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Tissue engineering with gellan gum

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Abstract

Engineering complex tissues for research and clinical applications relies on high-performance biomaterials that are amenable to biofabrication, maintain mechanical integrity, support specific cell behaviours, and, ultimately, biodegrade. In most cases, complex tissues will need to be fabricated from not one, but many biomaterials, which collectively fulfill these demanding requirements. Gellan gum is an anionic polysaccharide with potential to fill several key roles in engineered tissues, particularly after modification and blending. This review focuses on the present state of research into gellan gum, from its origins, purification and modification, through processing and biofabrication options, to its performance as a cell scaffold for both soft tissue and load bearing applications. Overall, we find gellan gum to be a highly versatile backbone material for tissue engineering research, upon which a broad array of form and functionality can be built.

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Tissue Engineering with Gellan Gum

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Engineering complex tissues for research and clinical applications relies on high-performance biomaterials that are amenable to biofabrication, maintain mechanical integrity, support specific cell behaviours, and, ultimately, biodegrade. In most cases, complex tissues will need to be fabricated from not one, but many biomaterials, which collectively fulfill these demanding requirements. Gellan gum is an anionic polysaccharide with potential to fill several key roles in engineered tissues, particularly after modification and blending. This review focuses on the present state of research into gellan gum, from its origins, purification and modification, through processing and biofabrication options, to its performance as a cell scaffold for both soft tissue and load bearing applications. Overall, we find gellan gum to be a highly versatile backbone material for tissue engineering research, upon which a broad array of form and functionality can be built.

Introduction: The Tissue Engineering Imperative

Tissue engineering (TE) is a field of research that aims to combine cells and biomaterials into functional structures that have the potential to repair, replace or replicate living tissues. The field has progressed markedly over recent decades, and stands poised to provide viable clinical therapies for the repair of skin[1], cartilage[2, 3], bladder[4, 5], vasculature[6] and bone[7]. Research is increasingly being focused on the regeneration of 'complex' tissues with multi-layered, multi-cellular and highly vascularised structures[8]. The scientific advances that are required to progress the field of TE towards complex tissue fabrication have been regularly reviewed[9-11], with biomaterials design[12-14] and processing[15-18] being frequently highlighted as areas of priority.

The primary function of biomaterials in TE is the encapsulation and support of living cells. For this reason, many of the materials utilised in TE are either sourced from, or functionally replicate, those present in the natural extracellular matrix (ECM)[19]. The ECM is a complex network of proteins and polysaccharides that surrounds and supports living tissues (Figure 1). As well as providing mechanical support, many of these ECM components also play critical roles in cell signalling and cell adhesion processes that modulate cell behaviour[20]. To ensure artificial scaffolds provide an effective platform for TE, it is critical that they replicate both the structural and functional roles exhibited by the natural ECM[21, 22]. Additionally, TE biomaterials must be biocompatible, and induce minimal immunogenic or fibrotic response upon implantation[23, 24]. Unlike inert implants, biomaterials are also expected to controllably biodegrade such that the engineered scaffold progressively gives way to natural ECM components as the recovering tissue repairs and remodels its surroundings[24]. Finally, biomaterials for TE need to be processable. This broad term is determined by a number of specific material properties including viscosity, surface tension, cross-linking mechanisms, pH and phase transition temperatures. All such properties must fall within certain tolerable ranges for the material to be reliably processed using the emerging suite of biofabrication technologies [14]. Ideally, the materials employed in TE would possess all of these favourable characteristics, rendering them processable, mechanically robust, biocompatible, biofunctional and biodegradable. Realistically, this array of requirements will require a broad spectrum of materials that work in concert within the engineered tissue. One class of biomaterials with significant potential to contribute to engineered tissues are the polysaccharides, of which several are known to be both processable and biocompatible. Reviews that directly compare the various polysaccharides used in TE have been published by Khan and Ahmad[25], as well as Bacáková *et al.*[26]. This review focuses on gellan gum, an emerging polysaccharide with potential as a versatile and processable scaffold material suitable for a broad range of engineered tissues.

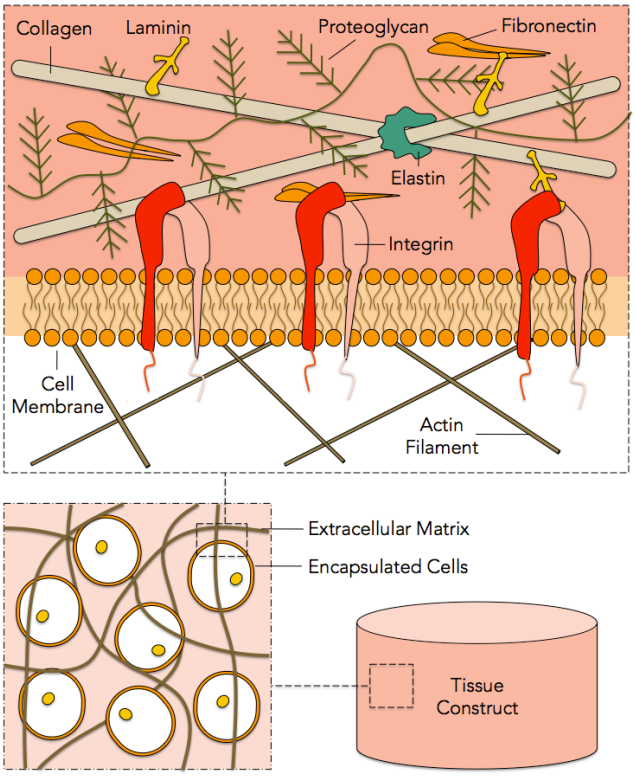

Figure 1: The Extracellular Matrix: The natural ECM is a complex and multi-component system containing collagen, elastin, proteoglycans, polysaccharides and glycoproteins. Collectively these elements provide mechanical integrity, cell adhesion and signalling, roles that must be replicated in synthetic extracellular matrix mimics.

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Gellan Gum

Source

Gellan gum is an exopolysaccharide produced by bacteria from the *Sphingomonas* group as a major constituent of their extracellular polymeric substance (EPS). Commercially, gellan gum is usually produced by batch fermentation using the *Sphingomonas paucimobilis* strain, due to the higher yields and purity of GG achieved with this species[27]. GG is extracted from this broth by a multi-step process. Firstly, the broth is heated at elevated pH, followed by centrifugation or hot filtration to separate cell bodies from the EPS. Multiple rounds of precipitation, resuspension and dialysis are then used to purify GG, which is then dried or lyophilized and distributed as a powdery, off-white solid. The quantity, purity, and physical characteristics of extracted gellan depend on many factors including cell population, nutrient feedstock, temperature, pH, and extraction procedure[28]. A detailed review of gellan gum biosynthesis can be found in Fialho *et al.*[29].

Structure

The gellan gum polysaccharide is a repeating tetramer comprised of L-rhamnose, D-glucuronic acid and two D-glucose subunits. In its native state, these tetramers also contain glycerate and acetate functionalities, however the hot alkaline conditions employed in GG extraction leads to their reduction to hydroxyl residues (Figure 2). Works exploring the functional impacts of deacylation may be found in[30-33], however TE with gellan gum is most commonly performed using the deacylated form due to the relative ease of isolation and processing. In hot aqueous dispersion, both high and low-acyl gellan gum chains are randomly distributed in solution, but self-assemble into helix pairs upon cooling through a sol-gel transition temperature (~50 ˚C). This process is a critical step in the gelation of GG, which will be discussed later.

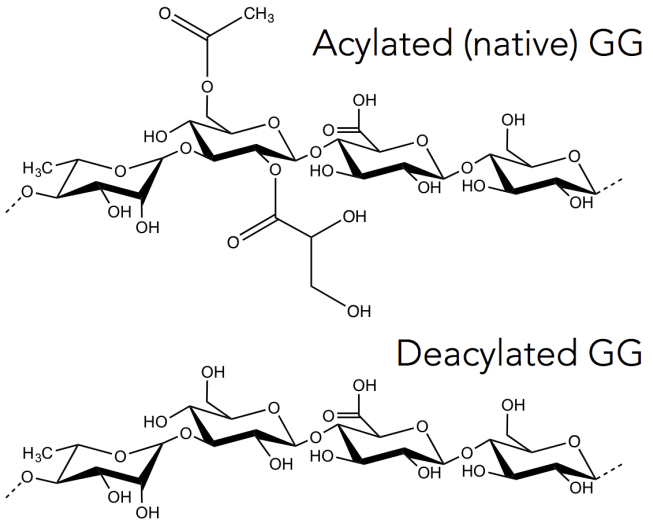

Figure 2: Gellan Gum: The chemical structures of gellan gum as produced by *Sphingomonas sp.*, and its deacylated form that is more commonly employed for GG tissue engineering.

Purification and Sterilisation

All materials intended for tissue engineering applications require a high degree of purification and quality control to ensure the product is both safe and reliable. This is particularly true for scaffolds intended for implantation, where infection, inflammation and immunogenicity are of concern. In most cases, GG products need to be sterilised prior to use in cell culture and TE. Various techniques are in use for this purpose including autoclaving[34-36], ethylene oxide treatment[37], micro-filtration[38], and antibiotic supplements[39]. Although no studies directly compare these methods with respect to their impacts on GG, equivalent investigations using other polysaccharides[40-43] and ECM materials[40, 44, 45] provide a good indication of the likely effects on GG. In general, these studies report that common sterilisation procedures are generally effective at removing or deactivating contaminants without loss of the biological activity. However, heat-based techniques may decrease the molecular weight of polymer chains and thereby alter the rheology of polysaccharide solutions and gels. Whilst such effects in no way preclude the use of heat-sterilisation, GG scaffolds and handling procedures need to be designed in a manner that accounts for the likely impacts of sterilisation.

As well as live-cell contamination, bacteria-derived materials such as GG are susceptible to contamination with endotoxins, which have the potential to trigger immunogenic reactions, and thereby compromise implanted scaffolds. For this reason, scaffolds intended for cell culture, particularly *in vivo* testing, should be purified of endotoxins. Several methods exist for endotoxin removal, including phase separation, chromatographic and ultrafiltration techniques[46], and the levels of remnant endotoxins can be quantitatively determined using limulus amoebocyte lysate (LAL) tests. However, low-endotoxin GG products such as Gelzan are commercially available[47], and will likely be an appropriate feedstock material for most GG tissue engineering purposes.

As well as biological contamination, GG is highly sensitive to the presence of salts, particularly divalent and multivalent cations[48, 49]. Whilst not biologically detrimental, these cations can substantially alter the physical properties of GG hydrogels as well as the processing behaviours of GG solutions. Recently, Kirchmajer *et al.* reported that the removal of divalent cations from GG greatly increased the ease of hydrating the polymer, improved solution clarity and lead to a more controlled and homogeneous gelation[50]. Ferris *et al.* noted the purification of commercial GG to its sodium salt form, NaGG, was a prerequisite for further functionalisation[51]. For optimum processability and material consistency, researchers should consider including salt purification as a routine step in their application of GG. Both Kirchmajer, and earlier works by Doner *et al.*[52, 53] provide procedures for the purification of GG to monovalent salt forms.

Gelation

One of the defining properties of the GG polysaccharide is the ability to form pseudo-solid structures known as hydrogels. These structures are formed from the interlocking of GG chains into a single, coherent network that entraps a very high (~99 %) volume fraction of water. The formation of this network is driven by the spontaneous aggregation of GG chains from a 'random coil' state, to paired helical structures as they cool through the sol-gel transition temperature[49, 54]. Multiple GG helices then aggregate into junction zones that act as cross-linking sites for the network. This structure was initially resolved by Gunning and Morris using light scattering[55], and later observed directly using atomic force microscopy[56-58]. Importantly, the junction zones of GG networks are strongly stabilised by multivalent cations, commonly Ca^{2+} or Mg^{2+}, and the presence of these species renders the gelation of GG practically irreversible[48]. Many authors leverage this phenomenon to generate GG hydrogels that are 'physically cross-linked' through the presence of cation-stabilised junction zones of helical GG chains. Tailoring the concentrations of GG and cations present in solution prior to cooling allows for a modulation of many physical properties of the resulting hydrogels[59]. A thorough review of the structure, physical properties and gelation mechanism of gellan gum can be found in Morris *et al.*[54].

Degradation

For tissue engineering applications, the pathways, timeframe and by-products of biodegradation are important in the design and application of cell scaffolding biomaterials[23]. A number of authors have attempted to investigate the degradation behaviours of GG both *in vitro* and *in vivo.* Long-term degradation behaviour in PBS for pure GG[60], and blended GG networks[61] has been reported. In both cases, gels were observed to lose 10–15 % of their starting mass over periods of up to 168 days. Lee *et al*. studied weight loss in pure and blended GG scaffolds during immersion in cell culture media as part of a study optimising gels for fibrocartilage tissue engineering, reporting 10–20 % weight loss over 20 days[62]. Degradation is substantially accelerated under alkaline conditions, with Coutinho *et al.* reporting methacrylated GG to loose between 20–100% of starting mass over 24 h in 0.1 mM NaOH, with rates depending on the degree of photo-cross-linking[63]. Degradation rates may also be influenced by the presence of enzymes, with Singh *et al*. reporting accelerated viscosity loss and drug release from GG beads in simulated colonic media containing the enzyme galactomannanase[64]. Finally, Jahromi *et al.* studied the degradation of GG, alginate and pectin after seeding each material with bone marrow stromal cells, finding GG degradation to be the least rapid of the three materials [65].

A number of studies have assessed the performance of GG scaffolds *in vivo*. For example, Oliveira *et al.* implanted GG-based scaffolds in rabbit cartilage defects for 8-weeks[66], reporting that adipose stem cells and chondrocytes encapsulated in GG scaffolds both remodelled that scaffold and integrated it with surrounding cartilage tissue. In another study Silva-Correia *et al*. implanted GG and methacrylated GG subcutaneously for 10-18 days. The authors reported minimal inflammatory response and endothelial cell infiltration, however methacrylated GG was also observed to impede vascularisation[67, 68].

Overall, these studies present positive indications that GG biodegrades over a period of weeks to months, and may be suitable for a wide range of tissue types. However the studies have so far been directed towards a limited range of tissue types and conditions, providing only a partial understanding of GG's degradation pathways and kinetics. Studies that more completely explore these pathways would be of benefit to future GG tissue engineering.

Blends of Gellan Gum

Engineered tissues are complex and highly sensitive systems that require application-specific tailoring of the physical, biological, morphological and processing properties of component materials[13]. GG was recently highlighted for its significant capacity for tissue engineering applications, particularly after blending or chemical modification[69]. Here, we will discuss the numerous modifications and blends that have been employed to tailor the biological, mechanical, chemical and electrical functionality of GG.

Blending for Biofunctionality

Many mammalian cells are 'anchorage dependant', meaning that their survival and phenotypic behaviour is strongly dependant on successful attachment to a matrix material. Although the GG polysaccharide is cytocompatible, it does not participate in the specific cell binding interactions[69]. As a result, anchorage dependant cells grown in pure GG matrices may exhibit low levels of cell attachment or anomalous differentiation behaviours. One approach for resolving this issue is to blend GG with bioinformative materials that induce regular cell behaviour. For example, Cencetti *et al.*[70], Bellini *et al.*[34], Kang *et al.*[71], and Cerquiera *et al.* [72] have each blended GG with hyaluronic acid (HA), a polysaccharide present in the mammalian ECM, to form engineered tissues for bone, intevertebral discs and vascularisation, respectively. The ECM protein fibronectin has also been employed to improve cell binding and differentiation in GG scaffolds for endothelial cell entrapment[73]. Gelatin, a derivative of the ECM protein collagen, has also been incorporated into GG matrices using enzymatic binding[74] and genipin cross-linking[75], improved both the cell-GG interactions and mechanical strength of the resulting hybrid gels. Such approaches that simultaneously improve cell-interaction and mechanical integrity are notable because they address one of the other factors limiting the application of water-swollen hydrogels in TE, being their lack of inherent strength[76].

Blending for Strength

Engineering load-bearing tissues requires scaffolds that possess a high level of mechanical integrity, however different strategies are required **if** these load bearing tissues are 'soft' like cartilage, or 'hard' like bone. For bone tissue, researchers have used combined blending and bioconditioning processes to mineralise GG scaffolds**.** Douglas *et al.* mineralised GG scaffolds using the alkaline phosphatase enzyme to improve strength and stiffness of the material, as well as promote cell attachment and differentiation[35, 77]. Douglas and others have also explored incorporating bioglass into GG hydrogels to improve mineralisation, bone regeneration and antibacterial properties[78, 79]. Jamshidi *et al.* achieved similar effects through the direct blending of GG with nanocrystalline hydroxyapatite[80], whilst Veira enhanced biomineralisation using gold nanorods that had been surface-functionalised with GG[81]. Shin *et al.* also found elevated osteogenesis when GG microgels were employed for the reinforcement of a gelatin hydrogel intended for bone tissue engineering[82]. Finally, hard-soft tissue interfaces have also been explored with GG hydrogels set alongside brushite cement, forming structures akin to the bone-cartilage interface[83].

The bioengineering challenge of forming cartilage and other soft, yet load bearing, tissue scaffolds is **another** highly active sub-discipline of TE. Towards this goal, numerous 'tough hydrogels' have been formed through the combination of network elements with differing failure mechanisms that collectively distribute applied stresses. Reviews of these approaches and future perspectives can be found in Zhao[76] and Costa *et al.*[84]. Gellan gum has commonly been employed in 'ionic covalent entanglement' (ICE) systems. Various secondary networks have been reported including poly(acrylamide)[85], epoxy-amines[86], gelatin[74, 75] and hyaluronic acid[87]. It is considered likely that incorporating secondary networks with biofunctional or cell attachment behaviours, notably gelatin or hyaluronan, present the most promising routes towards engineering cartilage replacements.

As well as ICE networking, other reinforcement approaches have also been reported. Park *et al.* blended GG with the PLGA microspheres to create a reinforced scaffold for intervertebral disc scaffolds[88]. Microgels formed from blends of low and high-acyl GG have also been tested as a means of reinforcing GG hydrogels for intervertebral disc TE [89]. Thorvaldsson reinforced GG with electrospun nanofibers of poly-ε-caprolactone for nucleus pulposus engineering[90]. Silva *et al.* reported a GG, starch and poly-ε-caprolactone system for spinal cord injury repair[91]. Finally, wet spun chitosan fibers have been employed as a reinforcing material for high-acyl gellan gum hydrogels[92].

Blending for Conductivity

Blending has also been employed as a route for the formation of conductive hydrogels, which may be of use in forming, stimulating and recording from electrically excitable tissues such as muscle and nerve[93-96]. Carbon nanotubes[97, 98], carbon nanofibers[99] organic conducting polymers[99, 100], and graphene[71], have all been successfully dispersed into GG systems, whilst gellan gum has also been used as a dopant for electropolymerised conductive polymer surfaces for neural electrodes[101]. To date, there are few examples of conductive GG systems being applied *in vivo* towards clinical application, however the systems have generally appeared most promising when applied as electrode surfaces with low water content, rather than as water-swollen hydrogels.

Chemical Modification of Gellan Gum

An alternative pathway for altering the functionality of GG is through covalent chemical modification. Broadly speaking, chemical modifications of GG have been targeted towards similar applications as for blending, notably improved cell binding and mechanical integrity. However, chemical modification also provides a route for adding new functionality to the main gellan gum chain, including photo-cross-linking, self-assembly and drug delivery.

Methacrylation and Cross-Linking

One of the most frequently performed covalent modifications of gellan gum is methacrylation. This moiety is popular because it enables photo-initiated cross-linking, providing an alternative cross-linking mechanism that can also be employed to covalently link GG to other polymers and molecules. Methacylated gellan gum (MA-GG) is generally produced using methacrylic anhydride[82, 87, 102-104]. An optimisation of methacrylation density and UV dose time can be found in Bartnikowski *et al.*[105], and the mechanical and biological performance of MA-GG has been reported by Silva-Correia *et al.*[67, 106]. Because it provides GG with a secondary cross-linking action that is somewhat controllable, MA-GG is most often applied for the fabrication of load bearing tissues such as intervertebral discs[37, 63, 102, 104] and cartilage[105]. As with unmodified GGs, MA-GGs have also been blended with other polymers including hyaluronic acid[87] and gelatin[82, 103], to improve cell attachment and differentiation.

As well as methacrylation, a number of alternative covalent cross-linkers have been applied to GG. For example, a photo-cross-linking effect similar to MA-GG has been achieved by binding GG with cinnamate[107], in this case for the purpose of anti-adhesion coatings. Lee *et al.* reported a carboimmide (EDC) coupled GG that spontaneously cross-links to form gels with enhanced mechanical strength for wound healing applications[108]. Finally, Hamcerencu *et al.* reported a series of esterified GGs with cross-linking and copolymerisation potential[109].

Peptides and Biofunctionality

As with blending, numerous authors have sought to apply covalent modification techniques to tailor the biofunctional behaviours of GG. For example, Silva *et al.*[110] and Ferris *et al.*[51] have both covalently coupled GG with short peptides

containing the arginyl-glycyl-aspartic acid (RGD) sequence, a motif derived from the cell attachment sites of ECM materials[111]. The covalent attachment of RGD to GG induces cells to actively bind with the polysaccharide as they would for natural ECM, improving growth and differentiation behaviours of neural stem/progenitor cells[110], mesenchymal stem cells[39], muscle cell lines[51] and primary cortical neural tissues[38]. It is evident from these reports that the inclusion of cell-binding moieties in GG hydrogels has significant and positive impact on encapsulated cells, however, it is less clear whether such covalent attachment of cell binding peptides is more or less effective than directly blending GG with ECM materials. Nevertheless most engineered GG tissues will likely benefit from the application of at least one of these strategies.

Other Chemical Modifications

As well as peptide binding and cross-linking, a number of other functional groups have been attached to GG. D'Arrigo *et al.* covalently coupled GG with prednisolone, yielding a self-assembling nano-hydrogel that retained the anti-inflammatory properties of prednisolone[112]. Novac *et al.* reported the synthesis of aminated carboxymethyl-GG derivatives[113], targeted towards central nervous system drug delivery. This work was later extended to create an antibacterial GG hybrid that supported the controlled release of ciprofloxacin towards dermal repair applications[114]. Hamcerencu produced three esterified GG derivatives, with the new functional groups providing potential active sites for a wide variety of further modification and cross-linking processes[109]. Individually, the modification processes recounted here are designed to enhance the material's usefulness towards a particular target. In many cases, however, there is potential for cross-fertilisation, where GG reportedly modified for soft tissues may be useful for bone regeneration and *vice versa*. The continued exploration of GG, and the broad spectrum of chemical derivatives, will likely identify many untested applications for this highly versatile TE platform.

Physical Modification of Gellan Gum

Finally, tailoring the properties of GG for TE application can also be achieved through physical modifications of GG chains or hydrogel macrostructure. In general these processes are undertaken to optimise the mechanical, rheological and processing properties of GG.

Molecular Weight

The elasticity of tissue scaffolds plays an important role in influencing the behaviour of encapsulated cells[115, 116]. In a laboratory setting, the elasticity of GG scaffolds can be readily tailored by controlling the concentration of cations, and therefore cross-linking density. However, scaffolds applied *in vivo* will generally equilibrate to physiological ion concentrations, limiting the scope for controlling cross-linking through ion availability. An alternative means of modulating the elasticity of GG hydrogels is to alter the molecular weight (MW) of the polysaccharide, which influences many aspects of hydrogel strength and rheology. To this end, Gong *et al.* employed sodium periodate ($NaIO_4$) as a chemical scissor of GG chains, tailoring chain lengths for application in cartilage repair[117]. Goh *et al.*[118] and Moxon *et al.*[119] have both achieved a similar effect using ultrasonication, allowing for modulation of gel properties whilst retaining physiological cross-linking rates[119].

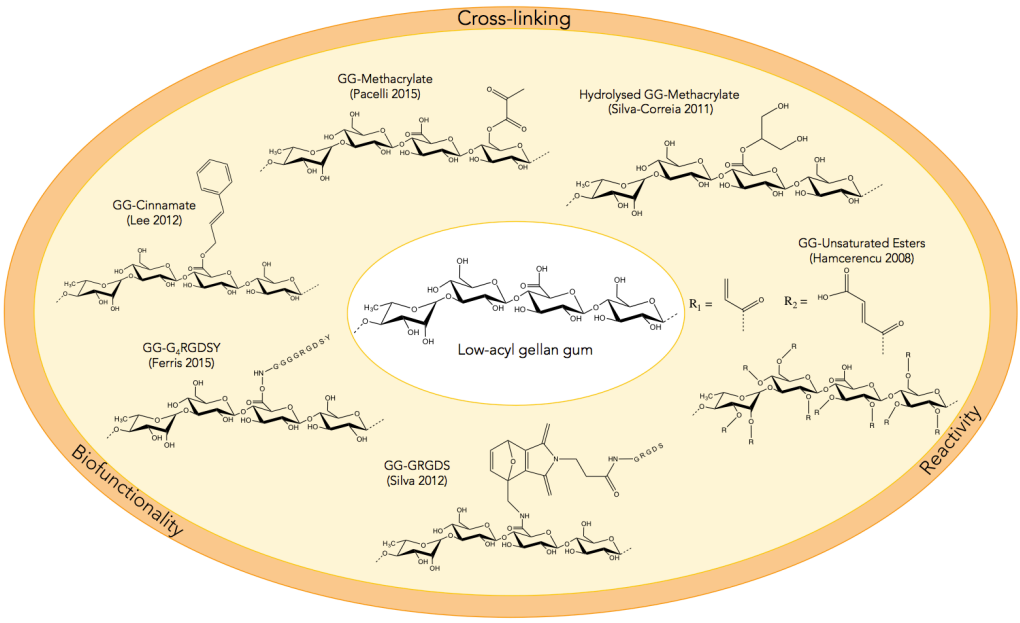

Figure 3: Chemical Modifications of Gellan Gum: A selection covalently modified GGs that have been reported to tailor GG's mechanical strength, cross-linking behaviour, biofunctionality and reactivity. The majority of GG functionalisation reactions target the carboxylate and hydroxyl residues. Presented structures are collated from original reports in Lee *et al.* [107], Pacelli *et al.* [104], Silva-Correia *et al.* [37], Hamcerencu *et al.* [109], Silva *et al.* [110] and Ferris *et al.* [51]

As noted previously, heat treatments induce a similar chain-shortening effect. To date no studies have employed heat treatment as a means of intentionally tailoring chain lengths, however autoclaving could conceivably be employed to simultaneously sterilise and optimise rheology in GG dispersions.

Altered Gelation

Finally, several authors have drastically altered the form and functionality of GG hydrogels by disrupting the network either during, or following gelation. For example, cast GG hydrogels that are subsequently freeze-dried and rehydrated have been reported to have increased strain recovery and support better differentiation in encapsulated human adipose stem cells[73]. Similar lyophilised 'GG sponge' systems have also been suggested for use as a dental filling material[120]. Microgel suspensions of GG have also been variously reported, including the creation of a bio-ink from GG that was gelled under high shear to form a loosely bound microgel suspension[121]. The bio-ink was employed to prevent cell settling during ink-jet printing, allowing high accuracy cell patterning down to single-cell resolution. Shin *et al.* created similar microgels from GG using a water-oil emulsion technique[82], which were utilised for reinforcement. Pereira *et al.* have also described the formation of microgels by cross-linking blends of low and high-acyl GG in a PBS bath under agitation[89]. Montanari *et al*. employed an autoclave to form even smaller 'nanogels' from a cholestorol derivative of GG[36]. The technique was highlighted for its simultaneous formation and sterilisation of the nanogels, which were intended for drug release.

Overall it can be seen that a diverse array of modifications can be successfully applied to GG though physical, chemical and blending processes. By virtue of these modifications, GG has been rendered suitable for the formation of tissues as diverse as bone, muscle, cartilage, dermis and the neural cortex. A summary of the modifications made to GG for tissue engineering applications is presented in Table 1. These examples highlight the incredible versatility of GG, which is probably best considered as a processable and biocompatible backbone material, onto which a wide variety of application-specific functionality can be built. The next section will focus on how these materials may be successfully patterned into complex tissue structures using biofabrication technologies.

Table 1: Summary of the blending and modification processes performed on gellan gum to improve its usefulness in tissue engineering applications. Acronyms are defined as follows: CHPTMAC (N-(3-chloro-2-hydroxy-propyl)-trimethyl ammonium chloride), CNS (Central Nervous System), EDAC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide), EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide)), LAGG (Low-acyl gellan gum), HAGG (High-acyl gellan gum), hASC (human Adipose Stem Cells), NHS (N-hydroxysuccinimide), NMP (N-methyl-2-pyrrolidone), PAA (poly(acrylic acid)), PAH (poly(allyamine hydrochloride)), PBS (phosphate buffered saline), PCL (poly-ε-caprolactone), PLA (poly(lactic acid)), PVA (poly(vinyl alcohol),

Category	Material / Modification	Key Method / Reactant	Impact	Target Tissue / Application	Reference
Blending	Hyaluronic acid	Mixing at RT	Cell binding	Bone	Bellini 2015
	Hyaluronic acid	Mixing at 90 °C	Cell binding	Vasculature	Cerqueira 2014
	Gelatin/genipin	Mixing at 80 °C	Reinforcement + Cell binding	Unspecified	Kirchmajer 2014
	Poly(acrylamide)	Mixing at 70 °C	Reinforcement	Unspecified	Bakarich 2012
	PLGA Microspheres	Mixing at 45 °C	Reinforcement	Vertebra	Park 2015
	Gelatin/enzymes	Microbial transglutaminase	Reinforcement + Cell binding	Unspecified	Wen 2013
	Gold nanorods	PAA/PAH Pretreatment	Strength + Microstructure	Bone	Veira 2015
	Bioglass	SiO_2, CaO, Na_2O, P_2O_5	Strength + Biomineralisation	Bone	Douglas 2014 Gantar 2014
	Carbon nanotubes	Ultrasonication	Conductivity	Unspecified	Ferris 2009
	Graphene oxide	Ultrasonication	Conductivity Reinforcement	Unspecified	Kang 2015
	PEDOT + Carbon nanofibers	Ultrasonication	Conductivity	Unspecified	Warren 2014
Chemical Modifications	HAGG/LAGG blends Methacrylation + Hyaluronic acid	Mixing at 70 °C	Photocross-linking, Reinforcement, Cell binding	Intervertebral discs	Khang 2015
	Methacrylation + Gelatin-methacrylate	Methacrylic anhydride	Photocross-linking Reinforcement	Load bearing tissues	Shin 2012
	Methacrylation + GG Microspheres + Gelatin	Methacrylic anhydride Water-oil emulsion	Reinforcement	Load bearing tissues	Shin 2014
	Methacrylation PEG-DMA	Methacrylic anhydride	Photocross-linking Reinforcement	Unspecified	Pacelli 2015
	Methacrylation	Glycidyl methacrylate	Photocross-linking Reinforcement	Intervertebral discs	Coutinho 2010 Silva-Correia 2011
	Cinnamate	Cinnamyl bromide	Photocross-linking Anti-adhesion	Wound healing	Lee 2012
	Esterification	Acryloyl chloride Acrylic acid Maleic anhydride	Reactivity Cross-linking	Drug delivery	Hamcerencu 2008
	Aminated carboxymethylation	EDAC	Unspecified	Drug delivery	Novac 2013
	Quaternary amine	CHPTMAC	Drug Release Antibacterial	Dermal repair	Novac 2014
	Prednisolone	NMP	Self-assembly Anti-inflammatory	Drug delivery	D'Arrogo 2012
	G_4RGDSY Peptide	EDC, NHS coupling	Improved cell binding	Muscle	Ferris 2015
	GRGDS Peptide	Diels Alder	Improved cell binding	Spinal chord/CNS	Silva 2012 Silva 2013
	EDC Cross-linking	EDC	Reinforcement	Wound healing	Lee 2010
	Reduced Chain Length	$NaIO_4$ Scissoring	Hydrogel Softening	Cartilage	Gong 2008
Physical Modifications	Increased pore size	Freeze Drying	Improved cell scaffolding	Human adipose stem cells	da Silva 2014
	Microgel	Vortex Mixing	Controlled cell settling	Cell Printing	Ferris 2013
	Microgel	HAGG/LAGG PBS Coagulation	Reinforcement	Intervertebral discs	Pereira 2011
	Reduced Chain Length	Ultrasonication	Hydrogel Softening	Unspecified	Goh 2015 Moxon 2016

Biofabrication with Gellan Gum

Hydrogel forming biopolymers can be processed into 3D structures using a variety of emerging biofabrication technologies[17, 122, 123]. Reviews on the principles and applications of these technologies can be found in Mota *et al.*[16], Arslan-Yildiz *et al.*[124] or Murphy and Atala[10]. When compared with other hydrogel forming biopolymers, GG has received very little attention in the biofabrication literature[14, 125], and most of the reported GG cell scaffolds have been produced by casting approaches (Fig. 4A). Despite it's relatively niche status in biofabrication science, GG is in fact amenable to a wide array of handling processes, demonstrated by Oliveira *et al.* through the formation of cast discs, films, fibers, spheres and lyophilized scaffolds of GG[126]. A clear pathway forwards for GG tissue engineering is through applying the material across the many emerging biofabrication technologies. The following section covers the limited present literature on GG biofabrication, and highlights possible directions for advancing the field in the short term.

Bioprinting

Beyond casting approaches, the formation of GG scaffolds has been most reliably been achieved with bioprinting techniques. In this broad space, GG has been applied as a cell carrier, rheology modifier or structural material, and processed across both ink-jet and extrusion printers. Across all of these approaches, a unifying theme is the need to control the rate and timing of cross-linking. For example, Ferris *et al*. produced a novel GG bio-ink by pre-gelling GG under high shear to form a suspension of GG microgels[121]. When supplemented with biocompatible surfactants, these GG microgel suspensions were shown to be highly amenable to ink-jetting, and reportedly stabilised live cell cultures to enabling single-cell patterning (Fig. 4B). This bio-ink has been applied for the patterning of single cell arrays for lipidomics [127] and for the placement of cells within wet-spun alginate fibers [128]. Applying these GG microgels alongside the delivery of bulk scaffolding materials may present a means of forming complex and high-resolution tissue structures. Another bioprinting technique applied in the patterning of GG is reactive extrusion printing, whereby GG is delivered simultaneously with Ca^{2+} ions. Lozano et al. applied this technique in the fabrication of a multi-layered cortical tissue mimic from RGD-peptide modified GG[38]. Although extrusion based techniques are generally of lower resolution than ink-jetting, this reactive printing approach could conceivably be applied at high resolutions using microfluidic approaches, which have been successfully applied in for the patterning of other polysaccharide materials [129-133]. Nevertheless, reactive extrusion printing represents a fast and efficient means of forming large, cell laden and self-supporting GG hydrogel scaffolds.

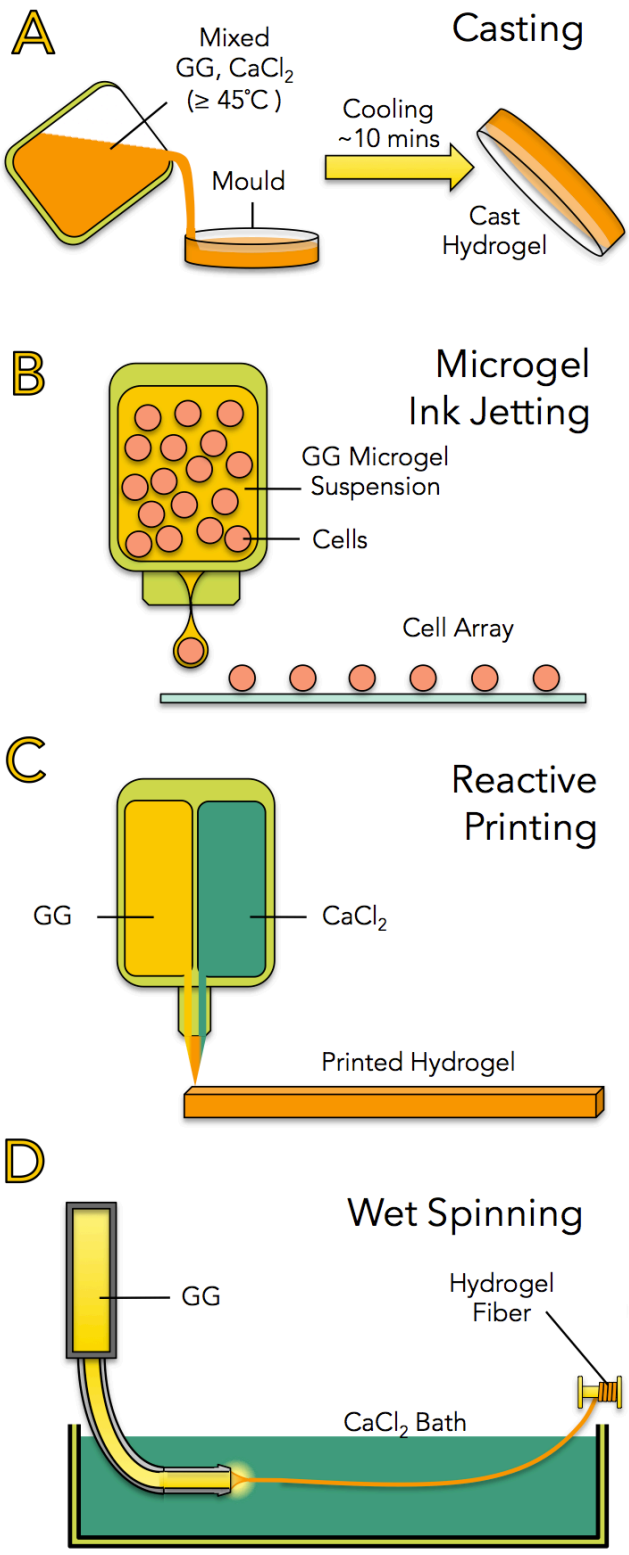

Figure 4: Biofabrication options for gellan gum: Schematic representations of the formation of hydrogel scaffolds from gellan gum including casting, reactive printing, ink-jet printing and wet spinning. To date, casting has been the primary means of forming GG scaffolds, however emerging biofabrication techniques are potentially applicable for a wide range of tissue engineering applications.

Gellan gum has also been extrusion printed as a component of blends with gelatin. Visser *et al*. incorporated GG/gelatin-methacrylate hydrogels as part of a multi-material printing approach for the formation of complex tissue-shaped structures[134]. Levarto *et al.* also printed blends of gelatin methacrylamide and GG, in this case seeding cells into the scaffold with the aid of poly(lactic acid) (PLA) microcarriers[135]. In both cases, the researchers applied photo-crosslinking of the methacrylated gelatin as a key step in scaffold fabrication. Such UV cross-linking approaches were recently highlighted as a promising means of accurately controlling bioprinted scaffolds[123]. Although methacrylated gelatin has been the principal material applied for this purpose to date[136, 137], the methacrylated gellan gums that have so far been photo-polymerised whilst confined in a casting mould[37, 63, 104] could be readily applied in UV-bioprinting. Extending MA-GG into the field of UV-bioprinting presents a new mode of GG biofabrication with significant future potential.

Overall, bioprinting presents a promising route towards GG tissue fabrication, and GG has already been utilised in both ink-jet and extrusion printing techniques. There remains significant opportunity for further developments in GG bioprinting, and the formation of complex tissues and whole organ structures will likely require a combination of high-resolution and high-throughput techniques.

Wet-Spinning

Wet spinning is a technique in which a feedstock material is continuously fed into a coagulation bath, forming fiber structures (Fig. 4D). Pure GG fibers may be formed by directly spinning GG solutions into baths containing divalent cations, akin to the fabrication of a wide range of alginate fibers[138]. Oliveria *et al.* demonstrated the formation of GG fibers through spinning alkaline GG solutions into a bath containing ascorbic acid (vitamin C)[126]. Several authors have also reported the formation of poly-ion complex fibers, whereby the polyanionic GG is spun alongside poly-cationic materials[139]. For example, Granero *et al*. reports that GG fibers spun into a bath containing chitosan (and *vice versa*) generating complex fibers with enhanced mechanical properties[140]. Meier *et al*. created similar poly-ion complex fibers between GG and amyloid protein nanofibers, reporting the bio-fibers to have high strength, and potential for use in drug release and cell scaffolding[141]. Finally, Schirmer *et al.* recently applied GG microgels to deliver cells in defined channels within wet-spun alginate fibers [128]. Although this study, and others [138], apply alginate as the primary hydrogel material, the similarity in the rheological and cross-linking behaviours of alginate and GG suggests that these wet-spinning techniques could be applied for the formation of GG fibers as well. Such wet-spun fibers may be highly applicable for the formation of tissue structures that require directional growth, such as muscle and nerve.

Other techniques

Alongside the work in bioprinting and wet-spinning, a number of niche approaches to GG biofabrication have also been reported. For example, Vashisth *et al*. recently reported the electrospinning of blended GG-poly(vinyl alcohol) (PVA) into fibrous mats which may have potential as biodegradable cell scaffolds[142]. A combined electrospinning and air-brush GG delivery technique has been reported for the formation of GG-polycaprolactone hybrids targeted towards *nucleous pulposus* regeneration[90]. 'Spongy' GG-based cell scaffolds have been fabricated through multi-step casting and freeze drying processes[72, 73], which de Silva *et al*. reported to improve hASC differentiation, particularly when fibronectin was swollen into the GG matrix during rehydration[73]. Similar freeze-dried GG scaffolds have been covalently cross-linked with EDC to render them suitable for dental filling applications [120].

A summary of the biofabrication processes applied to GG is presented in Table 2. Although the array of processing options for GG has grown markedly over the last five years, many areas are yet to be explored. Notably, many of the biofabrication techniques already reported for materials other than GG may be readily applicable for GG. More specifically, the rheology and gelation mechanisms of GG are closely related to that of alginate, a seaweed-derived polysaccharide that is already extensively employed in biofabrication and TE research. For this reason, many techniques in use for alginate processing could be directly applied to GG with minimal alteration. Applying conditions used in alginate research, and optimising from there, represents a highly efficient means of establishing reliable processing pathways for GG and its derivatives.

Table 2: Summary of the biofabrication processes performed in the patterning of gellan gum. Acronyms are defined as follows: EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide)), GG (Gellan Gum), hASC (human Adipose Stem Cells), PCL (poly-ε-caprolactone), PLA (Poly(lactic acid)), PVA (Poly(vinyl alcohol), RGD-GG (GG modified with the peptide G_4RGDSY), UV (Ultra Violet)

Technique	Gelling Mechanism	Materials	Cell Processing	Target Tissue (Cell Type)	Reference
Extrusion Printing	Photo (UV) cross-linking	GG Gelatin methacrylate	Encapsulated in GG:GelMa	Cartilage (Chondrocytes)	Visser 2013
	Photo (UV) cross-linking	GG PLA cell carriers Gelatin methacrylamide	On PLA microspheres	Bone (Mesenchymal Stromal Cells)	Levarto 2014
	Reactive Printing (Ionic)	GG $CaCl_2$	Encapsulated in GG	(Fibroblasts L929)	Stevens 2016
	Reactive Printing (Ionic)	RGD-GG $CaCl_2$	Encapsulated in GG	Cortex Mimic (Cortical Neurons)	Lozano 2015
Inkjet Printing	Thermal / Ionic (Under Shear)	GG $CaCl_2$ Novec FC-4430 Poloxamer	Individual cell deposition	Various (Fibroblast L929) (Muscle C2C12) (Nerve PC12)	Ferris 2013 Ellis 2012
Wet Spinning	Ionic / Neutralisation	Alkaline GG Ascorbic acid	Unspecified	Cartilage	Oliveira 2010
	Complexation	GG Chitosan	Unspecified	No Cells	Granero 2009
	Complexation	GG Amyloid protein nanofibers	Unspecified	No Cells	Meier 2011
	Complexation	GG Chitosan	Unspecified	No Cells	Amiake 1998
Freeze Drying	Thermal / Ionic Lyophilized	GG $CaCl_2$	Post Seeding	Cartilage (hASC)	Da Silva 2014
	Thermal / Ionic Lyophilized	GG Hyaluronic Acid $CaCl_2$	Implantation	Skin Repair / Vascularisation	Cerqueira 2014
	Lyophilized Covalent Linkage	GG EDC	Implantation	Dental Filling	Chang 2012
Electrospinning	Solvent Evaporation	GG:PVA (50:50)	Post Seeding	Unspecified	Vashisth 2014
	Reactive Air Brushing (Ionic)	GG $CaCl_2$ PCL	Post Seeding	*nucleus pulposus*	Thorvaldsson 2013

Examples of Engineered Tissues using GG

As GG materials design and processing technologies continue to mature, research interest is progressing towards the application of GG in biofabrication, tissue engineering and regenerative medicine. To conclude this review, we will highlight several works that exemplify the potential of GG in tissue engineering, including the creation of implants, tissue mimics and cell arrays.

Cartilage repair is a major target application for GG, a research area that has been pursued intensively by the Reis group[66, 126, 143, 144]. In one report from 2010, the group created several injectable formulations of GG that were used to treat defects in rabbit knee joints[66]. When these GG formulations were prepared as blends with growth factors and articular chondrocytes or adipose stem cells, the material was observed to visibly repair the knee cartilage over a period of 8 weeks *in vivo* (Figure 5A). In this case, Oliveira *et al.* applied a commercial GG material without further modifications, however several other works have employed blended or modified GGs for cartilage tissue engineering, particularly hyaluronic acid blends[37, 87, 88, 117].

A second example regards the ink-jet printing of cell microarrays using GG microgel bio-inks. As described earlier in this review, Ferris *et al.* developed GG microgel suspensions that prevented cell settling and protected cells during ink-jet printing, factors that allowed for highly controlled delivery down to single cell resolution[121]. Ellis *et al.* utilized this

approach to ink-jet print single-cell microarrays of L929, PC12 and C2C12 cell lines (Figure 5B), and then probed the molecular profiles of individual cells using a combination of liquid micro-extraction and nano-electrospray mass spectrometry techniques[127]. The authors conducted lipid profiling that positively identified cells through their lipid 'finger print', allowing a conclusive identification of the three tested cell types. However the technique's true value likely lies in its potential for furthering the fundamental understanding of cell behaviours through single cell testing, providing a valuable alternative to the multi-cellular assays and PCR techniques applied routinely in cell biology.

More recently, the chemically modified GG's have been applied in tissue engineering contexts. Lozano *et al.* reported on the formation of a multi-layered proto-tissue reflecting the structure of the cortex (Figure 5C)[38]. The work utilised a peptide modified GG material initially developed by Ferris *et al*.[51], which Lozano further demonstrated to be highly supportive of primary neural cell differentiation. In addition to peptide-modification, the cortical mimic was formed with the aid of a novel reactive extrusion system that allowed for the simultaneous and hand-controlled delivery of RGD-GG, $CaCl_2$ and cortical neurons in a spatially controlled manner. Whilst not intended for regenerative medicine, the authors reported these cortical proto-tissues to have potential as an *in vitro* model of brain tissue function and development, which could conceivably be applied for fundamental studies on a wide range of neurological processes and disorders.

Jamshidi *et al.* have recently reported another GG derivative, formed by blending GG and nano-hydroxyapatite, as an excellent substrate for regenerating bone tissue (Figure 5D)[80]. Their structures were reported to lead to significant rates of osteogenesis, and stimulated osteogenic differentiation of bone marrow stromal cells even in the absence of osteogenic media. This work compliments several other reports that have shown the potential of GG for bone tissue engineering and regeneration with alternative blending materials including hyaluronic acid[34], gelatin [82] and gold nanorods[81], and GG blending appears to provide a promising route towards bone tissue engineering

The above examples have been selected to represent the use of GG across a variety of different tissue types and application pathways, however these examples are not exhaustive. Gellan gum and its derivatives have also been applied for adipose stem cell culture[73], angiogenesis and vasculature promotion[68, 72], anti-adhesion and wound healing[107, 108, 114] and drug release[114, 145, 146]. The versatility of GG to perform across each of these roles is a major rationale for its ongoing exploration and development. It is hoped that further study will reveal yet more applications for this versatile polysaccharide.

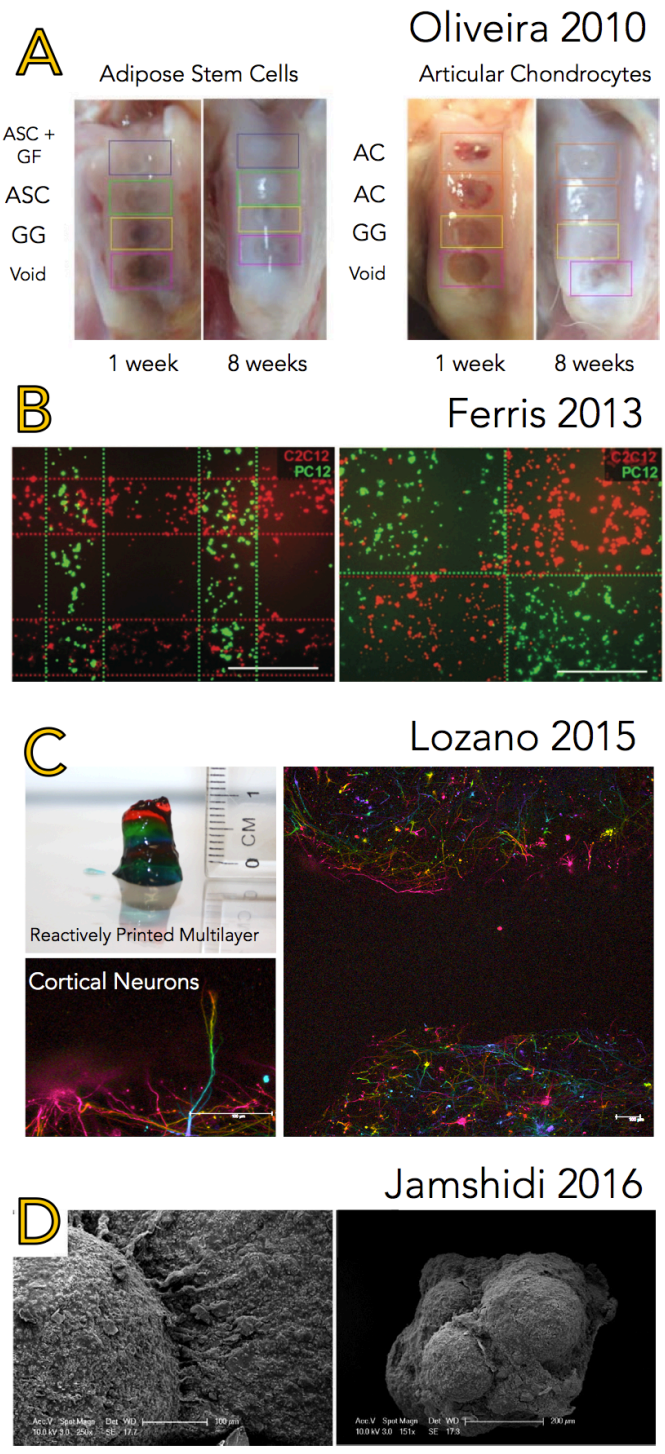

Figure 5: Examples of the use of GG in implantation, bioprinting and tissue engineering. (A) The repair of rabbit cartilage with injectable GG hydrogels laden with predifferentiated adipose stem cells (ASC), articular chondrocytes (AC) or as acellular controls (GG), reported by Oliveria *et al*.[66]. (B) The inkjet bioprinting of muscle (C2C12) and nerve (PC12) modelling cell lines using a GG microgel bioink developed by Ferris *et al*.[121] and applied for single cell lipidomics by Ellis *et al.*[127]. (C) A multi-layered cortical tissue mimic formed by the reactive printing of RGD-modified GG and $CaCl_2$ with encapsulated primary mouse cortex[38]. (D) A bone proto-tissue formed by MC3T3-E1 cells grown on the surface of GG/nano-hydroxyapatite composites[80]. Copyright material reproduced with permissions from John Wiley & Sons (A,D), Royal Society of Chemistry (B) and Elsevier (C).

Conclusions and Future Directions

This review has sought to inform readers about the polysaccharide gellan gum, and in particular its emerging potential as a biomaterial for tissue engineering. On its own, gellan gum provides a readily synthesised, purified and processed biopolymer able to encapsulate mammalian cells with minimal cytotoxicity. However, the most promising aspect of gellan gum is its extensive capacity for modification and optimisation to suit particular applications. As part of this review we have documented the reported pathways for tailoring the biological, mechanical, electrical and physical properties of GG, including chemical modifications, physical modifications, blending and controlled processing. Following these modifications, GG has been shown to be a viable substrate for the engineering of tissues as diverse as bone, cartilage, muscle and brain, and has additionally been employed for drug delivery, anti-fouling, soft conductors and stem cell culture. This versatility provides a rationale for furthering GG research, and it is anticipated that many avenues of application for the material are yet to be explored. Based on the literature reviewed here, we make several recommendations for extending GG research towards the fabrication of complex engineered tissues.

Recommendation 1 – Purify gellan gum to a monovalent salt forms prior to use. Purification of divalent cations has been shown to significantly improve the solubility, processability and modification potential of the material over commercial grades of GG.

Recommendation 2 – Apply relevant modification and blending processes of GG to improve strength, biofunctionality and cross-linking behaviours. Required modifications will differ between applications, however tissues scaffolds may benefit from GG methacrylation, peptide modification, or the blending of GG with hyaluronic acid or gelatin.

Recommendation 3 – Apply biofabrication machinery and processes developed for alginate for the processing of GG. The processing behaviours and cross-linking mechanisms of alginate and GG are closely related, and utilising the significant foundation laid by alginate research presents an efficient means for accelerating the progress of GG biofabrication.

Recommendation 4 – Apply sterilisation procedures cautiously. Heat-based sterilisation processes, notably autoclaving, are likely to reduce the molecular weight of GG chains and impact the mechanical, rheological and biological behaviours of GG hydrogels. Whilst such changes may be beneficial for many tissue applications, the impacts of sterilisation should nevertheless be considered.

Recommendation 5 – Elucidate the biodegradation pathways of GG and GG hydrogels. Research focused in this area would provide information on expected lifetimes of GG implants, and be beneficial for GG tissue engineering as a whole.

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