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Structuring of Hydrogels across Multiple Length Scales for Biomedical Applications

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The development of new materials for clinical use is limited by an onerous regulatory framework, which means that taking a completely new material into the clinic can make translation economically unfeasible. One way to get around this issue is to structure materials that are already approved by the regulator, such that they exhibit very distinct physical properties and can be used in a broader range of clinical applications. Here, the focus is on the structuring of soft materials at multiple length scales by modifying processing conditions. By applying shear to newly forming materials, it is possible to trigger molecular reorganization of polymer chains, such that they aggregate to form particles and ribbon-like structures. These structures then weakly interact at zero shear forming a solid-like material. The resulting self-healing network is of particular use for a range of different biomedical applications. How these materials are used to allow the delivery of therapeutic entities (cells and proteins) and as a support for additive layer manufacturing of larger-scale tissue constructs is discussed. This technology enables the development of a range of novel materials and structures for tissue augmentation and regeneration.

1. Introduction

Hydrogels are extensively used in regenerative medicine research as a consequence of their high-water content, meaning that they can be placed into a biological system without compromising viability.[1–3] Furthermore, given that they are formed from large, organic molecules, there is considerable scope for customizing the materials through modifications in the chemistry of the polymer backbone (the introduction of cell adhesion moieties, etc.), or by manipulating the gel cross-linking process.[4,5] The first application of encapsulation to protect mammalian cells was in the 1980s, to enable the delivery of pancreatic islets as a treatment for diabetes.[3] This has ultimately spawned a very active research field, where gels are often used for the delivery of cells or other therapeutics.[6–8] In the last ten years, gels have been used widely to study how cells are able to respond to their local environments, enabling ground-breaking work that not only starts to reveal how cell fate can be determined by tailoring stiffness,[9,10] the geometry of moieties distributed around the cells,[11,12] and the viscoelasticity of cell attachments/environments,[13] but can be exploited to synthesize unique delivery systems.[14,15] All of this points to the fact that there should be a panapoly of novel gel-based materials that are making their way to clinical application. Despite all of this progress, however, this is not the case and one of the major reasons for this is that new medical materials must pass a very large number of biological safety tests before they are used. In addition, materials to be used in clinical trials or for sale on the market must be supplied via a manufacturing process that is developed, validated, and operated in accordance with Good Manufacturing Practice (GMP). This requires engagement with appropriate GMP-licensed pharmaceutical/medical device manufacturing facilities. The use of bespoke chemistries and formulation conditions can preclude the use of processing methods and equipment that are standard to the pharmaceutical/medical device

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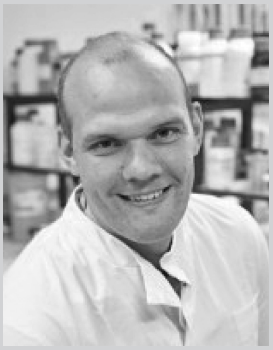
manufacturing industry. Thus, this furthers the increasing level of innovation required to develop a finished product. In many cases, this means that it is not economically viable to move these materials to the point of clinical trial and so clinical researchers tend to stick with a very small number of materials, which they use to deliver a multitude of therapeutic entities. In an attempt to take a much more rational approach to the use of hydrogels in clinical applications, we have pushed forward research on the structuring of materials that have already secured MHRA/EMA/FDA approval so that they can exhibit distinct physical properties. We have done this by modifying gelation conditions through the application of shear and by the absorption of reactive molecules into the gel structure in order to enable the formation of third phases within and between the gel particles. Although not completely void of the substantial costs required to deliver new therapeutic molecules, which require significant investment in toxicological and safety testing, the significant reduction gained from previously obtained data sets allows a more direct route to translation, which may result in higher degrees of adoption. Therefore, this paper summarizes the work that we have done in this area in the past few years and suggests areas for further research and development opportunities in the coming years.



Liam M. Grover is the Director of the Healthcare Technology Research Institute at the University of Birmingham. He is a materials scientist by training and his research focuses on the interactions between materials and biological systems. He has a particular interest in controlling the assembly of the extracellular matrix as a means to enhance regeneration, prevent scarring and inhibit the pathological formation of bone. His group currently works on developing treatments for these conditions and translating these technologies to clinical settings.

2. Structuring Hydrogels by Shear—Formation of Fluid Gels

The diverse and controllable characteristics of gels, in particular hydrogels (water-based gels), have initiated great interest across a multitude of industries and applications. Within the field of biomedical research, many FDA/MHRA/EMA-approved synthetic polymers have been studied for their ability to form gelled networks.[16] However, more recently, there has been a push toward more natural biopolymers such as polysaccharides and sugar-based hydrogelators[17] for both their ready availability and frequent similarity to the extracellular matrix (ECM). It is of no surprise therefore, that polysaccharide hydrogels have become widely used in the field of tissue engineering: predominately used as scaffolds, presenting a mimetic of ECM with the intrinsic ability to stimulate growth and the formation of new tissue.[18]

Their key ability to structure large volumes while creating an elastic system arises through the material microstructure; where coupling of long, structurally regular chains forms intermolecular junction zones that pack in a fashion comparable to those of solid-state materials.[19] However, to better understand the origins of the macroscopic properties, it is necessary to consider structure–property relationships from the molecular level. It is common to distinguish protein structures at multiple levels of organization.[20] These descriptions are also apt for polysaccharides: the primary structure detailing the sugar chain sequence, secondary structure defining geometric arrangement (helices and ribbons), and tertiary structure highlighting the 3D association of secondary structures.[21] This structuring across the polymer chains leads to varying levels of chain associations during the disorder–order transition upon gelation. For example, in the case of carrageenan (a galactopyranose polymer), domains of linked helices (tertiary structures) are formed through intermolecular association, but further require ionic-mediation to develop a continuous gel structure (**Figure 1**ai).[22] Therefore, inevitably, the number and density of junctions formed in this way between polymer chains is a direct function of the final gel strength and elasticity.[23]

This results in an array of material behaviors varying from strong gels, with relatively large moduli independent across a large range of frequencies, indicative of self-supporting structures, to weak maleable gels, where weakly associated chains lead to high-frequency sensitive systems with comparatively low moduli (Figure 1b).[24] However, the reliance on molecular configuration to achieve certain properties is a major problem, since when implanted or formed in situ the large number of other molecules that are present in the in vivo milieu interfere with these processes and can make them unpredictable. Such unpredictability is a significant barrier to clinical translation.

In an effort to produce more predictable systems, microgel suspensions known as "fluid" or "sheared" gels are being employed for their unique flow behaviors,[25] engineering the ability to self-structure post-shearing.[26–28] This presents a much more dynamic scaffold, which can be prepared exogenously for potential injection into the body.[29,30] Patented in 1990 as a system of microgels for use in food and cosmetics, derived upon shearing throughout gelation, fluid gels are substantially less rigid than their quiescently formed counterparts.[30] Shearing during the sol–gel transition results in networks of weakly flocculated, discrete gel entities (Figure 1aii), whereby particle sizes become dependent on the applied shear, and suspension rheology based on particle volume fractions.[31,32] However, in reality, such systems are more complicated, as interactions between particles on a mesoscopic level (structuring between particles) effectively dictate the bulk rheology of the systems. The particulate nature of these networks engenders a level of self-healing following manipulation, which is a key property that can be exploited in a number of biomedical applications.

The differences between quiescent gels and the properties of the microgels formed on shearing again lie in the microstructural changes upon gelation. One such example is the significantly reduced enthalpies of melting in carrageenan fluid

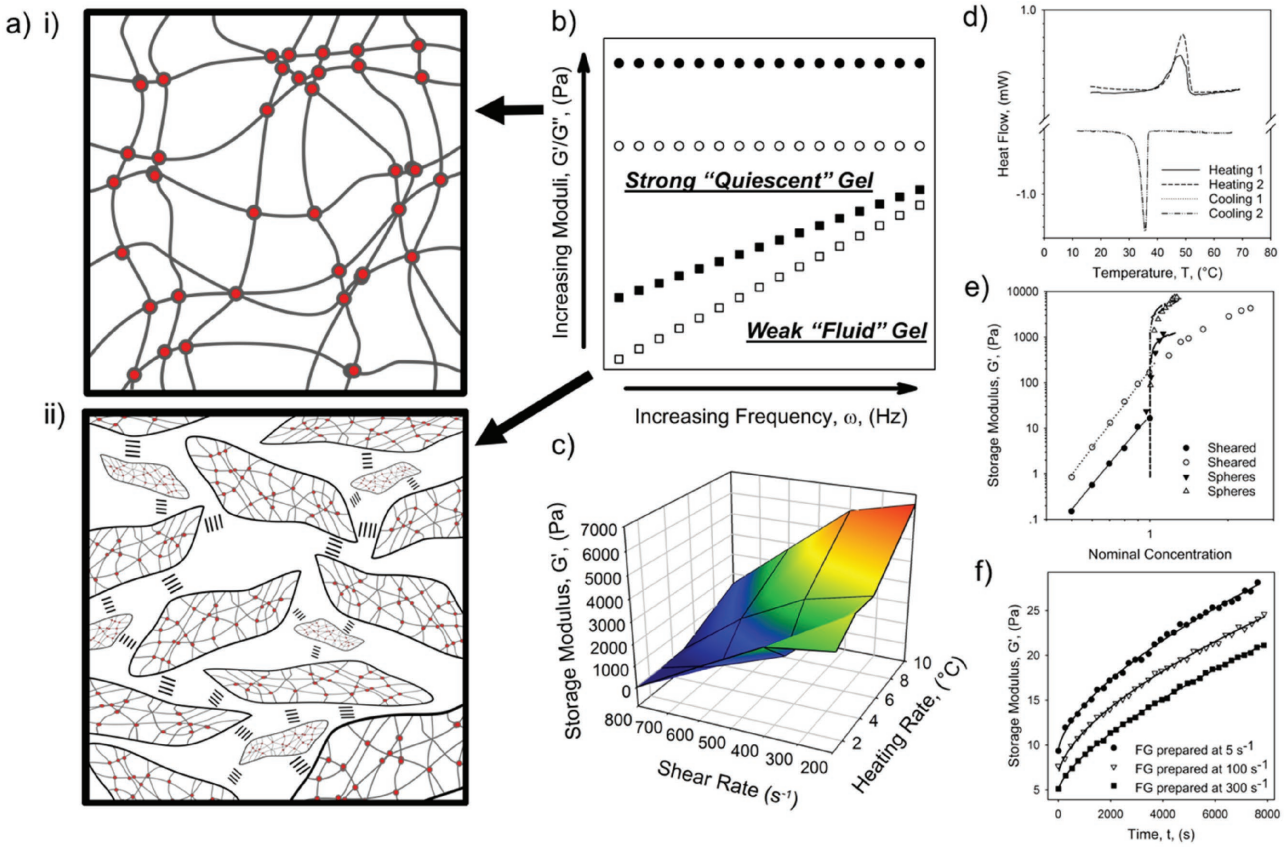


Figure 1. a) i) Schematic showing a typical gelled network where polymers interconnect to form junction zones (highlighted using red dots). ii) Diagram of a fluid/sheared gel system, where anisotropic gelled particles interact to form a weak continuous network. b) Idealized mechanical spectra showing G' and G'' dependency on frequency for both a strong "quiescent" and weak "fluid" gel system. c) 3D plot depicting the effects of processing on resulting suspension elastic response for protein fluid-gel particles. d) Differential scanning calorimetry (DSC) curves for carrageenan fluid gels. Note the change in peak height for the first and second melts, showing a change in degree of ordering between the fluid gel in the first heat and quiescent gel in the second heat. Adapted with permission.[34] Copyright 2012, Elsevier Ltd. e) Comparison of elastic moduli for sheared and spherical microgel systems: (●) 0.75% agar sheared gel, (○) 1.75% agar sheared gel, (▼) 2% agar microgel spheres, and (△) 5% agar microgel spheres. Trend lines show both power and Hertz fits to sheared and spherical systems respectively. Adapted with permission.[25] Copyright 2000, the Royal Society of Chemistry. f) Structuring as a function of time for carrageenan fluid gels (FG) prepared at various shear rates: (●) 5 s^{-1}, (▽) 100 s^{-1}, and (■) 300 s^{-1}. Reproduced with permission.[27] Copyright 2009, Elsevier Ltd.

gels when compared to their quiescent form (Figure 1d). Such data infers a heterogeneous polymer density across the gelled entities, with fewer ordered helices toward the particle peripheries.[33] This is not true for all polysaccharides however, as agar systems do not show such disparities.[32] Here, rapid gelation kinetics result in more uniform helical domains across the particles.[34,35] This suggests a mechanistic change in the microgel formation, driven by competition between two major factors: gelation kinetics and shear separation time/length scale (Figure 1c). Particle morphology also demonstrates a dependency on the competition between the two phenomena. In systems where gelation kinetics "k" greatly outweighs the time scale of separation "γ" ($k > \gamma$), rapid aggregation followed by subsequent shear breakdown develops large anisotropic morphologies. Whereas, in the counter case, $k < \gamma$, large shear forces confine growth, leading to more regular particles.[27] The consequential effects of such changes in particle morphology upon the aforementioned mesoscopic structuring have been clearly identified for both linear and nonlinear rheological properties. In the case of spherical particles, suspensions agree with Hertzian models resulting in elastic moduli arising through particle deformation, as systems become closely packed (Figure 1e).[25,36] Elastic response for anisotropic particles however, occurs at much lower volume fractions.[25] This is also reflected in the nonlinear measurements, with particles enhancing viscosities at volume fractions as low as $\Phi = 0.2$.[37] In these cases, large effective hydrodynamic volumes associated with anisotropic morphologies allow a vast degree of continuous phase to become structured by very few particles.[37–41]

Although some of the literature highlights the differences between quiescent and fluid gels, projecting the design rules in which to engineer systems with specific intrinsic properties, the actual nature of the interstitial space between particles is yet to be adequately defined. One theory proposes that disordered "hairy-like" polymers at the particle interfaces interlink to form a weak network between particles.[32] This may be the case for closely packed particles, where the interstitial layer is of the order of several hundred nanometers. However, an alternative explanation proposed here would be a combination of entropically driven structuring,[40,41] resulting in steric

confinement of particles, and/or weak electrostatic bridging between microgel interfaces at smaller length scales. Such a theory would encompass hysteretic effects, where self-structuring postshearing through entropic interweaving and electrostatic bridging, results in the observed recovery of an elastic network (Figure 1f).[26–28]

3. The Delivery of Biological Therapeutics Using Hydrogels

Many hydrogel materials have been used for the encapsulation and culture of cells in vitro, both for continued immunoisolation[42,43] and for the controlled delivery of biotherapeutics (both cells and proteins).[44–48] With the use of traditional "quiescently" gelled materials this can be very challenging, since gelation in a complex biological environment rich in ions and proteins can modify the kinetics of the gelation process and result in a material that gels unpredictably or behaves unpredictably following the gelation process – something that is completely unacceptable in a clinical environment. Up until relatively recently, there was also little consideration to how the local environment has a strong influence on the dynamic mechanical properties of these materials. Alginate, for example, is now known to lose mechanical integrity when placed into a physiological environment,[49,50] due to ion exchange of the divalent cations that enable the formation of "egg-box" junctions for monovalent ions such as sodium.[51] This causes the dispersion of the polymeric material and a loss of its ability to structure the surrounding liquid. This unpredictable behavior may explain why immunoisolation using polymers like alginate has proven ineffective when applied clinically, unless other polymers are incorporated as mechanically protective and permselective coatings.[42] What is also becoming very clear is that the materials are not "biologically inert", but rather provide subtle environmental cues that strongly drive cellular behavior. Initially, this was thought to be principally a consequence of the modulus of the materials,[9,10] but more recently reports have suggested that this can be modified by changing the distribution of grafted adhesion moieties and the nature of the interactions that may be formed with the polymer chain.[4,11,12] It has become clear that the level of entrapment that can be provided by the relatively stiff quiescently gelled matrix can have a significant influence on biological properties, including the capacity of cells to proliferate, maintain specific phenotypes or even secrete functional protein molecules as shown in **Figure 2**.[52–54]

3.1. Sheared/Fluid Gels as Delivery Agents

The shear processing of gels offers an additional degree of freedom for the production of cell and protein-delivery devices. From the perspective of handling, the reversible structural properties of fluid gels allow it to liquefy upon the application of shear, but may also be combined with biological fluids such as platelet-rich plasma or bone marrow aspirate and still thicken/solidify in situ. This is often very difficult to achieve with injectable hydrogels that solidify through a process of cross-linking, in situ. As such, fluid gels have the capacity to act as delivery agents for a great number of different cell and protein products (**Figure 3**).

Mesenchymal stem cells alone have been proposed to have beneficial effects in patients suffering a multitude of pathologies across body systems, including: the lungs,[55] brain,[56] musculoskeletal system,[57,58] peripheral nervous system,[59] skin,[60] and the heart.[61] Although some have reported that direct injection of these cells into the affected tissues can have beneficial therapeutic effects, the level of engraftment of the cells to the site of application typically ranges between only 1% and 10%,[62–65] and has been proposed to be as low as 0.001%.[66] As a result, the number of cells that are typically used in these therapies tend to be very large, ultimately creating the potential for carcinogenesis and other negative outcomes. The cost of such a treatment is, of course, considerable and as has been shown with autologous chondrocyte implantation (ACI), the clinical outcome and patient benefit over standard of care is unclear.[67,68] There are a number of ways to overcome these issues, which include the retention of the cells at the site of the implant within a material or through the use of cheaper autologous therapies. These are classified as minimal interventions by the regulator.

We have been able to develop materials for the repair and regeneration of cartilage, the cornea, and the skin that can be delivered in a way that is amenable to eventual clinical application. Through careful formulation, we have been able to produce materials that may be delivered using a range of different applicator technologies already used by clinicians, including cannulas/needles, sprays, and eyedroppers. Below, is a brief description of how we have used fluid gels to produce these therapeutics along with our progress in taking these to the point of clinical application. Furthermore, we have outlined major challenges that still need to be overcome in order to enable transition to the clinic. For each example, we have also outlined the clinical problems that we are attempting to address in order to place the technologies in context.

3.1.1. Cell Delivery for Cartilage Regeneration

Articular cartilage is a specialized avascular connective tissue layer covering the ends of bones that come together to form joints. It provides a smooth lubricated surface to minimize friction between the contact areas of the bones, and facilitates efficient load distribution through to the subchondral hard tissue. Chondrocytes, the sole cell type within cartilage, are sparsely distributed within a dense extracellular matrix that is mainly composed of collagen, water, proteoglycans, and glycoproteins. The high concentrations of hyaluronan and proteoglycan aggrecans allow for large volumes of aqueous medium to be entrapped within the matrix, and for covalent binding to other structural proteins, respectively, providing articular cartilage with its shock-absorbing properties.[69] Collagen organization, as well as relative matrix composition, cell density, and cell morphology, vary as a function of depth from the articular surface and act collectively to resist the range of forces experienced by the joint.

The avascular nature of cartilage and the low number of cells that are found within articular cartilage matrix result in a tissue

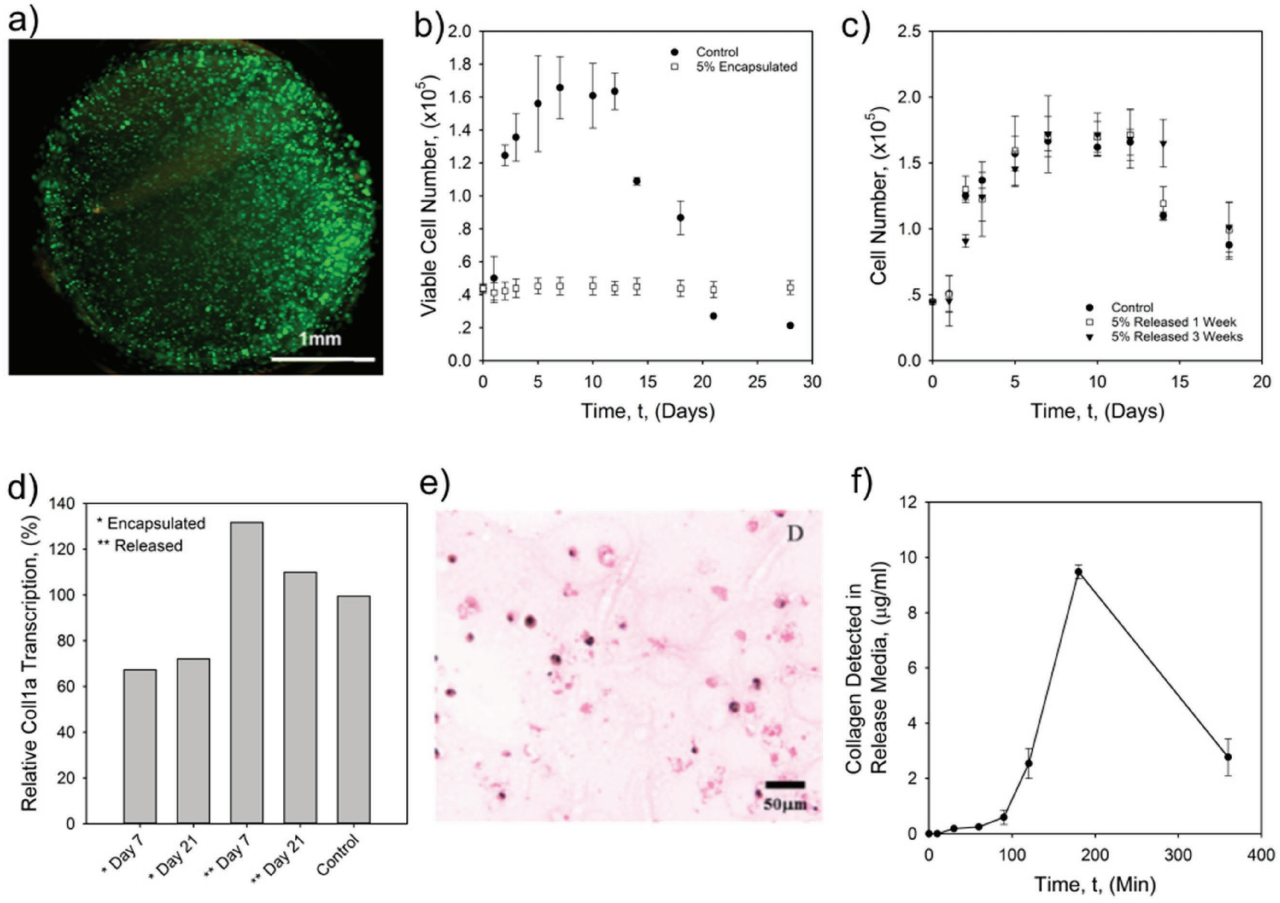


Figure 2. Effect of entrapment on cellular activity. a) Live/dead staining of encapsulated fibroblasts 4 d postencapsulation in 5% w/v alginate quiescent gel. Live cells stain green and dead cells stain red. b) Growth curves of the encapsulated 3T3 fibroblasts shown in (a), compared with nonencapsulated 3T3s (control). The encapsulated fibroblasts remain at a constant viable cell number up to 33 d postencapsulation, rather than exhibiting the normal bell-shaped growth curve seen with monolayer cultured fibroblasts. c) Fibroblasts released from encapsulation in 5% w/v alginate quiescent gel display normal growth in monolayer culture after both 1 and 3 weeks encapsulation. Adapted with permission.[52] Copyright 2009, Elsevier Ltd. d) Relative transcription of Coll1a by 3T3 fibroblasts encapsulated in 2% w/v Ca–alginate quiescent hydrogel for 7 and 21 d. Expression is shown for encapsulated cells and those that are released and grown as monolayers for 48 h and control fibroblasts, grown as monolayers having never been encapsulated. Col1a transcription was found to be only approximately 25–30% lower than before encapsulation. e) Despite the relatively high Col1a transcription levels while encapsulated, Haematoxylin Van Gieson (HVG) staining of the samples revealed very little collagen matrix had been produced by the encapsulated cells even after 3 weeks. f) However, soluble collagen was detected within the alginate structures and could be released into physiological media. Overall, this suggested that while the alginate gel system allowed for the passage of essential collagen precursor molecules and their assembly into soluble collagen, it sterically hindered collagen fibrillization. Adapted with permission.[53] Copyright 2012, the American Chemical Society.

with poor regenerative capacity following damage. Articular cartilage is one of the most challenging tissues to engineer or mimic synthetically due to the complex transitions in composition and ultrastructure, which are vital to its overall mechanical function. Consequently, most therapies for the repair of damaged cartilage involve tissue grafts or exploiting the local biology to facilitate new tissue formation. The current gold standard treatments in the repair of focal cartilage defects are microfracture or mosaicplasty. Microfracture involves removing the damaged cartilage and making a series of small holes in the subchondral plate causing bleeding from the marrow, and the formation of a clot that fills the focal defect. The aim here is to introduce and retain progenitor cells (present in marrow) into the defect site, a fraction of which have the potential to develop into chondrocytes and facilitate new cartilage formation. In mosaicplasty, small portions of cartilage are transplanted from nonarticulating regions of the affected joint into the cartilage defect site. This approach enables the engraftment of an already fully developed native cartilage structure. A common drawback with both methods is the formation of a fibrocartilaginous matrix as opposed to hyaline cartilage.[70,71] Fibrocartilage does not exhibit the same specialized surface structure as hyaline cartilage. As a consequence, the new tissue is mechanically inferior to the surrounding intact cartilage (particularly in resisting shear forces), which increases the risk of failure. A range of cell-based therapies, such as ACI, have been developed for the production of a native articular cartilage. To date, none of these methods have been able to surpass microfracture as the method of choice for orthopedic surgeons. Although there has been some success with the use of bone-derived mesenchymal stem cells for cartilage regeneration, there is a recognized need for culture and delivery systems that successfully maintain the

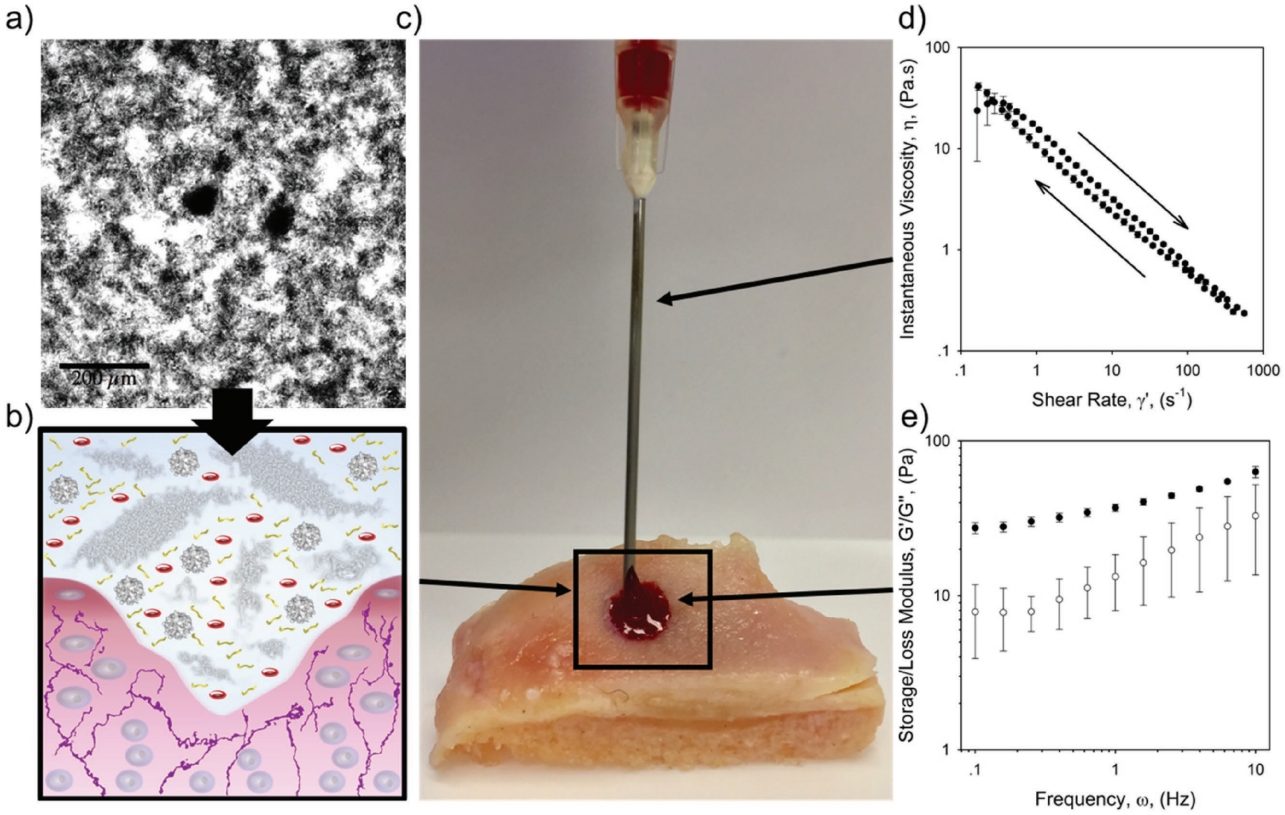


Figure 3. a) Optical microscopic image of diluted alginate fluid gel negatively stained with blood. Red blood cells (black) become sterically localized by the sheared alginate gel (white). Scale bar represents 200 µm. b) Schematic diagram depicting the stabilization of PRP (platelet rich plasma) via fluid-gel particles for in situ cartilage repair. c) Section of a tibial plateau with defect plugged using alginate/BMAC (bone marrow aspirate concentrate) fluid gel using a syringe. d) Flow profile obtained for 10% blood serum in 1% alginate fluid gel. The plot shows data for both increasing and decreasing shear, highlighting its reversible shear thinning behavior, crucial for application via syringe. e) Frequency sweep obtained for the same sample showing the typical weak solid-like structure exhibited by fluid gels under static conditions.

required chondrogenic phenotype during culture expansion in the lab, and upon implantation – especially for maintaining chondrogenic phenotype during the healing process. Alginate-based gels have been used as a biomaterial in chondrocyte culture since the 1980s as demonstrated by Guo et al. in 1989.[72] The general popularity of alginates in bioengineering stems from their good biocompatibility/tolerance with a range of tissues, low cost, and ease of gelation using divalent ions (many of which are endogenous to the human body). However, it is the similarility between the ultrastructure of gelled alginates and that of native extracellular matrix that has made alginates favorable for use in research and development into chondrocyte culture.[51,73] The 3D structure of the gels is thought to help the chondrocytes exhibit more in vivo-like behavior.[74] Furthermore, the ability to form 3D structures with tunable bulk mechanical properties, incorporate cell binding moieties, and allow the diffusion of soluble factors have proven to be important features in supporting chondrogenesis and maintaining chondrogenic phenoptype. Thus, their regenerative potential is maximized.[51,75] Hwang et al. demonstrated that the porosity in a gel system can be tuned by the addition of gelatin microbeads as a means of enhancing the mass transport of oxygen, nutrients, secreted biomolecules, and waste products.[76] Promising human cell-based models have also been developed with good medium to long-term cell viability. Choi et al. successfully cultured human articular chondrocytes in alginate beads over 15 d, and Loeser et al. successfully cultured human articular chondrocytes in alginate exposed to the chondrogenic growth factor insulin-like growth factor-1 and reported 95% survival at 21 d.[77,78]

Beyond the successful culture of chondrocytes in a lab, a major issue is the delivery and retention of chondrocytes into a focal defect using a non-invasive approach. Many have attempted to form in situ gelling materials that are capable of solidifying in the focal defect, and there are clinical products on the market that form a polymerized biological scaffold within the defect (BST-CarGel, Smith & Nephew; GelrinC, Regents Biomaterials; Chondron, RMS Regrow). We have demonstrated that it is possible to use "sheared" or "fluid" gels as vehicles to deliver and retain populations of chondrocyte cells. These materials can be injected into an aqueous medium and will retain their mechanical integrity over a period of 21 d of ageing (**Figure 4**). Importantly, the material can be injected through a narrow gauge needle while maintaining the viability of the incorporated cell population (Figure 3). Once injected into a defect, unlike gel systems that are gelled in situ or before implantation, the cells are able to move within the structure of the fluid gel and can migrate down into the bottom of the tissue

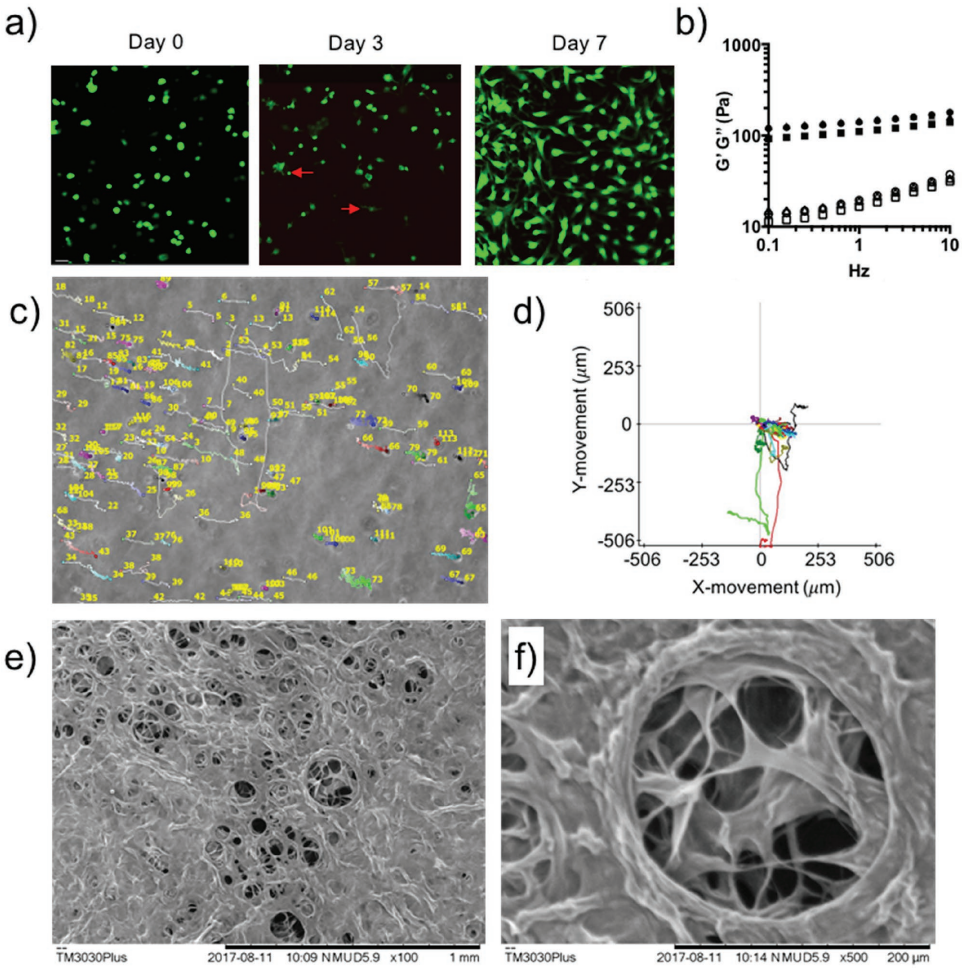


Figure 4. a) Sedmination of cells through a fluid-gel network over 7 d. b) Mechanical stability of fluid alginate over 3 weeks (day 7: circles; day 14: squares; day 21: triangles; *G′*: closed symbols; *G″*: open symbols). c) Cell tracking showing lateral movement of cells through fluid alginate. d) Quantification of the cell movement in the *x*–*y* plane. e) Fluid alginate architecture showing voidage regions in the polymer network.

defect (Figure 4). However, recent work within our group has also suggested that the lack of physical entrapment of the cells can result in the dedifferentiation of the encapsulated cells into a fibroblastic phenotype. While this lack of physical entrapment may ultimately hinder the use of fluid gels for the delivery of differentiated chondrocyte cells, we have demonstrated that fluid gels are effective in the delivery of other biological agents, such as platelet rich plasma and bone marrow aspirate concentrate, and their stabilization within a model joint defect. Our current work aims to translate this technology into a usable minimal intervention for cartilage augmentation (Figure 3). The ability of fluid gels to facilitate cell migration can actually be an advantage when designing therapies for other tissue applications. In the case of the skin, the ability for cells to migrate through the surface of the material would enable a topically applied cell population to fully colonize the wound bed.

3.1.2. Skin—Keratinocyte Delivery to Aid Wound Closure

Skin exhibits a multilayered structure with the uppermost layer of the epidermis consisting of layered keratinocytes that differentiate from the basal lamina over a period of several weeks.[79] The layer below the epidermis, known as the dermis, consists of a population of fibroblast cells that play a role in secreting extracellular matrix molecules such as collagen types I and IV and glycosaminoglycans.[80]

Serious damage to the epidermal and dermal layers break this fragile interplay until healing occurs and the wound is closed. In the case of large and deep burns, wound healing without intervention can take several weeks, wherein a patient is likely to develop acute systemic dysfunction with high risk of death through systemic infection and dehydration.[81] In order to speed the process of conventional skin grafting, it is possible to grow a multilayer sheet of keratinocytes in the laboratory in a period of up to 3 weeks.[82–85] These cells may then be applied to the surface of the wound. The keratinocytes then accelerate the closure of the wound by secreting factors that are able to stimulate collagen deposition (TGF-β1) and differentiation of the cells in the wound bed into myofibroblasts, which are able to fully close the wound. Although this process was pioneered in the 1970s and has found use in the clinic, the cost of the treatment, in addition to the amount of time it takes to generate sufficient cells, limits utility.[86] Poor outcomes in terms

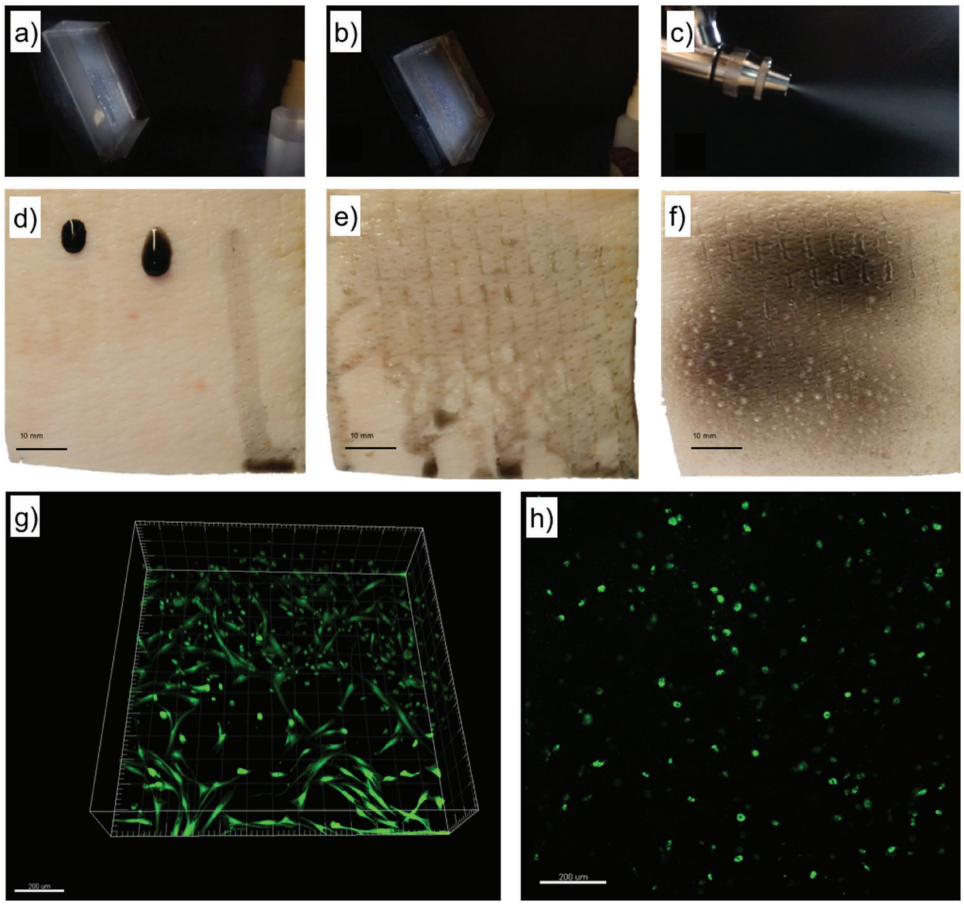


Figure 5. Cell encapsulation and spray deposition of gellan gum fluid gel. Video of a) spray delivery with handheld spray pump of material to tilted gelatin substrate demonstrated poor adherence of water b) compared to gellan gum fluid gel. c) Airbrush spray delivery system was used to standardize air pressure at delivery (15 PSI) and nozzle size (0.75 mm). d) Comparison of dyed gellan fluid gel (left), quiescent gellan gel (middle) and sodium chloride (right) droplets to porcine skin indicates a low rate of spreading of the gellan explained by the higher viscosity and structural stability of the material. e) Airbrush spray delivery of dyed sodium chloride to porcine skin showed rapid run-off with pooling of material at the bottom of the substrate, f) whereas uniform distribution without run-off was achieved by gellan fluid-gel spray delivery. g) Fluorescence cell viability assay of human dermal fibroblasts (HDF) 7 d after encapsulation in gellan fluid gel (live cells green, dead cell red), cells are viable and attach to the bottom of the well and seem to migrate out of the gel. h) High viability of cells at day 1 following spraying of encapsulated HDFs in gellan fluid gel.

of graft take, formation of bullae (blisters), poor handling due to fragility of sheets, and wound contraction have been reported. More recently, spray technologies have been used to deliver autologous and allogenic keratinocytes to the surface of wounded skin. Preconfluent cells have demonstrated utility in regenerating the skin and require a similar culture period of approximately 2–3 weeks.[87–89] Consequently, there is now a drive to develop allogeneic therapies from immunogenically privileged neonatal foreskin fibroblasts and keratinocytes.[90] However, a phase 3 clinical trial in nonhealing wounds was stopped prematurely due to low efficacy.[91] There are also products available that eliminate the in vitro culturing step by separating epithelial cells from patient biopsies in the clinical setting, and these cells may then be dispersed and sprayed onto the wound surface in a single procedure.[92,93] Although these technologies have been available for some time, there are as yet few clinical trials that show a conclusive benefit for using this approach.[94,95] One potential reason for this is that the cells themselves are delivered using a low-viscosity spraying system and anecdotally the level of "run-off" from the wound surface following spraying is high.[86] Although some products now try to remedy this using fibrin glue as an adjunct, the technology has yet to find strong traction in the clinic.[96,97] One of the major advantages of using a fluid gel system for the delivery of keratinocytes is that the material itself shear-thins considerably under the rapid shear forces experienced in a spraying system. We have utilized fluid-gel systems that are formed from a polysaccharide called gellan gum. Populations of cells may be incorporated into this system and can then be sprayed through a nozzle and deposited onto a surface as shown in **Figure 5**a–c. The soft solid then rapidly structures as it impacts on the skin, where the cells are retained on the surface, rather than running off, maintaining high-levels of viability. Importantly, the structure of the fluid gel allows cell movement within it, and we have shown that the viscosity of the material can be tailored to allow sedimentation of cells onto the upper surface of the tissue. In addition to being sprayed onto the surface of the skin, the gellan fluid gel may also be used to spread cells

across a surface, yet be retained in place once the spreading process has been completed. Figure 5d–f demonstrates how the material is retained on the surface of the skin. The material is compared against sodium chloride and a gellan quiescent gel. When the surface of the skin was inclined, the fluid gel was retained on the surface of the skin in exactly the same way as the gellan quiescent gel. It is worth noting that in order to get the quiescently gelled gellan to spread and then reset on the surface of the skin, the material was heated to around 50 °C before being allowed to cool, which would not have been conducive to cell survival. Shear structuring of the material was a successful way to enhance functionality without compromising cell viability (Figure 5g–h). The next challenge with this material will be to develop a manufacturing and packaging process that will not compromise the properties of the finished product. At present, the delicate structure tends to breakdown when exposed to doses of gamma irradiation considered low by industry standards. The development of non traditional sterilization methods may prove essential in the transit of these materials to the clinic. Although autoclaving is a possibility without causing a significant change in the properties of the final product, this method is unsuitable for the delivery of delicate therapeutic proteins that may be required to enhance the regenerative capacity of the tissue.

3.1.3. Cornea—Scar Prevention

The cornea is a layered structure exhibiting very high levels of organization in order to both protect the inside of the eye and provide the majority of the eye's refractive power.[98] The cornea is composed of five layers, with the anterior surface of the cornea consisting of epithelial cell layers that overlay a tough collagenous layer (Bowman's layer), mostly consisting of tightly woven collagen type I meshworks. The corneal stroma is an optically transparent layer composed of heterotypic collagen type I and V fibrils of around 30 nm in diameter, embedded within a proteoglycan matrix.[98,99] The stroma makes up 90% of the overall corneal thickness and is maintained by resident keratinocytes.[98] The collagen fibrils are arranged in flattened tightly spaced bands called lamellae that traverse the diameter of the cornea and are superimposed over one another making up most of the stromal thickness.[100] Further structuring occurs from the interlacing of the lamellae, which is most prominent within the anterior and mid-stromal layers and less so in the posterior stroma, where they are stacked in a similar fashion to layers in plywood.[98] The posterior stromal region, particularly in the central corneal region, is more hydrated than the rest of the stroma and thus the corneal stroma overall is akin to a highly structurual and mechanical anistotropic hydrogel system. The predominant collagen orientation within the stroma also varies as a function of depth. In the anterior region, the collagen does not exhibit any preferred orientation, whereas the mid- and posterior regions demonstrate preferred orthogonal arrangement of collagen along the nasal-temporal and superinferior directions.[101] The key structural factors of the cornea, leading to high optical transparency, are the uniformity of the collagen fibril diameters and tight regulation of the distances between adjacent collagen fibrils.[98,99,102,103] The fourth layer of the cornea, Descemet's membrane, is composed of a thin layer of collagen type IV and is designed to support the corneal endothelium layer that makes up the posterior surface of the cornea. When the corneal surface is damaged, it is typically capable of healing itself. However, in the case of infection (e.g., microbial keratitis), a significant inflammatory process is triggered, resulting in the upregulation of growth factors such as TGF-β1, which is a potent fibrogenic factor leading to disorganized deposition of collagen and extracellular matrix within the stroma.[104] Dysregulated collagen and ECM deposition prevents light transmission through the cornea and ultimately leads to blindness.[105] Treatment of diseases such as microbial keratitis may result in a corneal transplant if standard treatments with antibiotics and steroid fail.[106] Therapeutic proteins such as decorin have been shown to regulate the deposition of collagen by binding the inflammatory cytokine TGFβ-1.[107] This prevents collagen upregulation as well as the differentiation of keratocytes to the myofibroblastic phenotype that causes wound contraction and ECM deposition.[108] Topical delivery of therapeutic proteins/molecules to the surface of the eye has previously been attempted, however with limited success.[109–112] Gellan fluid-gel based eyedrops allow for the initial retention and then tailored release of the therapeutic agents onto the surface of the eye over a period of hours, as opposed to seconds/minutes as experienced with the majority of eyedrops (**Figure 6**a–c).[113] Since gellan is optically transparent, a patient's vision would not be compromised by the gellan when placed on the eye. During blinking, the eyelid sweeps over and applies shear to the surface of the gellan eyedrop, causing layers of the material to be removed. The eyedrop is cleared from the surface of the eye over a period of around 2 h. This technology has been exploited to deliver a sustained dose of decorin to the surface of the eye, which we have demonstrated facilitates reepithelialization of the eye surface (Figure 6d,e), both ex vivo in an organ culture model of corneal abrasion and when challenged in an in vivo corneal abrasion model. In addition to delivering the molecule to the surface of the eye over an extended period of time, we believe that the eyedrop will provide some relief to patients with this very painful condition, by lubricating the surface. Indeed, it has previously been reported that fluid gels, if formulated correctly, can be used as an adequate lubricant for surfaces.[114]

4. Bioprinting

In addition to enabling the encapsulation of cells and proteins for in situ delivery, the "self-healing" structure of fluid gels means they have found application as supports for additive layer manufacturing (ALM) of soft material structures, with complexity that is far beyond what has previously been reported. For many years, researchers have sought to replicate tissue structure and composition by using a combination of isolated cells and polymeric hydrogels that have a structural resemblance to ECM, to create an implantable living construct.[115] Most constructs have been fabricated using a gel casting process that provides little control over the microscale geometry of the deposited material, or the local mechanical properties so important to controlling cell fate. Innovation in

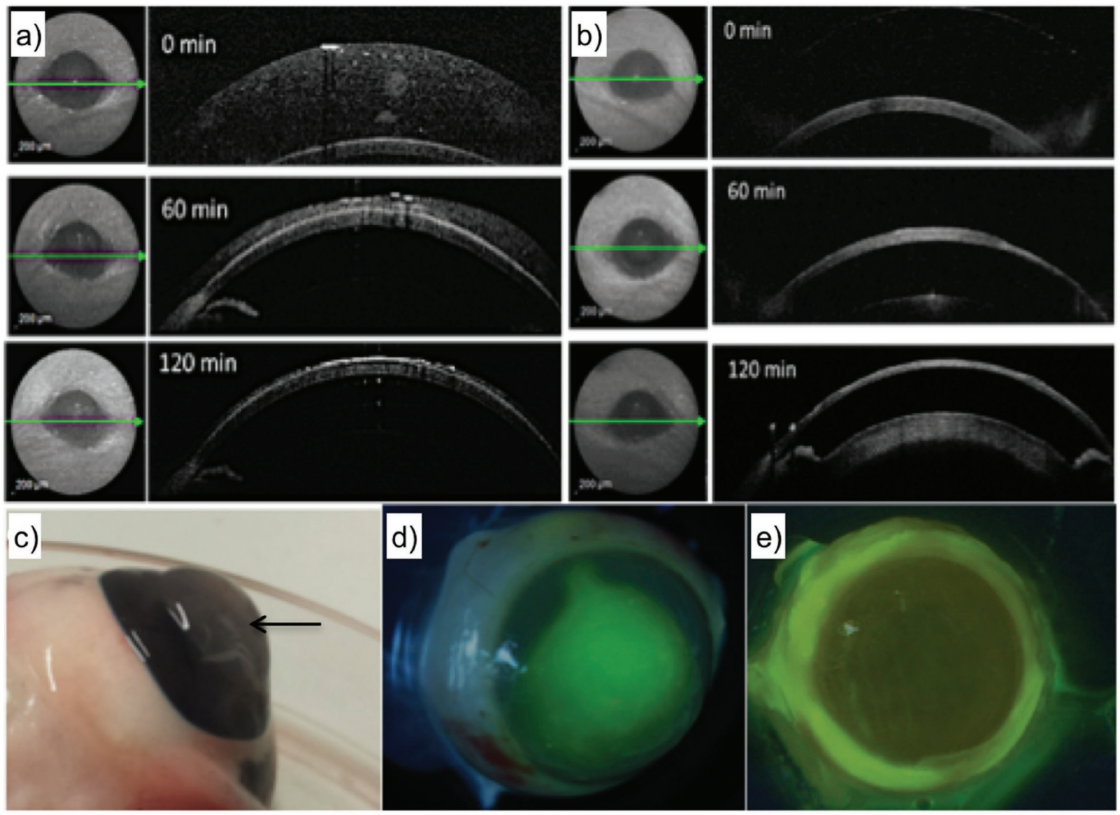


Figure 6. Optical coherance tomography (OCT) images illustrating the flow and clearance rate of fluid-gel eyedrops: a) when compared to PBS drops. b) On the surface of intact rat eyes over a 2-h period, fluid gel remained on the surface of the eye for nearly 60 min and some residue was noticed at 120 min. The PBS drop however cleared within 60 min. Fluid-gel thickens on the surface of freshly enucleated pig eyes, indicated by the black arrow. c) Eyes treated over 4 d with fluid gels containing decorin demonstrated significant reepithelialization in ex vivo organ culture models e) when compared to d) fluid gels without decorin.

this area has been driven largely by the desire to develop therapies that come close to offering the same key advantages as auto- and allografts, in that they are structurally and biologically the same as the surrounding tissue, while moving away from harvesting graft material itself. This is because the grafts are only suitable for relatively small defects and the graft approach in general is unlikely to be able to meet future clinical demand.

3D bioprinting using ALM has become a useful approach for creating structures with a greater level of complexity than traditional processing methods, such as casting, with some degree of control over the distribution of biological material throughout the structure.[116] While ALM of hard materials is relatively mature and adopted by a number of industries, ALM of soft mateials remains challenging. One of the major challenges for 3D bioprinting is the lack of suitable materials that are both able to replicate tissue material behavior and compatible with current 3D printing technologies. The advantage of biopolymer materials for tissue engineering applications is their similarity to native ECM. However, controlling their physical properties to be optimal for both the tissue engineering application and the mechanics of the printing process is more difficult than of synthetic materials. Biopolymers used for tissue engineering tend to have relatively low viscosities in the pregelled state that facilitates mixing with cells. Furthermore, many of these materials exhibit pseudoplastic (shear thinning) flow behavior that also can be advantageous for extrusion, when fabricating scaffolds. Unfortunately, low-viscosity materials can be problematic when 3D bioprinting, as the printed structure can collapse and lose its shape before solidification can be initiated.[117] Another problem when 3D printing biopolymer hydrogels is the inability to integrate multiple layers of material once gelled, preventing the production of integrated gels with regional variations in mechanical behavior. To overcome these problems, researchers often incorporate highly viscous materials to maintain a 3D shape after deposition,[117–119] which is not ideal as highly viscous materials can impede homogeneous cell mixing and often require increased extrusion pressure in order to print. This can lead to reduced cell viability as a result of the shear stresses inflicted on cells and has therefore limited their use in 3D bioprinting.[120]

We recently developed a technique that overcomes some of the issues associated with additive layer manufacturing when using low-viscosity materials, allowing them to be used as a bioink to create relatively complex soft-solid structures.[116] This was achieved by extruding a gelling biopolymer solution into a self-healing fluid-gel matrix, which suspends the fragile printed construct in the liquid state to prevent flow and thus retaining the deposited morphology. Additionally, as the printed construct remained in the liquid state, it was possible to build the construct layer by layer and interface two different materials

with dissimilar mechanical properties, creating a structure with distinct regions of anisotropic physical behavior. To demonstrate the potential for clinical application of the technique, we created a structure that recapitulated the osteochondral region as directed by microcomputed tomography (CT) imaging.[116]

Native osteochondral tissue has a gradually changing microstructure that extends from disordered mineralized collagen in subchondral bone[121] to cartilage, across which there are distinct variations in the relative concentrations of noncollagenous proteins, and orientation of collagen fibers as a function of depth.[122] This graded structure allows applied stress to be distributed across the interface without specific stress localization, helping to prevent delamination from occurring.[123] In our work, femoral condyle tissue was donated from patients following total knee replacement surgery and a full-thickness osteochondral defect was introduced using a surgical drill. The tissue containing the defect was scanned using micro-CT to generate a 3D model. Chondrocytes and osteoblasts were isolated from the tissue samples and, following primary cell culture, were added to 1.5% gellan and 1.5% gellan mixed with 5% nanohydroxyapatite (HA), respectively. Using the 3D model as a guide for accurate dimensions, an osteochondral implant was manufactured with the lower layer loaded with gellan, HA, and osteoblast cells, while the upper layer of the construct was manufactured using gellan gum loaded with chondrocytes. The manufactured cell-laden construct was then implanted into the defect of the ex vivo femoral condyle. After 4 weeks in culture, the construct maintained morphology and the encapsulated cells retained their phenotype within the distinct layers of the manufactured structure.

Our suspended additive layer manufacturing process has since been integrated into a commercially available extrusion-based 3D bioprinter (Cellink, Sweden), adding the ability to manufacture more geometrically intricate structures. To demonstrate the ability to retain the structural integrity of printed low-viscosity materials, an S-hook shaped construct was printed from a G-code file using 1% w/w gellan gum (**Figure 7**a). When printed onto a planar surface, the structure collapsed as the material began to flow under gravitational force (Figure 7b). However, when printed into a fluid-gel bed (prepared from 0.5% agarose), the structure retained the S-hook shape, clearly demonstrating protective support afforded by the surrounding fluid gel on the printed construct (Figure 7c). We then demonstrated the ability of the process to manufacture graded interfaces between soft-solid structures over relatively large length scales. Our model for this was printing conjoined doublet and triplet cuboids from single cuboid units measuring 10 mm × 10 mm × 5 mm, using gellan gum and gellan gum containing an orange dye (Figure 7d–f). Once the first cuboid was printed and remained in the liquid state, a second cuboid was printed with a 1 mm overlap with the first cuboid in the lateral plane to create an interfacial region. Once printed, the structures were crosslinked in situ using 100×10^{-3} M $CaCl_2$ for 30 min, and recovered as a single structure from the suspending particulate gel by gently washing with distilled water. This technique could potentially be used to model diffusion of molecules from one gel system to another, or fabricate discrete molecular reservoirs within a larger structure. Finally, we explored the potential to integrate different materials each with different gelation mechanisms using suspended bioprinting. Figure 7g–i shows the manufacture of cylindrical constructs consisting of a layer of gellan gum and a layer of type 1 collagen (PureCol EZ Gel, Advanced BioMatrix, USA), each layer with dimensions of 5 mm × 5 mm × 2.5 mm. In this example, the fluid gel base was maintained at 37 °C while the gellan cylinder was deposited first and left in the liquid state. The collagen was then deposited at 20 °C and left for 60 min to allow thermal gelation to occur, as the collagen equilibrated to 37 °C temperature of the supporting particulate bed. To initiate gelation of the gellan cylinder, 100×10^{-3} M $CaCl_2$ was added to the construct and left to gel for a further 30 min. Once fully crosslinked, the construct could be removed as one piece (Figure 7h). The finished construct exhibited a clear interfacial region between the two layers, which could not be physically separated, thus suggesting the formation of an interpenetrating polymerized network across the interface (Figure 7i). The order in which the different materials are gelled during fabrication of a construct can strongly influence the stability of the interface between them. Successful integration of collagen and gellan layers required the gelation of the collagen layer before gelation of the gellan layer. This is attributed to a mechanism reported by Gillette et al. when attempting to integrate collagen into crosslinked alginate, whereby the small pore size of the gelled alginate inhibited the formation of a network of collagen fibers within the alginate structure.[124]

In summary, the easily removable physical support offered by the fluid gel bed enables the deposition of biological materials in the liquid phase in 3D, with complex geometries and interfaces, before solidifying into a single construct with graded mechanical, chemical, and structural properties. This approach effectively decouples the viscosity of the starting material from the desired mechanical properties of the final printed construct. Biopolymers can be prepared at the relatively low viscosities required for low-pressure extrusion-based bioprinting, with the final mechanical properties engineered into the materials by gelation postextrustion.

Suspended bioprinting is, therefore, an enabler for a range of soft-solid materials, for which their full potential in tissue engineering is well established but not fully realized to date, to be used in the ALM of implantable biological constructs.

5. Conclusions and Outlook

Soft-solid materials have long been used as basic building blocks in the fields of tissue engineering and regenerative medicine, with certain materials having found use as wound dressings, eyedrops, and for the immunoisolation of cells. Despite extraordinary amounts of innovation around the manipulation of chemistry and local mechanics, which has significantly improved our understanding of how local environments can influence cell behavior, relatively few new so-called "smart materials" have made the transition to full clinical use. One of the reasons for this relative lack of progress is that the costs of taking new compositions to the clinic, through toxicity testing and characterization, is so high as to make it uneconomical. We have focused a large amount of research on modifying material structures by making changes

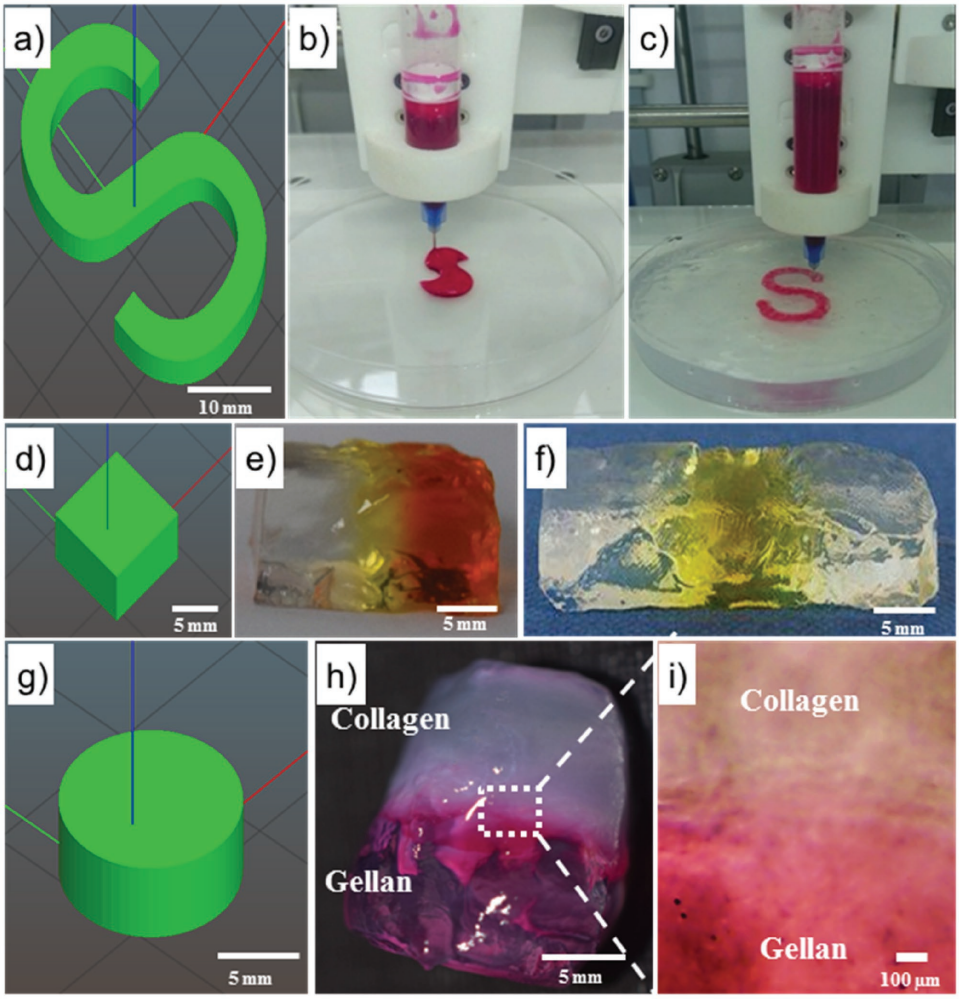


Figure 7. Suspended bioprinting of various biopolymer structures. a,b) G-code image for an S-shape hook with dimensions of 25 mm × 40 mm × 2.5 mm was printed using 1% w/w gellan gum onto a planar surface and imaged at the end of the printing process. c) The same structure printed into a 0.5% w/w agarose particulate gel bed. The construct printed onto the planar surface lost all structural integrity, collapsing under its own weight and flowed across the surface, resulting in a distorted structure. The construct printed within the particulate bed retained accurate dimensional conformation. d–f) Design and manufacture of multilayered constructs using suspended bioprinting. d) G-code file of a 10 mm × 10 mm × 5 mm cuboid. Two cuboids integrated using suspended bioprinting process; one contained a dye highlighting the ability to interface two separate biopolymer structures e) and another incorporating a double interface of gellan/dyed gellan/gellan to illustrate the potential for creating compositional gradients. g–i) Suspended bioprinting of a composite hydrogel cylindrical structure. g) G-code image of the single cylindrical structure with dimensions of 10 mm × 10 mm × 5 mm. h) Composite structure prepared from 1.5% gellan with 0.5% type 1 collagen using the suspended bioprinting process. i) A microscopic image of the interfacial region between the two dissimilar materials. These example constructs illustrate the potential of this approach to 3D bioprinting to manufacture biopolymer constructs with distinct anisotropic chemical and mechanical properties.

to processing conditions, without modifying composition. In the case of hydrogels, this has allowed us to take existing regulator-approved materials, and process them in a manner that allows us to produce structures that exhibit very distinct properties from the conventionally processed materials. Of particular use is the capacity of these materials to self-heal following the application of mechanical shear. This means that the materials are able to be delivered through a narrow aperture, which generates local shear and liquefies the material sufficiently that it may be deposited over a surface, where it solidifies. This has allowed us to produce eyedrops, cell spraying devices, and injectable delivery devices. Importantly, these materials are ready for translation to the clinic having undergone less cumbersome toxicity testing than for a completely new material. We have also used the self healing capacity of these materials to provide support in a new method for the additive layer manufacturing of cell containing soft-solids. This method differs to other soft-solid additive layer manufacturing methods, in that both the support phase and the cell-bearing phase can exhibit exactly the same composition. When the part has been removed from the print-bed, the support material can be dispersed by the application of gentle shear. This enables us to produce complex structures with a supporting medium without the worry of contaminating the implant surface.

These systems are still far from being exploited to their full potential and also still need considerable refinement

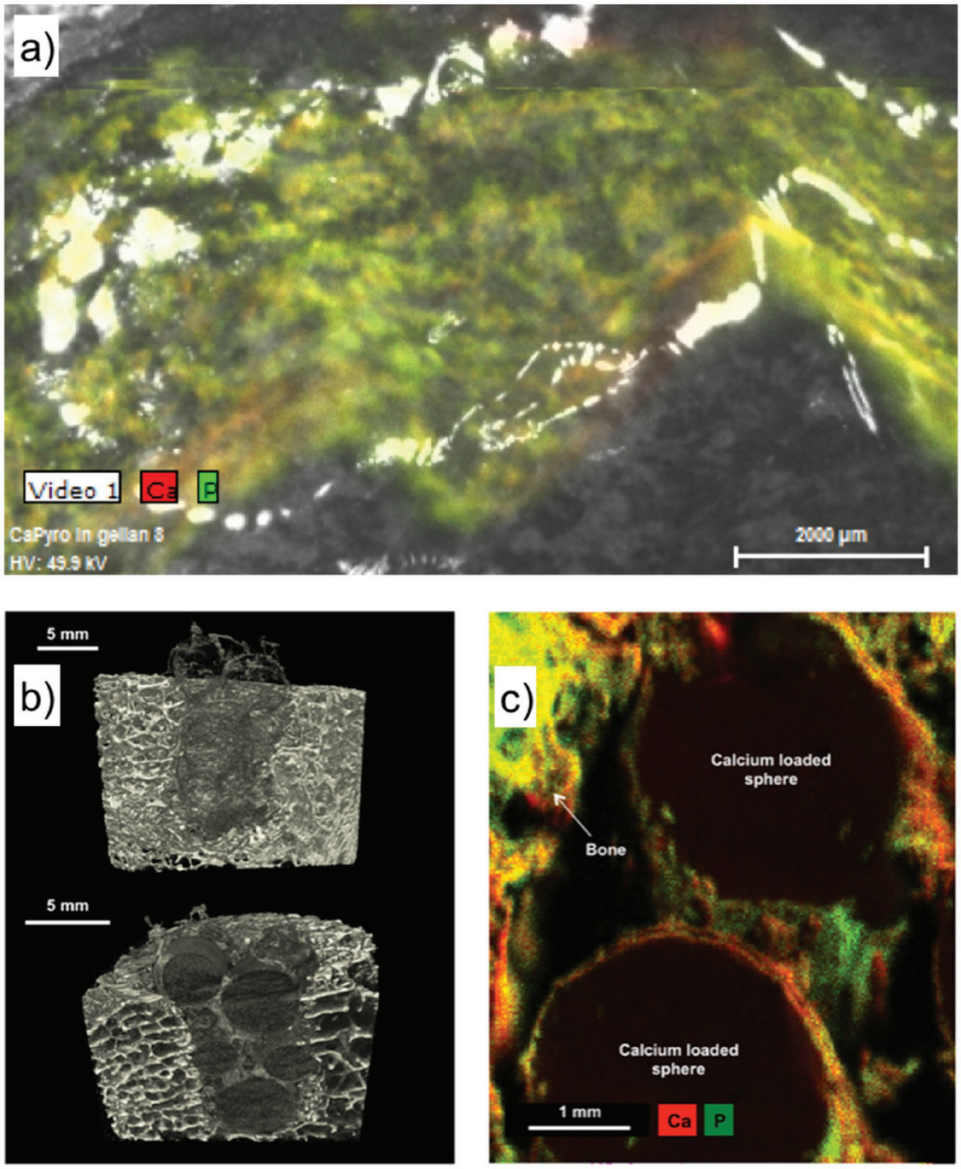


Figure 8. Fluid-gel materials may be loaded with osteogenic ceramics materials and extruded through a syringe, before they thicken upon a surface. a) The ceramic particles remain evenly distributed throughout the process. b,c) It is also possible to deliver the precursors of bone mineral formation within gel materials such that they harden in situ to form a relatively stiff composite matrix, but remain liquid prior to administration. This flexibility enables the tailoring of local environments to create more regenerative localized conditions. b,c) Reproduced with permission.[125] Copyright 2018, Wiley-VCH.

before eventually finding widespread use across medical fields. The polymeric gels are very difficult to sterilize using standard methods, and at present require non-cost effective low-throughput processes that allow for sterile-filling. Future innovations and collaborative work with the regulators should seek to develop new, more gentle sterilization methods, that are not likely to cause destruction of the polymeric material from which the structure is formed. Gamma irridation, ethylene oxide, and high temperatures are all very useful for sterilizing metallic materials, but impart enough energy into hydrated polymer systems to cause significant breakdown, and therefore significantly modify the properties exhibited by the materials. The use of alternative light sources (UV and blue light) and supercritical processing technologies may well provide the answer to this significant problem, but need to be validated and widely accepted by regulators worldwide.

We have also only just begun to explore the potential of these materials for delivering complex, composite structures. The incorporation of secondary materials into the fluid gel, which are capable of inducing osteogenesis in codistributed bone marrow aspirate and other sources of cell populations, has significant potential for shaping the local cellular environment in vivo, thereby enabling superior tissue regeneration (**Figure 8**a). Indeed, providing the reactants to generate a third phase between the polymeric components of the gel, enabling hardening into a comparatively stiff structure has already been achieved with some success (Figure 8b,c).[125]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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