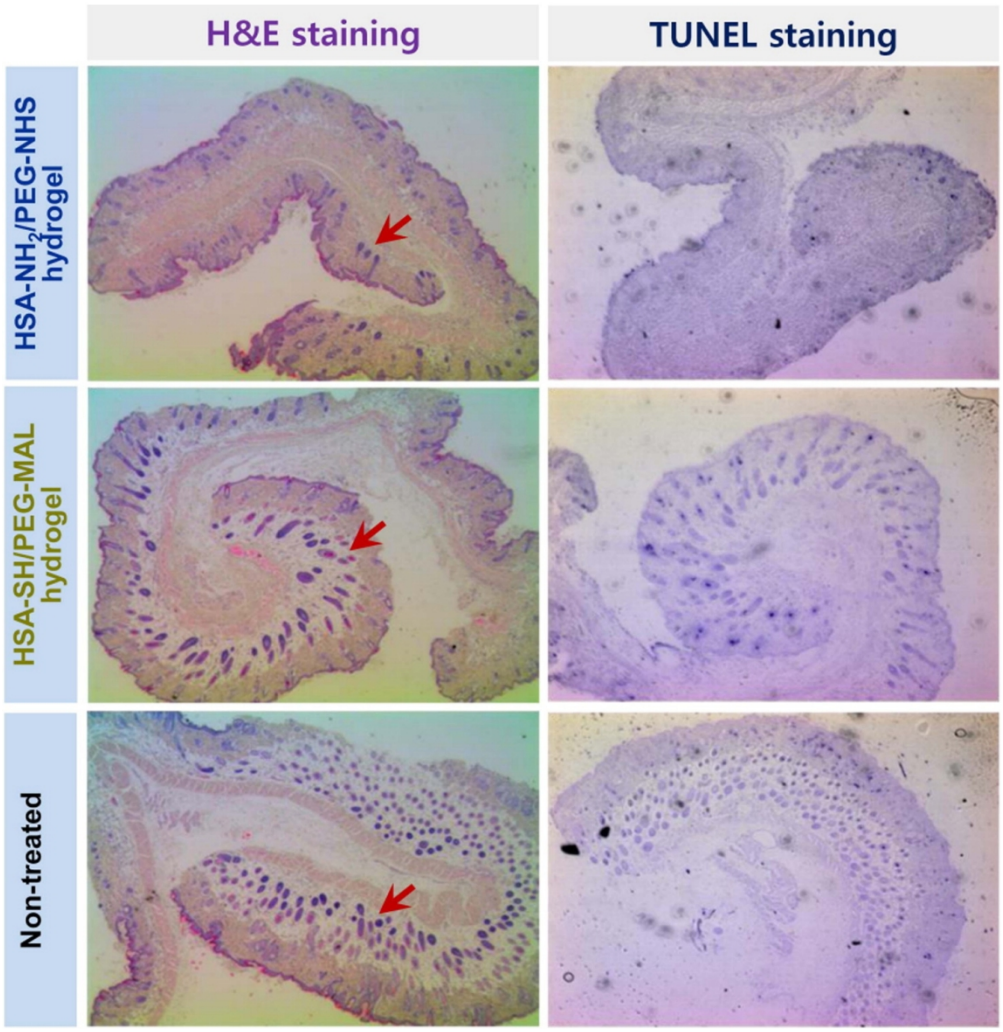
H&E staining
TUNEL staining
HSA-NH2/PEG-NHS hydrogel
HSA-SH/PEG-MAL hydrogel
Non-treated

Figure 4

Table 1: Summary of Albumin-Based Hydrogel Properties

Method of gelation vs properties	**pH-induced albumin hydrogels**	**Thermally-induced albumin hydrogels**	**Chemically crosslinked albumin hydrogels**	**References**
Structure of albumin	pH < 2.3: E form (extended) pH 2.3-4.2: F form (fast migrating) pH 4.3-8: N form (normal) pH 8-10: B form (basic) pH > 10: A form (aged)	Variable: heat causes a range of changes from monomeric structural differences such as unfolding and disruption of secondary structure, dimerisation, oligomerisation and polymerisation.	Dependent on crosslinking process and materials.	Amiri et al., 2010; Barone et al., 1995; Chen et al., 2019; Leggio et al., 2008; Molodenskiy et al., 2017
Mechanical Properties				Amdursky et al., 2018; Baler et al., 2014; Zhou et al., 2018
Young's modulus	~46 kPa for 20 wt% BSA hydrogel, measured using indentation (Baler et al., 2014)	~34 and ~67 kPa (pH 3.5) respectively for 17 and 20 wt% BSA hydrogels, measured using indentation (Baler et al., 2014) 5-17 kPa for 3-9 wt% BSA hydrogels, measured under tension. Under confined compression, the values varied from ~0.2-4.4 kPa for 3-9 wt% BSA hydrogels (Amdursky et al., 2018).	Not reported	
Storage *G′* and Loss Modulus *G′′*	*G′* & *G′′*: ~5-10 and ~60-80 kPa respectively for 16 and 20 wt% BSA hydrogels after 2300 and 340 s (Baler et al., 2014)	*G′* & *G′′*:~120 and ~60 kPa respectively for both 16 and 20 wt% BSA hydrogels (80ºC, after 30-50 s) (Baler et al., 2014) *G′*: 3-5 and 8-13 kPa respectively for 4.5 and 9 wt% BSA hydrogels. *G′′*: 0.7-1.5 and 2-4 kPa respectively for 4.5 and 9 wt% BSA hydrogels (0.1-10 Hz) (Amdursky et al., 2018)	Not reported	
Tensile Strength	Not reported.	~2-5 MPa for 3-9 wt% BSA hydrogels (Amdursky et al., 2018)	~40 MPa for 10 wt% HSA hydrogel (Zhou et al., 2018)	
Hydrogel Turbidity	Clear to translucent.	Translucent to opaque (white). Highly dependent of ionic content, type of albumin e.g. BSA vs HSA, and albumin concentration.	Clear to opaque; dependent on crosslinking process and materials.	Amdursky et al., 2018; Arabi et al., 2018; Baler et al., 2014; Murata et al., 1993

Biocompatibility	Cells cannot survive in the bulk of a strongly acidic or alkali hydrogel unless it is leached. Once leached, cells can be seeded on the surface or within pores of the hydrogel.	Cells cannot survive the thermal gelation process. Once gelled, hydrogels are biocompatible but cell attachment is often poor. Functionalisation of surfaces can be explored.	Almost all studies report good biocompatibility (cell survival and growth).	Baler et al., 2014; Hirose et al., 2010
Biodegradability (duration)	Rapid: 1 day to 1 month	Long: > 1 month	Variable: 2 weeks to > 1 month	Baler et al., 2014; Feldman & McCauley, 2018; Gallego et al., 2010; Kim et al., 2015; Raja et al., 2015
Immunogenicity	Low	Low to moderate - fibrous capsule round transplanted scaffolds	Low to high: dependent on crosslinking process and materials. E.g. With glutaraldehyde, a fibrous capsule around transplanted scaffold and evidence of local inflammation were noted. With PEG and species-specific albumin, the above complications were absent.	Amdursky et al., 2018; Baler et al., 2014; Feldman & McCauley, 2018; Gallego et al., 2010; Kim et al., 2015; Ma et al., 2016; Raja et al., 2015
Printability	Difficult: low and high pH albumin solutions are very viscous. Maybe problematic at high resolution and high pressures may be needed.	Possible: high heat required to induce gelation of printed construct	Possible: allows new gelation methods e.g. gelation by photo-crosslinking of PEGDA-albumin conjugates.	No articles identified at time of review.
Current applications	Cardiac tissue engineering	Cardiac tissue engineering	Bone and cardiac tissue engineering, skin and wound healing, toxicology studies for liver disease models, stem cell derived nerve cells, drug delivery.	See main text.