Injectable cryogels for biomedical applications

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Abstract

To prevent postoperative complications, there has been a substantial interest in designing syringe injectable hydrogels. To date, cryogels remain the only viable option for preformed and large-scale hydrogels to be delivered through a conventional needle–syringe injection. Cryogels, a type of hydrogels with exceptional features, are fabricated at subzero temperatures. This process typically results in a biomaterial with a unique macroporous network, shape-memory properties, and exceptional flexibility allowing syringe injectability. These advanced biomaterials have been used for a number of biomedical applications, including tissue engineering, drug delivery, and more recently immunotherapy. This review summarizes the recent progress on the design of injectable cryogels, their current limitations, and strategies to further improve their properties for translatability into the clinic.

For a number of biomedical applications, including cell therapy and tissue engineering, there is an increasing need to engineer advanced three-dimensional (3D) scaffolds to provide a structural and mechanical support for cells and facilitate tissue regeneration [1-3]. For the latter, these constructs need to mimic the complex physical and biochemical properties of the native extracellular matrix (ECM) [4-6]. Additionally, the scaffolds must promote cell survival, proliferation, motility, and differentiation, as well as tissue integration within host tissues [7-9]. In this context, 3D scaffolds should be fabricated from biocompatible and resorbable polymers and have large interconnected macropores, ranging from 10-400 μm based on the targeted tissue. Additionally, they should provide a physical framework as well as a large surface area to enhance cell housing and encourage tissue formation. [10, 11].

Hydrogels have been extensively used as polymeric scaffolds due to their high water content, biocompatibility, and physical properties similar to soft tissues (Figure 1, Key Figure) [12]. However, the small pore sizes (usually in the nanometer range) of conventional hydrogels have hindered their biomedical applications due to limited cellular motion, cell spreading, and molecular diffusion of proteins, oxygen, and nutrients/waste products [13, 14]. To address these challenges, porogens such as sacrificial particles and organic solvents have been investigated to increase hydrogel porosity [15, 16]. However, due to insufficient pore interconnectivity and concerns over toxicity associated with incomplete porogen removal, their clinical applications have been impeded. Alternatively, a simple and eco-friendly technique known as cryogelation has recently attracted much interest as it produces macroporous hydrogels without the need to use toxic organic solvents [17]. Cryo-hydrogels (or cryogels) are typically formed in water (solvent) at subzero temperatures. When the solvent freezes, ice crystals form and subsequently expel the gel precursors (monomers, polymer, cross-linker and initiator), which concentrate into an unfrozen phase (Figure 2). The cryopolymerization or gelation occurs around ice crystals generating a dense, highly cross-linked polymer network. When thawed, ice crystals leave behind a continuous and interconnected macroporous system (Box 1) [18, 19]. These advanced hydrogels have recently drawn great attention for use in a wide variety of biomedical applications such as bioseparation, drug delivery, tissue engineering, and regenerative medicine [17].

Minimally invasive delivery of hydrogels is a critical aspect to bypass open surgery and associated post-surgical complications once these scaffolds are implanted in the body [18, 20, 21]. Therefore, engineering injectable hydrogels has become an active field of research (Figure 1) [22, 23]. To enable injection, most *in situ* forming hydrogels are delivered in a liquid form that will subsequently solidify in the body [24, 25]. However, injecting a liquid presents a number of limitations, including suitable gelation time, formation of gels with inadequate mechanical properties, biocompatibility, and the ability to protect the cargo of biomolecules or cells in complex biological environments [26]. Additionally, liquid precursor solutions may leak into surrounding tissues or dilute within the body fluids, which may not only limit hydrogel formation but also alter gel properties [21, 22, 27]. To overcome these limitations, it is essential to engineer solid (pre-formed) and well characterized scaffolds capable of being injected without any structural damage through conventional syringes [17, 18, 26]. Our 2012 publication disclosing the first cryogel scaffold to be injected through a conventional small-bore needle while recapitulating aspects of the native cell niche has sparked massive interest in the field.

These cryogels with unique features have created a new class of injectable materials applicable for biomedical applications [17-19, 28].

In this review, we discuss in detail recent efforts in the development and applications of state-of-the-art injectable cryogel scaffolds. Their inherent and unique features are a subject of considerable scientific research interest, which should accelerate and expand their potential for various hi-tech biomedical applications. To date, injectable cryogel scaffolds prepared from natural and synthetic polymers have been investigated in several biomedical applications including cell therapy, drug delivery, biosensing, wound healing, and tissue engineering (i.e. bone, skin, neovascularization, neural and adipose tissues). This review summarizes recent strategies, as well as challenges, to further improve their properties. Furthermore, a brief outlook on the future prospects of injectable cryogels is presented at the end.

Development and applications of injectable cryogel scaffolds

Over the last decade, injectable cryogels have been extensively investigated for the minimally invasive implantation of 3D scaffolds. Both micro- and macroscale cryogel scaffolds have been utilized to provide a porous construct in 3D, help protect encapsulated biological agents against degradation, as well as to control the delivery of mammalian cells and/or biomolecules to host tissues [29]. However, particle-based biomaterials may show inadequate retention at the injection site due to their small size [30]. This limitation increases the need for repeated injections, potentially leading to severe side effects and increased healthcare costs. To overcome these limitations, macroscale cryogels are being increasingly investigated as they can create a confined and localized construct remaining at the injection site [29]. With their remarkable sponge-like macrostructural characteristics combined with a set of unique features (e.g., shape-memory properties), injectable cryogels have emerged as a biomaterial of choice for a number of biomedical applications [17]. Their suitability for applications in tissue engineering and immunotherapy will be discussed in the next sections.

Hosting cells within the macroporous framework of cryogels

Injectable cryogels hold great promise for tissue engineering and immunomodulation [17, 31]. Their macroporous structure and high porosity promote cell survival, adhesion, infiltration, and proliferation (Figure 3). In 2012, the first published article on injectable cryogels clearly demonstrated their great potential as scaffolds for 3D cell culture and a carrier for cell transplantation and drug delivery [19]. In this work, the cryotreatment of methacrylated-alginate (MA-alginate) revealed new physical properties of cryogels, including shape-memory features and injectability, as compared to their conventional hydrogel counterparts. Additionally, this study showed the capacity of cryogels to control over several weeks the sustained release of large biomolecules such as bovine serum albumin and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Depending on the application, injectable cryogels can be fabricated from natural and/or synthetic polymers. For example, Rezaeeyazdi and colleagues have demonstrated that cryogel scaffolds can be made out of natural polymers while retaining their intrinsic biological characteristics even after cryotreatment [23]. Their hyaluronic acid (HA)-based cryogels displayed robust mechanical properties and injectability but lacked

NIH 3T3-fibroblast cell adhesion. On the other hand, gelatin-based cryogels exhibited weaker physical properties while promoting cell attachment, motility, and survival. However, when these two biopolymers were mixed together, HA-*co*-gelatin cryogels exhibited improved properties, combining the advantageous features of each polymer including syringe injectability. In another study, Bruns and colleagues have engineered injectable cryogels made out of synthetic polymers to support cell growth and delivery [32]. They showed that their injectable polyethylene glycol (PEG)-based cryogels were cytocompatible. Additionally, they demonstrated that the physical properties could be controlled effectively, highlighting the ability of cryogels to be fine-tuned with respect to a specific application.

Due to their ability to dissipate mechanical energy during compression, cell-laden cryogels are capable of protecting and retaining host cells upon injection [17]. These unique features make cryogels applicable for cell-related applications. For instance, Kim and colleagues demonstrated that heparin-*co*-gelatin cryogels could achieve high cell retention and survival following injection of NIH-3T3 fibroblasts. Due to these remarkable properties, injectable cryogels have also been used to restore blood flow by delivering fibroblasts in combination with endothelial growth factors [33]. Similarly, Béduer and colleagues found that alginate and carboxymethyl-cellulose (CMC) cryogels infused with primary neurons not only maintained high cell adhesion, but also protected the neuronal network during injection [15]. Furthermore, several approaches to functionalize cryogels with adhesion peptides or ECM proteins (e.g., RGD, laminin) have been investigated to further enhance cell-matrix interactions and cell retention at the injection sites [18, 32].

Although individual cell transplantation has many promising applications, tissue engineering often requires the delivery of more organized cell-based constructs. The technique of 3D printing has recently gained momentum as a promising approach to recreate complex layer-by-layer tissues [34]. However, emulating the biomechanical features of soft tissues while enabling minimally invasive delivery and integration of the printed tissue constructs into host tissues remain a major challenge. As shown by Béduer and colleagues, injectable cryogels can meet these specific requirements. They leveraged the technique of cryogelation with 3D printing to engineer sophisticated cryogels and encourage neovascularization [26]. In this study, they investigated the effect of pore size on blood vessel formation from 3D printed CMC-based cryogels. When tested in mice, subcutaneous injection of these cryogels with the largest pore sizes (~130 µm *vs* 16 µm) facilitated new blood vessel formation. In a second study, Qi and colleagues engineered injectable composite cryogels to manufacture large 3D bioprinted adipose tissues [35]. These composite cryogels were fabricated from a combination of gelatin, HA, and PEG, and displayed comparable stiffness to native adipose tissues. Furthermore, these constructs promoted attachment, proliferation, and the spontaneous adipogenic differentiation of human adipose derived mesenchymal stromal cells (HADMSCs). Additionally, human umbilical vein endothelial cells and HADMSCs were 3D printed on these cryogels. This strategy promoted formation of capillary-like networks within the engineered constructs and enhanced their integration into host tissues.

Another major advantage of cryogels is their capacity to allow cellular infiltration and trafficking as well as the controlled release of biomolecules. These attributes are of particular interest in the context of immunotherapy, where a construct can be used as a platform to manipulate immune cells in the body. Bencherif and colleagues have developed cryogel-based cancer vaccines to reprogram the immune system against melanoma [19]. Once subcutaneously injected into the body, these cryogel vaccines co-delivered irradiated tumor cells (antigens) and immunomodulatory factors (adjuvants and cytokines). Furthermore, these cryogel constructs enabled infiltration and activation of dendritic cells (DCs), the main regulator of the adaptive immune response [19]. Cryogel vaccines were also tested in the context of breast cancer [22]. In both studies, these cryogel vaccines delayed tumor onset and substantially improved survival (80%) in a prophylactic setting [19, 22]. When tested therapeutically, cryogel vaccines strikingly induced regression of established melanoma [19].

In a different approach, Shah and colleagues developed injectable alginate-based cryogels to support the generation of T-cells in mice following hematopoietic stem cell (HSC) transplantation [36]. Once introduced in the body, these cryogels loaded with cell-instructive cues induced T lineage-specific differentiation of transplanted cells. Mice treated with these bone marrow mimicking cryogels generated life-saving T-cells from the transplanted HSCs faster than untreated mice [36].

Injectable cryogels are a minimally invasive platform for biomedical applications

To prevent complications associated with surgical scaffold implantation, injectable cryogels are a viable alternative [18]. Zhao and colleagues fabricated injectable hemostatic cryogels for delivery into deep and bleeding wounds. Once in contact with blood, these cryogels expanded quickly and mitigated bleeding [37]. In comparison to commonly used gauze and gelatin hemostatic sponges, these cryogels exhibited improved hemostatic performance when tested in various mouse bleeding models. Furthermore, these cryogels also promoted wound healing in a rabbit model.

Injectable cryogels have also been considered for bone defect healing such as pathologic fracture or bone nonunion. Bone defects often require invasive surgery and are difficult to treat [38]. To tackle these challenges, Lu and colleagues functionalized injectable alginate-based cryogels with platelet-rich plasma (PRP) [39]. Although incorporating PRP negatively impacted the physical features of cryogels, several cycles of freeze-thawing improved their porosity and compressibility. These PRP-loaded cryogels enhanced the proliferation of human bone osteosarcoma cells and induced their mineralization. Another strategy relied on the encapsulation of biphasic calcium phosphate (BCP) to bestow cryogels with osteoconductive and resorptive properties for bone remodeling. Abueva and colleagues demonstrated that chitosan-BCP composite cryogels (CSGs) were injectable and displayed good protein absorption supporting pre-osteoblast cell attachment and proliferation [40]. Additionally, subcutaneously injected CSGs exhibited good biocompatibility and promoted recruitment of polymorphonuclear cells in rats.

As the cryogel dimensions and shape can be preset prior to injection, the desired geometry can be easily achieved to match a particular defect. These properties are especially useful for applications in soft tissue reconstruction, in which hydrogels are typically injected beneath the skin. To demonstrate their potential as dermal fillers for soft tissue augmentation, Cheng and colleagues subcutaneously injected heart-shaped HA-based cryogels in mice. These cryogels popped back to their original shape and size, and remained unaltered up to 30 days post-injection [41].

The minimal invasive delivery via syringe injection minimizes the risk of implantation-related complications (e.g., infections), which is a key consideration when designing biomaterials [37, 42]. The biocompatibility of a material is defined from its interaction with the host tissue. Capsule formation around implants indicates acute and chronic inflammatory responses [43]. For subcutaneously injected cryogels, host responses to implanted cryogels have been mixed: some cryogels induced capsule formation [26, 44], while others did not [40, 41]. These findings indicate that a number of important factors (e.g., type of polymer, polymer charge, degradation products) need to be taken into account when engineering cryogels [42, 45]. Although most injectable cryogels are designed to be biocompatible upon injection, a subset of immunomodulatory cryogels have been engineered to stimulate immune cells in the context of cancer immunotherapy [19, 22, 36, 44].

Strategies to enhance properties of injectable cryogels

To further expand their applications in the biomedical arena, various strategies are being investigated to improve the properties of injectable cryogels. One of their main limitations is the insufficient control over the release of biomolecules, especially low molecular weight components. Due to their inherent high porosity, drug-loaded cryogels have often been associated with a burst release, limiting their potential as a drug delivery carrier [46, 47]. To improve drug release kinetics, Koshy and colleagues hybridized cryogels with laponite nanoparticles (NPs) pre-loaded with immunomodulatory factors [46]. Unlike laponite-free cryogels, immobilizing laponite NPs in cryogels prevented a burst release. Additionally, varying laponite content further fine-tuned release kinetics from cryogels while preserving their syringe injectability.

During tissue regeneration, integration of cryogel scaffolds within host tissues may be hindered by the stable polymer walls. Cryogels fabricated from cell-responsive materials better recapitulate the dynamic nature of native ECMs. Cell-driven degradation can enhance ECM remodeling, promote cellular and developmental processes, and ultimately enhance integration of cryogels within host tissues. Koshy and colleagues fabricated gelatin-based scaffolds susceptible to enzymatic degradation by matrix metalloproteases (MMPs) [44]. Interestingly, the controlled release of GM-CSF from these cryogels accelerated their degradation due to the recruitment of MMP-producing immune cells. This concept of cell-mediated degradation could be further investigated and leveraged to better control the release of biomolecules and drugs from cryogels. For example, a number of strategies explored whether the design concept of MMP-sensitive hydrogels could be implemented to injectable cryogels. One of these strategies is based on using MMP-cleavable peptide cross-linkers or co-monomers to permit cell-mediated enzymatic degradation [45, 48].

Electrically conductive scaffolds can restore the electrical communication with biological systems. Recently, injectable cryogel bioelectronics have been developed by introducing electrically conductive components. For instance, polypyrrole-conjugated elastin-*co*-gelatin cryogels hybridized with carbon nanotubes exhibited electrical conductance upon compression [49]. Additionally, incorporating iron-oxide NPs turned these hybrid cryogels magnetically responsive, making them remotely controllable. These conductive cryogels can be useful for neuromodulation when electrical stimulations and mechanical softness are a prerequisite. In a different approach, conductive injectable cryogels have been used to promote wound healing. Zhao and colleagues developed quaternized chitosan-based cryogels hybridized with carbon nanotubes [37]. Once inoculated into wounds of mice and rabbits, these cryogels significantly enhanced wound healing and reduced blood loss. Furthermore, these chitosan-containing cryogels exhibited excellent antibacterial activity, which could further reduce the risks associated with infections and help patients heal faster.

Improving cryogel injectability

Improving injectability of large-scale or bulk cryogels

The injectability of cryogels is a result of their reversibly collapsible and elastic structure due to their cross-linking mechanism, nature of polymers used, and structural features such as interconnected macropores and dense polymer walls. To date, large-scale cryogels (with dimensions up to 8 x 8 x 1 mm) have been injected through 16-gauge (16G) needles. Although a 16G needle injection reduces invasiveness in comparison to surgical implantations, tissue damage can potentially be minimized to a greater extent with smaller needles are used. To reach that goal, cryogels require further optimization to exceed their compaction and improve injectability. One approach has been to reduce the polymer concentration. For instance, both the injectability and mechanics were tweaked by simply reducing the polymer content of gelatin-based cryogels, allowing their injection through 17G needles [33, 44].

The importance of cryogel elasticity during injection was confirmed by Liu and colleagues. They showed that the incorporation of a rigid polypyrrole network within gelatin-*co*-HA cryogels altered their elasticity and ultimately injectability [49]. Furthermore, Qi and colleagues demonstrated that cryogel robustness and mechanical properties are improved when a 4-arm PEG cross-linker is used. As a result, the cryogels were reinforced and able to be pushed through 16G needles [35]. While most injectable cryogels can be injected through large needles, injecting them through finer needles remains a major challenge. Shih and colleagues engineered tough alginate-based cryogels that simultaneously combine covalent and ionic cross-linking [22]. Unlike standard injectable cryogels that are typically covalently cross-linked, tough cryogels can be successfully injected through 18G needles. This type of cryogels exhibit high stretchability, in part due to reversible ionic cross-linking and self-healing properties. Another strategy to use thinner needles is simply based on injecting smaller cryogels. Bruns and colleagues investigated the syringe injectability of disc-shaped PEG-based cryogels while varying their overall diameters [32]. While all cryogels were successfully injected through 16G needles, they showed that smaller cryogels (diameters <2 mm) could also be injected through smaller 21G needles.

Small-scale cryogels for cell-therapy

For some specific applications, using large needles (<18G) during injections may cause tissue injury including bleeding and tissue disruption. The use of thinner needles (≥25G) is preferred for sensitive organs (e.g., brain) or tissues (e.g., nerves). Since bulk cryogels still cannot be injected through small-bore needles, microengineered cryogels (microcryogels) with diameters up to 600 µm have been investigated. Newland and colleagues fabricated ~300 µm diameter microcryogels by cross-linking 4-arm PEG and heparin in a water-in-oil emulsion [50]. These microcryogels were able to remain intact when injected through a 27G needle, and effectively maintained high cell viability when carrying neuron-like cells. Alternatively, other approaches have been investigated to fabricate microcryogels. Liu and colleagues generated PEG-based microcryogels on microarray chips that could be optionally loaded with human MSCs [51]. In contrast to large-scale cryogels, microcryogels can facilitate a more homogeneous cell distribution following seeding, lower the risks of cell necrosis, and be injectable through 27G needles. Another advantage of microcryogels is there potential to fill in large and irregular tissue defects [52]. For example, injectable gelatin-based microcryogels have been used to specifically adhere to liver lesions via transglutaminase-mediated binding. Furthermore, when infused with MSCs, these constructs effectively treated mice with local liver injuries [53].

Despite several advantages, microcryogels do not create a well-defined macroscopic environment and may diffuse out of the injection sites post-implantation. These limitations make large-scale cryogels more appealing scaffolds. That being said, microcryogels offer a number of advantages over traditional microgels due to their inherent porous structure. For instance, microcryogels made out of different polymers such as PEG, gelatin or alginate are injectable through 27G needles, while their microgel counterparts are not [51, 54, 55]. Additionally, Zeng and colleagues demonstrated that injecting MSCs with alginate along PEG-based microcryogels through 21G needles resulted in markedly increased cell viability when compared to alginate alone [56]. As microcryogels can effectively protect cells from shear-stress induced cell death experienced with narrow-bore needles, they have been applied for wound healing, intervertebral disc degeneration, bone repair, kidney disease and ischemic limbs [55-59].

Concluding Remarks and Future Perspectives

Over the last decade, injectable cryogels have gained a rapid and wide interest in the biomedical arena. They have been utilized as drug/cell delivery carriers, 3D scaffolds for tissue engineering, dermal fillers in cosmetic surgery, and cell-instructive platforms for immunotherapy (Table 1). Their unique interconnected macroporous architecture makes them fit to host and protect mammalian cells during syringe-injection, and to provide a microenvironment favorable for cell delivery, cellular infiltration, and neovascularization. The minimally invasive delivery of scaffolds can obviate the risks associated with surgical implantations. To date, being the only preformed and injectable large-scale scaffolds, cryogels are set to advance the field, more particularly in tissue engineering and immunotherapy.

Although significant advances in cryogel technology have been made, much more research is needed to address some of the current technical challenges such as control over material degradation, drug release, and cell-matrix interactions. Additionally, it is essential to better understand the impact of cryogels on tissue integration, cell differentiation and immunomodulation (see outstanding questions). Furthermore, the size of injectable cryogels could be a major limitation when moving from preclinical to clinical studies in humans, where larger scaffolds are usually required. Moreover, practical concerns of cost, manufacturability, reproducibility, and scalability of cryogels still remain.

Several strategies are currently being explored to improve cryogel injectability such as investigating various cross-linking mechanisms, but also optimizing cryogel porosity, pore orientation, toughness, and polymer wall flexibility. Looking into the future, engineering more compressible or self-healing cryogels for improved injectability, designing cryogels that can respond to a specific stimulus including pH, temperature, light, etc, and fabricating 3D printed multi-layered cryogels with more defined macrostructural features should further expand their biomedical applications.

Box 1: What are cryogels really?

Cryogels, a subclass of hydrogels, are biomaterials with unique physical properties [23]. They are characterized by a highly interconnected macroporous and elastic structure, resulting in a sponge-like morphology with high mechanical stability [60]. Cryogels (or freeze-thawed cryogels) are formed via cryogelation at subzero temperatures (typically between -5 to -20°C). Freeze-dried hydrogels, conventional hydrogels that undergo cryostructuration through several cycles of freeze-drying [61], are often inadvertently referred to as cryogels [17]. The process of freeze-drying leads to the formation of ice crystals within the gel matrix, creating pores in regions previously occupied by polymers. Although this technique enables the formation of an open macroporous structure [62], freeze-dried hydrogels tend to have a loss of structural integrity leading to weaker mechanical properties. As a result, freeze-dried cryogels are usually not syringe injectable [17]. In contrast, the physical or chemical cross-linking of cryogels takes place under freezing conditions, resulting in the formation of ice crystals that act as non-toxic porogens. Polymerization occurs in a liquid phase around ice crystals where the monomers/polymers and cross-linking systems are concentrated [18]. When the cryogelation is completed, a simple thawing at room temperature causes ice melting and unveils an interconnected macroporous network. The resulting cryogels are soft and elastic, can be compressed up to 90% of their initial volume without any permanent mechanical damage, and can withstand the compressive stress experienced during syringe injection [18]. Furthermore, cryogels are capable of rapid swelling and expansion, facilitating cellular infiltration, cell organization and proliferation [17], as well as promoting angiogenesis [63].

Additionally, cryogels are versatile biomaterials that that can be tailored selectively as per the intended application. They can be fabricated from synthetic and/or naturally-derived polymers, and other materials can be integrated within their polymer walls (nano- and macroparticles) to form composite or hybrid cryogels [17]. Cryogels could be ionic, non-ionic, amphoteric or zwitterionic [17, 64, 65], and can be engineered to be responsive to external stimuli such as electric [66], magnetic [49, 67], or thermic [68], as well as other signals such as pH variations [69].

Table 1. Biomedical applications of polymeric injectable cryogels.

Application	Polymer	Cross-linking Mechanism	Cryogel Dimension Needle Size	Description	Ref
In Vivo Applications					
Allogeneic Hematopoietic Stem Cell Transplantation	MA-alginate PEG	Covalent	• 8x8x1 mm • 16G	• Bone marrow mimicking gels stimulated T-cell generation from stem cells • Cryogels restored T-cell levels in immunodeficient mice after HSC therapy • Induced T-cells increased mice survival	[36]
Cancer Immunotherapy	MA-alginate	Covalent	• 4x4x1 mm • 16G	• Cryogel vaccines contain whole tumor cells, GM-CSF and CpG ODN • Gels induce recruitment, antigen uptake, activation and dispersion of DCs • Therapeutic vaccination increases mice survival in a melanoma model	[19]
Cancer Immunotherapy		Covalent and ionic	• Ø 5 mm h 1 mm • 18G	• Ionic and covalent crosslinking allowed cryogel injection through thinner needles • Cryogel vaccines integrate whole tumor cells, GM-CSF and CpG ODN • Prophylactic vaccination increased mice survival in a HER2/neu breast cancer model	[22]
Cell/Protein Delivery		Covalent	• 8x8x1 mm • 16G	• First study to demonstrate injectability of large-scale and preformed cryogels • Cryogels showed controlled release of proteins • Cryogels enhanced cell retention at the injection sites in mice	[18]
Cell/Protein Delivery	MA-gelatin	Covalent	• Ø 5 mm h 2 mm • 16G	• High DC numbers recruited in cryogels following GM-CSF release	[44]

				• Recruited MMP-producing immune cells accelerate cryogel degradation	
Tissue Engineering, Bone	Chitosan-gluconic	Physical	• Not reported • 23G	• Calcium phosphate-containing cryogels promoted attachment of pre-osteoblasts • Optimized calcium phosphate concentration enhanced cellular infiltration in rats	[40]
Tissue Engineering, Soft Tissue	MA-HA	Covalent	• Ø 5 mm h 2 mm • 16G	• Cryogels maintained skin firmness and preset geometry up to 30 days following injection in mice • Cryogels did not induce an inflammatory response in mice • Cryogels promoted neovascularization	[41]
Tissue Engineering, Vascularization	CMC	Covalent	• 30x40 mm • 0.8 mm catheter	• 3D cryoprinting can control cryogel porosity and cell density • Cryoprinted gels protected host cells during injection • Tissue infiltration is dependent on scaffold porosity • Larger pores enhanced vascularization in mice	[26]
Tissue Engineering, Vascularization	Gelatin, Heparin	Covalent	• Ø 5 mm h 3 mm • 17G	• Heparin allows sustain release of VEGF from cryogels • Low polymer content improved injectability and retention of fibroblasts at the injection site • Cell/VEGF delivery restored blood flow in a hindlimb ischemia model	[33]
Tissue Engineering, Wound Healing	MA-glycidyl, Quarternized Chitosan	Covalent	• Ø 5 mm h 20 mm • Ø ~1.5 mm	• Cryogels hybridized with carbon nanotubes increased electrical conductivity • Hybrid cryogels had a faster hemostatic time, less blood loss and higher	[37]

				vascularization than conventional gauze in various animal injury models	
In Vitro Studies					
Cell Delivery	PEG	Covalent	• Ø 2 mm h 2 mm • 21G	• Incorporating multi-arm PEGs fine-tuned cryogels' properties • Cell-adhesive cryogels increased fibroblast proliferation	[32]
Tissue Engineering	MA-alginate HAGM MA-gelatin	Covalent	• 4x4x1 mm • 16G	• Gelatin-based cryogels allowed strong fibroblast attachment and viability • HA-containing cryogels showed good physical properties and injectability • Composite HA-*co*-gelatin gels exhibited a combination of these favorable properties	[23]
Tissue Engineering, Adipose	MA-HA, MA-gelatin, PEG	Covalent	• Ø 6 mm h 0.8 mm • 16G	• Cryogels promoted adipose and endothelial cell proliferation and maturation • Cells were bioprinted onto cryogel scaffolds to create adipose tissue constructs	[35]
Tissue Engineering, Bone	MA-Alginate	Covalent	• Not reported • 16G	• Loading of regenerative factors in cryogels did not alter mechanical properties • Optimal regenerative factor and polymer concentrations were determined with respect to bone cell infiltration, proliferation and scaffold mineralization	[39]
Tissue Engineering, Neuronal	Alginate, CMC	Covalent	• Ø 6 mm h 3.5 mm • 16G	• Bioengineered cryogels maintained cell survival and protected neuronal network following syringe injection of mouse primary neurons • Experimental and theoretical properties of injectable cryogels were conducted	[15]

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Figure legends

Figure 1. Overview of the origin and applications of injectable cryogels. Hydrogels can be fabricated using synthetic or naturally-derived polymers. Within the injectable hydrogel repertoire, many *in situ* forming gels and small-scale gels have been developed. Cryogels are the only readily available large-scale preformed injectable hydrogels, making them suitable for applications in tissue engineering, cosmetics, cell/drug delivery and immunotherapy.

Figure 2. Fabrication process and injection of cryogels. (**a**) For the formation of cryogels, (1) a hydrogel precursor solution is frozen (T < 0°C). This process leads to a phase separation of the solvent (e.g., water) into a frozen phase (ice crystals) and a non-frozen phase around ice crystals where the gel precursors (monomers, polymer, cross-linker and initiator) are expelled. (2) Next, concentrated gel precursors are crosslinked around ice crystals (porogens). (3) Following cryogelation, thawed ice crystals give rise to a macroporous sponge-like hydrogel, known as cryogels. (**b**) Syringe injection of cryogels with shape-memory properties. Free water within the macropores of cryogel scaffolds (1) is released during injection and the polymer network is reversibly collapsed to a fraction of its original size, enabling the gel to be pushed and travel through the needle (2). Following injection, the initial shape and dimension of the gel are rapidly restored upon rehydration and spontaneous swelling (3). (**c**) SEM image of the macroporous architecture of cryogels and photos showing cryogel injection. Images were adapted with permission [18, 19].

Figure 3. Cryogels are suitable delivery systems and create a confined niche for several biomedical applications. The interconnected macropores are an ideal environment that allows cell attachment and protection during transplantation. Furthermore, the open macroporous structure facilitates recruitment and trafficking of cells. Finally, the inherent properties of cryogels promote neovascularization, stimulate native ECM formation, and facilitate tissue integration.

Outstanding Questions

- Can large-scale cryogels potentially be injected through small-gauge (>21G) needles?
- Can cryogels be fabricated with preset geometry and dimensions to match irregular defects? Would they fill in the space precisely?
- Can release mechanisms and kinetics of therapeutic agents from cryogels be shaped to recruit more effectively targeted cells? Should cryogels be hybridized with functional materials to further regulate the release of their cargo? Can one achieve high level of control with on-demand drug release?
- What are the optimal physical and biochemical properties of cryogels for tissue engineering? Can cryogels provide cell-instructive cues to enhance cell proliferation and differentiation in tissue regeneration?
- What strategies can make cryogels more susceptible to degradation? Can cryogels' properties be modulated to match tissue growth and new ECM formation?
- Can 3D printed cryogels emulate the architectural complexity of native tissues and organs? Could they retain such complex 3D structures post-injection?
- Can one controllably predict cryogel-host tissue interactions and prevent unwanted inflammation?
- Can bioengineered cryogels instruct the immune system to promote tissue regeneration?
- What is the required cryogel size to induce a protective immune response in humans? Can cryogels be scaled up to expand immunization coverage for cancer treatment?

Highlights

- Injectable cryogels were disclosed in 2012 as the first preformed large-scale hydrogels to be injected through a conventional hypodermic syringe, obviating the need for invasive surgical implantations
- Injectable cryogels have shape-memory properties and are reversibly compactible
- When syringe injected, shear-collapsed cryogels flow through conventional needles. Once released, cryogels instantly pop back to their initial shape and size
- Cryogels with their inherent interconnected macroporous network can host and/or deliver mammalian cells for tissue regeneration, cell transplantation, and *in situ* cell manipulation/reprogramming
- Biomolecules can be efficiently entrapped within the dense polymer walls of cryogels. Fine-tuning cryogel properties can control the spatiotemporal release of their payloads
- Enhancing cryogels' properties will expand their potential in the biomedical field

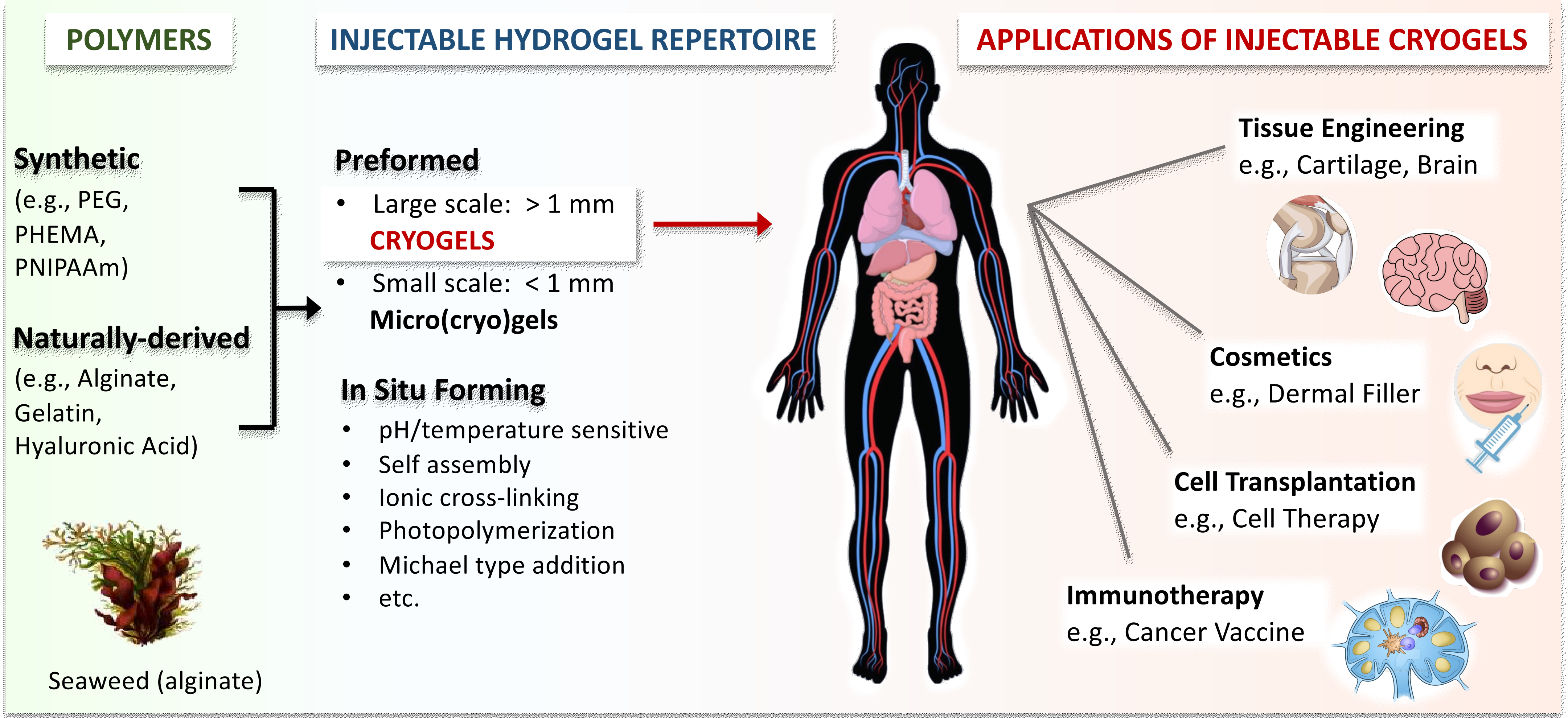
POLYMERS
INJECTABLE HYDROGEL REPERTOIRE
APPLICATIONS OF INJECTABLE CRYOGELS
Synthetic
(e.g., PEG,
PHEMA,
PNIPAAm)
Naturally-derived
(e.g., Alginate,
Gelatin,
Hyaluronic Acid)
Seaweed (alginate)
Preformed
• Large scale: > 1 mm
CRYOGELS
• Small scale: < 1 mm
Micro(cryo)gels
In Situ Forming
• pH/temperature sensitive
• Self assembly
• Ionic cross-linking
• Photopolymerization
• Michael type addition
• etc.
Tissue Engineering
e.g., Cartilage, Brain
Cosmetics
e.g., Dermal Filler
Cell Transplantation
e.g., Cell Therapy
Immunotherapy
e.g., Cancer Vaccine

Figure 2

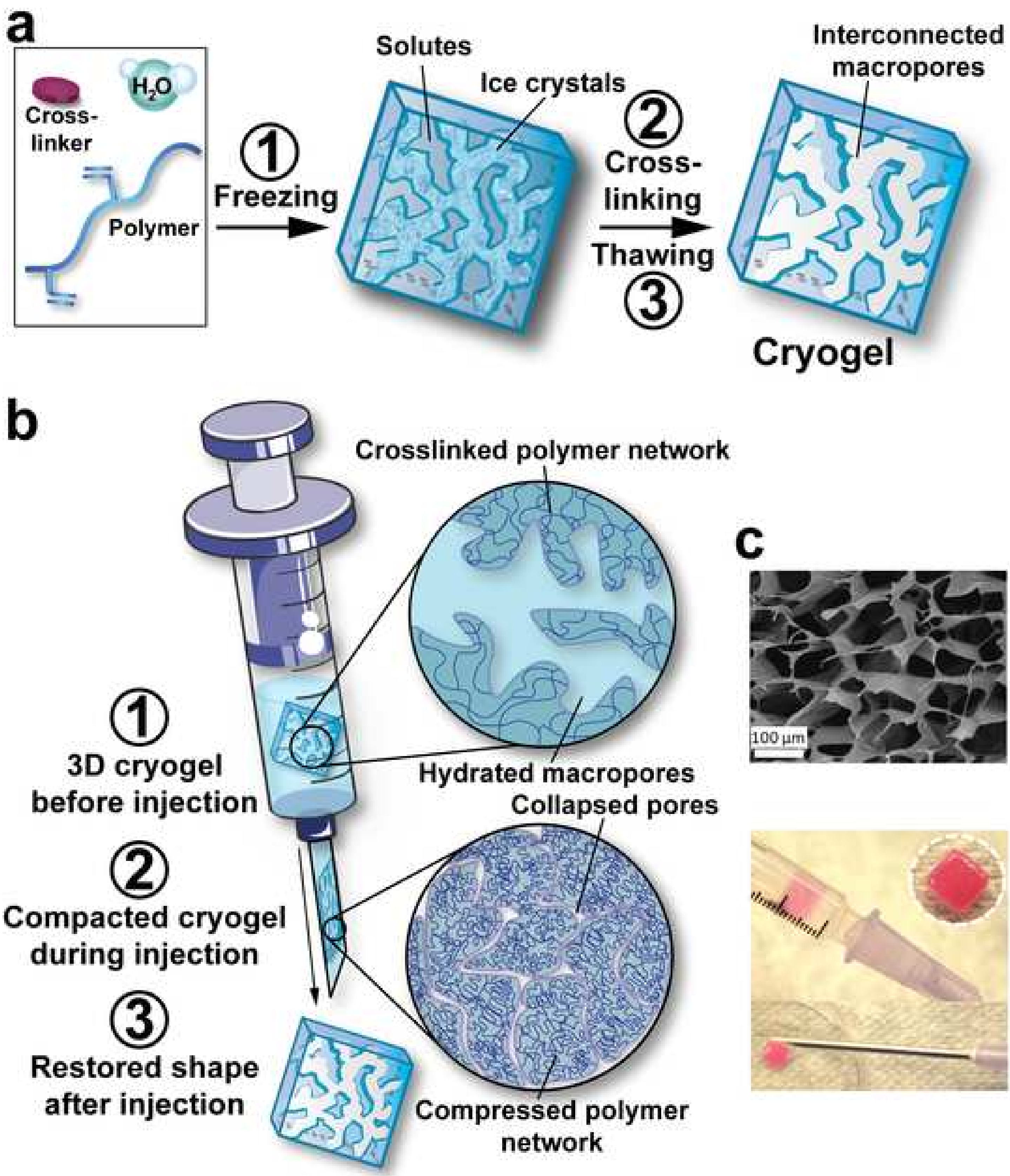
a
Cross-
linker
H_2O
Polymer
1
Freezing
Solutes
Ice crystals
2
Cross-
linking
Thawing
3
Interconnected
macropores
Cryogel
b
Crosslinked polymer network
1
3D cryogel
before injection
Hydrated macropores
Collapsed pores
2
Compacted cryogel
during injection
3
Restored shape
after injection
Compressed polymer
network
c
100 µm

Figure 3

Click here to access/download;Figure;Figure 3_Final.tiff