Review

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Review

Recent Trends in Cryogelation Phenomenon & Factors Affecting Cryotropic Gelation

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Abstract

Polymeric gels are a vital class of functional biomaterials for biomedical applications. Cryogels, formed through cryogelation of polymeric or monomeric precursors in aqueous solvents at sub-zero temperatures, provide a cost-effective and straightforward solution for incorporating porosity, mechanical strength, and chemical cues into gels. This review highlights recent advancements in cryogelation and factors influencing the synthesis of cryogels. The three key stages—freezing, incubation/polymerization, and thawing—are explored in depth, alongside crosslinking mechanisms (covalent, physical, or ionic). Critical parameters affecting cryogelation, such as ice nucleation, temperature, solutes, solvents, and precursor composition, are discussed comprehensively. By offering insights into the mechanisms and factors governing cryogelation, this review aims to inform the development of advanced cryogels tailored for biomedical applications. These findings are crucial for developing optimized materials to improve polymeric gel fabrication, enabling advances in regenerative medicine, drug delivery, and tissue engineering.

Keywords: cryogels; ice-templating; tissue engineering; polymeric scaffolds; porous materials

1. Introduction

Porous interconnected networks build some very intricate structures serving important structural and biological functions and are abundantly found in nature [1]. Several examples of porous structures exist in nature exemplifying the important role of such porous networks in every aspect of tissue properties and biochemical exchange. Porous structures are found in the most complex mammals to simplest microorganisms like diatoms surrounded by a protective cell wall composed of porous silica-containing shell [2,3]. They can also be observed in the form of aligned pores in plants which are responsible for water supply and in more complex structures like human spongy bone wherein pores provide flexibility to otherwise high-strength yet brittle structures [1].

Generating porous scaffolds with appropriate mechanical strength is a much sought-after goal, especially for biomedical applications [1,4]. Three-dimensional macroporous materials are increasingly becoming important for in vitro culture of cells or encapsulation of cells for cell delivery and tissue engineering [5–8]. These scaffolds provide the basic matrix which helps in constructing native tissue-like environments for the cells, thus being an important step for achieving the goal of tissue regeneration and repair. The pore morphology, surface properties, and pore structure besides polymer composition, all seem to enhance and modulate cell phenotype and physiology [5,9,10]. Recently they have also been applied for vaccine and drug delivery [11,12].

Macroporous polymeric scaffolds may be synthesized or molded by various fabrication techniques. Some of the commonly used methods are thermally induced phase separation, compression molding, particulate leaching, solvent casting, solid freeform fabrication and gas

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foaming, [5,13,14]. Almost all of these techniques use a method to generate pores by using two immiscible phases [6,13]. Thermally-induced phase separation relies on phase separation induced by the insolubility of one liquid in the other upon increase in temperature resulting in pore formation. In the case of particulate leaching or salt leaching method, particles of salt are used as porogens to create pores of a specific size corresponding to the size of the salt particles in the otherwise continuous polymer walls. The salt is ultimately washed off to create a three-dimensional (3D) network of pores [5,6,13,15].

These methods share several common drawbacks, including the addition of organic solvents during synthesis that can influence biocompatibility, incomplete removal of salt particles serving as porogen, resulting in non-optimal scaffold structures, formation of pores with limited interconnectivity, lengthy processing, and washing steps, and the creation of matrices with non-uniform material properties. Additionally, while solid freeform fabrication shows promise for generating scaffolds tailored to specific sizes and patients, it comes with its own challenges such as high cost, complexity, and the need for specialized equipment [5,6,13,16].

In the past few years, ice templating of porous materials has received significant attention. Freeze-casting or ice-templating is used not only for soft polymers but also for hard materials like ceramics and metals [17]. The method usually involves suspending the precursors in water,and freezing the solution starting at room temperature to obtain a solid ice block. Subsequent sublimation of ice gives rise to a macroporous structure. The method relies heavily upon ice nucleation and crystal growth which dictate the properties especially homogeneity and pore distribution of the resulting macroporous scaffold. In all the above-mentioned cases, the presence of a pore size gradient is observed in proximity to the cooling surface. The process is highly versatile, environmentally friendly, and biocompatible as it uses water converted to ice as the second immiscible phase or as a porogen. The pore structure of the network can be modified by tweaking the process that controls the nucleation conditions or additives that affect the morphology of growing ice crystals [17–20].

Closely related to ice templating as a method to develop macroporous scaffolds is 'cryotropic' gelation [21]. The super macroporous polymeric matrices synthesized through cryotropic gelation are termed "cryogels". Cryogels can be described as ice-templated macroporous polymeric networks where a negative imprint of the ice crystals formed during the gelation is obtained upon thawing [22,23]. The process of generating cryogels is inspired by the natural phenomenon of permafrost formation in frozen grounds, frost heave, or the formation of ice cream [24]. Cryotropic gelation involves the elimination of solute particles from increasing ice crystals in an aqueous solution that is under a semi-frozen state [22,25]. The process of cryotropic gelation in cryogels differs from the formation of macroporous materials made via freeze-drying for two main reasons: i) cryotropic gelation in cryogels typically involves crosslinking of the precursors while in a semi-frozen state, leading to the development of thick polymeric walls that builds edges of the pores; ii) Unlike freeze-dried macroporous substances, which tend to lose their macroporous structure upon thawing, cryogels obtained through cryogelation are stable and maintain their crosslinked macroporous structure even after thawing at room temperature [25,26]. This makes cryogelation to be a single-step process for obtaining the macroporous structure where no specialized equipment is necessarily required to ensure complete drying of the solvent as is the case for freeze drying [27,28]. Consequently, cryogels produced through cryotropic gelation offer an appealing and straightforward approach to fabricate macroporous scaffolds. Due to their simplicity of synthesis and utilization of eco-friendly chemistry, these cryogels have been increasingly embraced for diverse biomedical purposes. Therefore, this review aims to delve into the details of the method involved in the synthesis of macroporous cryogels, and various factors affecting their synthesis.

2. Phenomenon of Cryogel Synthesis

Cryotropic gelation (*cryogelation/cryostructuration*) (Greek *word* κριοσ [kryos] meaning ice) occurs as a consequence of cryogenic treatment of monomeric or polymeric precursors which can form crosslinked polymeric networks resulting in gel formation. [25,29] Cryotropic gelation involves

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crystallization of the solvent at sub-zero temperatures, which is in contrast to cooling-induced gelation, where the gel is formed due to a decrease in temperature and does not involve any phase transition [25].

The process of cryogel closely resembles the natural phenomena of ice templating or synthesis under sub-zero temperatures, a natural phenomenon that may have played a significant role in the origin of life. Life's building blocks, nucleotides and biomacromolecules, were likely concentrated by ice crystals in a saline environment. This process also applied to salt and microorganisms, trapped in high-salinity brine channels. Freezing-induced gelation or ice crystal growth can concentrate dilute solutions, enabling polymerization. Solute rejection by ice crystals can increase biomacromolecule concentration up to 200-fold in on frozen spacesn, accelerating polymerization without enzymes or catalysts. [30,31]

The phenomenon of cryogels synthesis can be divided into three specific phases: (Figure 1 A& B) [22]. The three phases are primarily, 1) a homogenous solution of monomeric/polymeric precursors, 2) a heterogenous frozen state consisting of "non-frozen liquid microphase" along with frozen ice crystals, and, 3) final stage with crosslinked cryogel walls forming a porous network wherein the pores are left behind by the thawed ice crystals. The phases are the same for figure 1 A and B, however, for figure A, the three stages are distinctly represented by the three diagrammatic representation images of the cryogel. Whereas, in figure B, the phases are represented by the micro-CT images wherein the first stage is represented by 0 minutes; second stage is represented as a transitional phase demonstrated by time points 12 mins, 36 mins, 1.8 hours, and 5 hours; and, third stage is represented by 12 hours' time point. [22] Each step of cryogel synthesis is adaptable, providing opportunities to modify the synthesis procedure and properties of the resulting cryogel. These three steps are further discussed in detail.

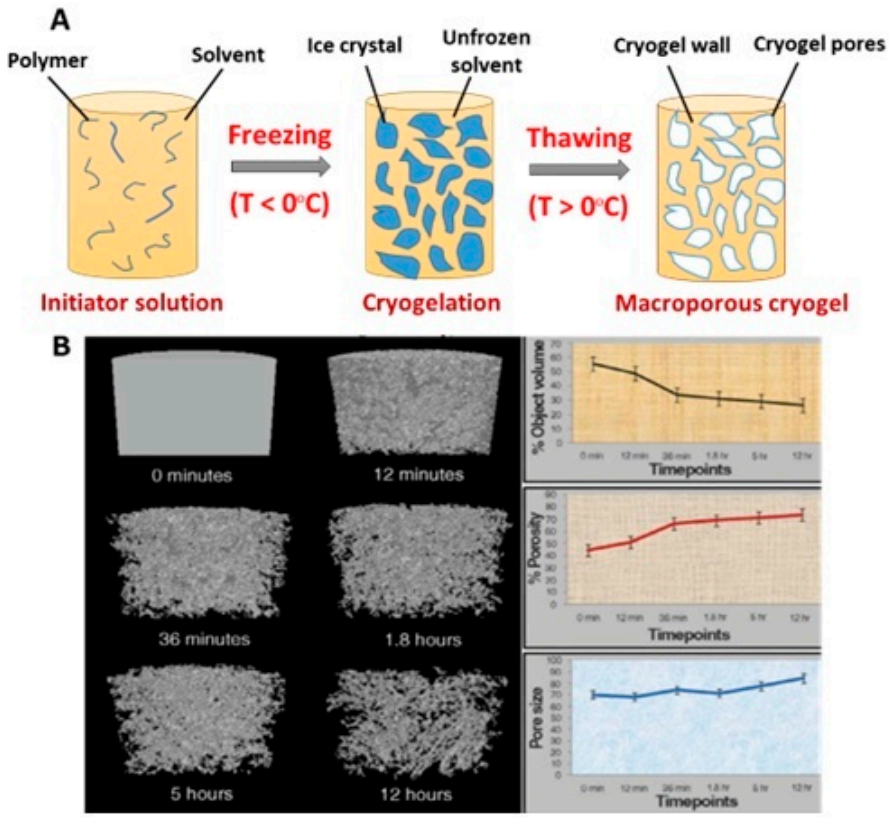

Figure 1. The process of cryogel formation. A) Schematic of the three phases of cryogel formation. **B)** Measurement of the three phases of cryogel formation using micro-CT. The process of cryogel formation is shown in the form of a change in three-dimensional structure (transverse cryogel sections), object volume, porosity, and average pore size. Reproduced with permission copyrights [2010/Elsevier].[22].

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2.1. Freezing of Precursor Solutions and Phase Separation with Ice Formation

Low or high molecular-weight precursors/solute particles are dissolved in a suitable solvent and frozen below the solvent crystallization point. It is essential for the formation of cryogels that the significant bulk of the solvent freezes to form ice crystals. Apparently, most of the liquid phase converts into solid crystals beyond the freezing point, though a small amount of solvent containing the dissolved solutes remains as "non-frozen liquid microphases" (NFLMP). Consequently, a heterogeneous two-phase system is formed that consists of NFLMP and frozen solvent crystals. The formation of NFLMP is an essential and characteristic feature of the first step in cryogel formation [32].

NFLMP formation starts when an aqueous solution with dissolved salts or precursor macromolecules freezes, leading to the growth of ice crystals. These crystals expel solute particles into a smaller non-frozen liquid volume, creating NFLMP. The phenomena of reduction of aqueous volume and concentration of solute particles into much smaller volume is called cryo-concentration [33,34]. Pioneering studies by Butler and Bruice [35] comparing the kinetics of chemical reactions in water and ice have confirmed the presence of such NFLMP in moderately frozen solutions.

Usually, the NFLMP is a few degrees colder than the freezing point of the solvent. As the name suggests "cryo-concentration" the solutes are present in a very high concentration due to a reduction in the volume of solvent available to keep the solutes dissolved. The volume of the NFLMP varies from 0.1 to 10% of the total sample volume [36,37]. The volume of the NFLMP is determined by a number of factors such as the cryogenic temperature regime, precursor concentration, properties of the solvent, and presence of other solute particles. Modulation of NFLMP volume not only guides the reaction efficiency but also the ultimate properties of the cryogel so formed [38].

2.2. Incubation Under Frozen Condition, Cryo-Concentration and Polymerization

Cryo-concentration of the precursor molecules in the NFLMP [39] leads to precipitation and re-dissolution of solutes as they are continuously consumed in a chemical reaction [37]. Several other studies investigating the detailed kinetics of the reactions in the cryo-concentrated NFLMP region confirm their existence and occurrence of further chemical reactions in the otherwise ice block using in situ Nuclear Magnetic Resonance Spectroscopy (NMR) [33,40]. Despite the low temperatures, owing to cryo-concentration and surge in the dielectric constant of the NFLMP upon cooling, chemical reactions are accelerated in a specific limit of subzero temperatures in relation to the used solvent. The reaction proceeds faster with a greater yield fraction of gel than in a liquid medium with the same initial concentration and at temperature above freezing point which is typical of cryogel synthesis at sub-zero temperatures [32,34,35].

Due to cryo-concentration, a higher concentration of the precursors leads to the formation of denser polymer walls around the ice crystals. The length of the reaction, as with any other reaction, is important in determining the total gel fraction yield and ultimately the physical strength of the polymeric network. It has been found that almost 80% of the polymerization occurs in the first 2 h of incubation while an additional 6 to 10 h of incubation is required for the gel yield fraction to reach 90% although the exact values can vary depending upon the type of polymeric network [38].

a. 2.3 Thawing and formation of interconnected pore network

b. During the freezing period, the polymerization continues in the NFLMP, while the solvent crystals keep growing from the vessel circumference towards the center, around the non-continuous NFLMP, until they join the sides of the other crystals leading to the formation of a continuous network of solvent crystals along the sides of the polymeric walls. The solvent crystals act as porogen that melts away upon thawing and leaves behind interconnected pores or cavities that were initially occupied by frozen solvent. Furthermore, due to the high concentration of the solutes in NFLMP (cryoconcentration), the cryogel has a dense polymer phase resulting in a heteroporous and heterophase system. The proportions and morphology of the pores are determined by a number of factors of which precursor concentration and temperature regime are the most important. The decrease in temperature decreases the entropy

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due to a lower Gibbs free energy leading to increased surface tension at the solid (ice)-liquid (polymer walls) interface. Thus, to overcome the increased surface tension the initially bent/sharp pores acquire a round and smooth shape after thawing [37]. The thawing rate is an important factor in the formation of certain types of cryogels and determines their ultimate properties [41].

3. Crosslinking Mechanisms for Cryogels

On the basis of the intermolecular bonding in the cross-linked polymers, the cryogels can be classified into three categories [29]; covalently cross-linked cryogels either chemical [42–51], Schiff base [46] or irradiation-induced [50,52–54], physically cross-linked cryogels with hydrogen or hydrophobic bonds linking the polymer chain [41,55–62] and lastly ionotropic cryogels whereby ionic crosslinking between polyelectrolytes [63–66], or crosslinking of polymer chains via ionic interaction with metal ion form the crosslinked network [65]. A summary of these different types of cross-linking, associated polymerization method, and, the types of polymers are mentioned in Table 1. However, each category is further discussed in the following sections:

Table 1. Summary of the different types of crosslinking methods for cryogel synthesis.

Type of Crosslinking	Polymerization Mechanism	Chemical Reaction or Initiating Agent	Advantages	Disadvantages
Chemical cross-linking	Free radical polymerization	Chemical initiators using redox pair	Highly efficient reaction yield in frozen conditions leading to high mechanical strength and better control over the polymerization reaction.	Residual initiators may cause toxicity. Uncontrolled reaction with side products
	Free radical polymerization	Gamma irradiation using low dose or high energy electron beam	Additional chemical initiators or crosslinkers traces of which may sometimes be toxic.	Limited penetration into the sample leading gradient of radiation dose and inhomogeneous reaction conditions.
	Free radical polymerization	U.V. cross-linking using photo initiators like hydrogen peroxide (H_2O_2), AIBN or Irgacure	Cryogels can be templated using both porogen that is ice and light to create complex architecture	Limited penetration into the sample leading gradient of radiation dose and inhomogeneous reaction conditions.
	Addition reaction	Click reactions such as Michael addition reaction.	Highly efficient reaction, biocompatible reaction conditions, no side products	Low reaction yield under frozen conditions.
Physical cross-linking	Physical interactions	Hydrogen bonding or hydrophobic interactions. Polymers rich in	High biocompatibility & biodegradability. Can be used as stimuli-response cryogels.	Cryogels usually have poor mechanical properties. Usually

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		hydroxyl, carboxyl, or amine groups are usually amenable to such reactions.		applicable to natural polymers.
Ionic cross-linking	Ionic interactions	Few examples such as alginate, chitosan, poly(ethylene imine)	High biocompatibility, biodegradability & mechanical strength.	Cryogels usually have poor mechanical properties. Usually applicable to natural polymers

3.1. Covalently Cross-Linked Cryogels:

Covalently cross-linked cryogels may further be subdivided as polymerizing systems, where the synthesis is done using low molecular weight monomeric precursors (e.g. acrylamide (AAm), N, N-diethylacrylamide) [37,67–74]. The other class comprises, cross-linking of macromolecules like proteins (gelatin cryogel) [43,75], polysaccharides (chitosan, agarose, or alginate cryogels) [12,42,64–66], or end-functionalized polymers like polyethylene glycol (PEG) under frozen conditions [76]. The most common reaction mechanism used for the formation of covalently crosslinked cryogels is free radical polymerization. Chemical initiators mostly ammonium persulphate and *N,N,N′,N′*-tetramethylethane-1,2-diamine (APS/TEMED) redox pair have been used very commonly to initiate free radical polymerization in cryogel systems. Polyacrylamide (PAAm) cryogels are a widely studied cryogel formed by free radical polymerization of the respective monomers and have been used for a multitude of applications [37,72,77,78]. Other examples include cryogels of poly-dimethylaminoethyl-methacrylate (pDMAEMA) [73,79,80], poly-ethyleneglycol (PEG) [81–83], poly-hydroxyethyl-methacrylate (pHEMA) [84,85], poly-*N*-isopropylacrylamide (pNIPAAm) [86,87] , poly-dimethylacrylamide (pDMAAm) [88] Other common methods that have been used more recently to induce free radical polymerization in cryogel systems include ultraviolet (UV) radiation [89], photoinitiators like hydrogen peroxide (H_2O_2) [90], 2,2′-azo-bis-isobutyronitrile (AIBN) or Irgacure [91], γ irradiation, and high energy electron beam [92].

Several examples exist where UV-initiated polymerization has been used to induce polymerization in semi-frozen systems to form crosslinked networks [93–95]. UV-crosslinking is a versatile technique applicable at various stages of polymer processing, contingent upon the specific polymer system and the required properties.[52] The literature identifies multiple approaches to UV-crosslinking, including those conducted under frozen, and partially frozen conditions. This approach has proven effective in methacrylamide-modified gelatin scaffolds, where high-energy irradiation at -5°C facilitates sufficient molecular mobility for chemical reaction to occur following cryogenic treatment [96]. Similarly, cryogelation reactions have been successfully executed at temperatures as low as -20°C, with the reaction system precooled before UV-initiated polymerization [52]. An innovative technique integrates directional freezing with frozen UV-induced crosslinking. Aqueous mixtures are directionally frozen in liquid nitrogen, then exposed to UV irradiation in dry ice, creating aligned porous stimuli-responsive hydrogels. [95]. An advantage of using UV-initiated polymerization or irradiation method is that the cryogels can be templated using both porogen that is ice and light to create complex architecture [97]. Moreover, photoinitiated polymerization cryogelations can be easily combined with 3D printing inks to generate cryogels of complex architectures and unique properties.[49,98,99]

To extend the use of free radical-initiated crosslinking, acrylated or methacrylated derivatives of polymers, particularly natural polymers have been synthesized. Acrylation of natural polymers like gelatin, alginate, *etc.* allows the formation of covalently crosslinked cryogels of polymers which otherwise cannot take part in free radical-mediated crosslinking. The use of free radical polymerization in such polymers allows for greater flexibility in the modulation of cryogel properties

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by tweaking the reaction parameters such as concentrations of polymer and crosslinker degree of acrylation which controls the degree of crosslinking [94,100–102]. This is particularly useful for natural polymers whereby the mechanical stability of the gels is increased by covalent crosslinking rather than physical crosslinking of the natural polymers [103,104]. Some of the examples where this approach is utilized include natural polysaccharides such as agarose[105] dextran[106], hyaluronic acid [103,107], alginate, and gelatin [104]. Moreover, due to the high molecular weight of the precursor polymeric chains free radical polymerization in such cases can also be initiated using irradiation by using either gamma irradiation [108] of low dose or high energy electron beam [107]. An advantage of using such irradiation for free radical initiation is that it does not require additional chemical initiators or crosslinkers traces which may sometimes be toxic in case the intended cryogel which is to be used for biomedical applications. Some additional advantages of using a high electron beam over gamma irradiation for initiating gelation of cryogels include a small reaction time of up to 10 min, inexpensive, and environmentally friendly because continuous radiation emission from the instrument is not required [53,109]. The technique has been used to produce cryogel of polymethacrylate [107,109], dextran and hyaluronic acid. The properties of the cryogel so formed are found to be dependent upon the irradiation dose and monomer concentration [53,107,109].

Recently, cryogels with covalent bonds have been developed using a multitude of click reactions to crosslink macromolecules like peptides, polysaccharides, or end-functionalized PEG polymers [110]. "Click"-reaction-based techniques for cross-linking polymers to create networks have gained popularity since the discovery of "click" chemistry, a class of chemical reactions that proceed with high yield in biocompatible conditions without the formation of toxic byproducts [111]. A commonly used click reaction is Michael-type conjugate addition which has been explored to make cryogels of end-functionalized polyethylene glycol (PEG) macromers [112]. The reaction is known to proceed under mild and non-toxic conditions. Different end-functionalized PEG macromers and their combination can be used including maleimide-amine, and acrylate-thiol. Such reaction chemistry has been used to generate redox-responsive PEG cryogels made by the reaction between PEG triamine and dithiobis(maleimido) ethane [113]. The cryogels so formed are responsive to the excess concentration of reducing agents like glutathione which reduces the disulfide bond in the maleimide-containing crosslinker leading to stimuli-responsive degradation [76]. Some other covalent reactions that have been successfully used to induce cryogelation include the Schiff base reaction [114], aldol condensation [115], carbodiimide crosslinking [116], and peptide ligation [117].

3.2. Physically Cross-Linked Cryogels:

Physically cross-linked cryogels have been extensively studied and applied in various areas [118]. The cryogel formation in these gels usually involves heating the polymeric solution, freezing the solution before the gel point of the polymer, and then thawing at a controlled rate. Once the gel is formed it is subjected to a repeated freeze-thaw cycle. Contrary to covalently crosslinked cryogels physically linked cryogels are reversible in nature as crosslinking between polymer chains is due to physical interaction rather than covalent. Repeated freeze-thawing and controlled rate of thawing of the system are generally seen to increase the mechanical strength [119]. The commonly studied cryogel formed by this method is polyvinyl alcohol (PVA) cryogels. As with any cryogel system freezing temperature is a critical factor in deciding the final properties of the cryogel. If the solution is frozen beyond the glass transition temperature (T_g) of the system, the gel stability decreases which can be attributed to decreased mobility of the polymeric chains at temperatures below (T_g) [120]. Other classes of polymer which have been made into cryogel by inducing physical gelation under freezing conditions include polysaccharides such as agarose[121], gellan[122], carrageenan[123], and locust bean gum [124]. Physically cross-linked cryogels are thermally reversible and dissolve at higher temperatures, repeated freezing/thawing of the solution further generates a cryogel. A common problem in the method is that it requires heating of the polymeric solution and then freezes quickly before the solution cools down to form a gel [61]. This generates heterogeneous cryogels if the gel point of the polymer is above room temperature for example in agarose cryogels [125].

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Chaotropic substances (urea or lithium chloride) which can break hydrogen bonds, weaken cryogel, or lessen its mechanical strength. Conversely, kosmotropic substances (trehalose, sodium fluoride, or amino acids) can strengthen mechanical strength and thermal tolerance. In similar lines, using amino acids of increasing hydrophobicity facilitates enhancing rigidity and heat endurance of resultant cryogels [126]. However, most of these studies have been done using PVA cryogels as model systems. It will be exciting to see if these results can also be applied to other physically crosslinked cryogels systems.

Similar mechanisms of physical gelation have been observed in hyaluronic acid (HA) cryogels. Cryogels of HA formed by physical crosslinking of HA in moderately frozen solutions lead to the formation of a relatively stable system in spite of the known non-gelling nature of the HA polysaccharide. The physical interactions between hydroxyl, carboxyl, and amine groups with possible hydrophobic regions can lead to the stabilization of crosslinks between the HA chains under frozen conditions [127,128]. The example demonstrates that crosslinking under moderately frozen conditions may facilitate the formation of rather stable systems that are otherwise difficult to obtain at room temperature. Alternatively, physical gelation in certain polymers can also be induced due to the re-arrangement of polymer chains, such as in polypeptides like silk-based cryogels [129]. Formation of silk cryogel usually involves the addition of an initiating agent that can induce a transition from α helices to β-chains which stabilizes the silk structure and causes gelation to occur [130]. A number of stimuli can be used in such cases such as the use of chemical initiators like ethylene glycol diglycidyl ether (EGDE), organic solvents like alcohol or dimethyl sulfoxide (DMSO), or physical initiators like sonication or vortexing [130]. Like other physically crosslinked systems, crosslinking interactions between chains arise from a combination of hydrogen bonding and hydrophobic interactions.

Physical gelation of cryogel usually is a highly biocompatible process as it does not involve the use of any chemical crosslinkers or initiators or the use of irradiation sources. However, one of the disadvantages is long processing times are required and repeated freeze-thawing at controlled rates which extends the length of the process. Moreover, it can only be used in polymers that render themselves to hydrogen bonding or hydrophobic bonding [131,132].

3.3. Ionically Crosslinked-Cryogels

Ionically crosslinked cryogels are difficult to sysnthesize as ionic reactions are very rapid and result in instantaneous gelation. Thus, prohibiting freezing of the precursor solution before the completion of the gelation [32,36]. A few common examples of cryogels formed by ionic gelation include chitosan cryogels/hydrogel gelled by the inter- and intramolecular hydrogen bonds [133]. One such example was presented by Krisebom and colleagues [65]. The group has made ionically crosslinked chitosan cryogels using the ionic interaction between chitosan, acetic acid, and gold (in the form of cholorauric acid). The cryogels were stable in high molar salt solution (0.2-0.8 M) and 4 and 5.5 pH. Increasing the ionic concentration of gold and increasing the incubation time from 18h to 48 h increased 3-fold the elastic modulus of the cryogels, indicating increased stability. Furthermore, chemical crosslinking using glutaraldehyde of the ionically crosslinked chitosan cryogel resulted in the formation of Au nanoparticles distributed with the pore walls of the cryogel which might hold potential for the cryogel to be used as a catalytic flow-through reactor. The authors also demonstrated the formation of chitosan cryogel with other noble metal ions like platinum and palladium [65].

3.4. Mechanical Stability of Physical and Covalently Crosslinked Cryogels

Inspite of several benefits of cryogels formed by physical or ionic crosslinking, an outstanding challenge is the mechanical fragility of physically crosslinked cryogels, which are formed through non-covalent interactions (Table 2). In contrast, covalently crosslinked cryogels exhibit enhanced stability and mechanical properties.[134–138] To address the mechanical limitations of physically crosslinked cryogels while maintaining biocompatibility, researchers have investigated various

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innovative strategies. One such approach involves the fabrication of double-continuous macroporous cryogels through sequential freeze-thaw cycles, resulting in enhanced mechanical strength and increased functional group content .[139] Another strategy employs a physico-chemical hybrid-crosslinking technique, as exemplified by chitosan/silk cryogels with silver and strontium co-doped hydroxyapatite, which exhibit remarkable resilience and flexibility.[140] Another example of the hybrid crosslinking technique includes a hybrid double network cryogels made with ionically crosslinked network of alginate and click crosslinked PEG acrylate network. The hybrid scheme enhanced the stability and mechanical strength of both networks, while using highly biocompatible chemical schemes for the cryogel synthesis.[141] The selection of an appropriate method for enhancing cryogel stability depends on the particular application and required properties. Each approach offers distinct advantages in terms of mechanical performance and biological compatibility, enabling researchers to tailor cryogels to meet the requirements of various biomedical applications.

Table 2. Comparative analysis of physically and covalently crosslinked cryogels.

Characteristic	Physically Crosslinked Cryogels	Covalently Crosslinked Cryogels
Crosslinking Mechanism	Non-covalent interactions (e.g., hydrogen bonding, ionic interactions)	Chemical bonds between polymer chains
Preparation Methods	Freeze-thawing cycles, ion-mediated gelation	Chemical crosslinking agents, photopolymerization
Stability	Generally less stable; more susceptible to dissolution or degradation	Enhanced stability; resistant to dissolution
Mechanical Strength	Typically, lower	Generally higher
Reversibility	Often reversible (can be melted and reformed)	Typically irreversible
Stimuli-Responsiveness	More likely to respond to environmental changes (e.g., pH, temperature)	Less responsive to environmental stimuli
Biodegradability	Often more biodegradable	Potentially less biodegradable, depending on crosslinking agents
Porosity	Can exhibit high porosity, but may be prone to structural collapse	High porosity with improved structural integrity
Biocompatibility	Generally good, due to absence of potentially toxic crosslinking agents	May be affected by residual crosslinking agents; requires thorough purification
Efficiency of Preparation	Often simpler and faster but less reproducible.	May require longer preparation times and additional purification steps, but may have more reproducible results.
Examples	Poly(vinyl alcohol) cryogels, alginate cryogels	Poly(acrylamide) cryogels, Poly(2-hydroxyethyl methacrylate) cryogels
Applications	Soft tissue engineering, drug delivery, wound dressing	Tissue engineering scaffolds, bioseparation, water treatment, cell culture substrates

4. Factors Affecting Cryotropic Gel Formation

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Several factors affect cryotropic gelation at different stages of cryogelation and thus guide the ultimate process of cryogel formation. Cryogels of significantly different physical properties pore morphology, total pore volume and mechanical strength can be obtained by interplay of the parameters affecting cryogelation. Some of these parameters include ice nucleation and crystal growth, type of solvent, ratio of aqueous to organic solvent, temperature gradient and direction of cooling, thawing rate, type and concentration of the solute, ionic strength of the solvent, pH of the reaction and precursor composition [36,86,135,142]. These factors are further discussed in detail

4.1. Ice Nucleation and Crystal Growth

Various factors influencing the properties or synthesis of cryogel primarily revolve around the regulating ice nucleation and crystal growth. Thus, understanding the process of nucleation or crystal growth is critical to successfully obtaining the cryogels with customized properties. X-ray radiographic and tomographic experiments [143] have shown that upon freezing an aqueous suspension starting at room temperature the suspension enters a super-cooled state before the beginning of ice nucleation and crystal growth, thus, taking the system away from equilibrium. Numerous investigations in the field of cryopreservation and ice-templating technology have explored the elements that influence ice nucleation. It is established that a major factor that affects ice nucleation is solute particle size and surface area, both of which serve as nucleation sites [38]. Smaller particle sizes and larger surface areas generate more nucleation sites. Another factor that influences ice nucleation is temperature. The larger the difference between the initial and final temperatures (lower temperatures), the more significant the degree of supercooling, leading to the creation of a greater number of smaller ice nuclei. Conversely, a slower cooling process results in fewer but larger nucleation sites (Figure 2A) [18]. The extent of supercooling is also associated with the rate of frozen phase formation, with a higher degree of supercooling leading to faster propagation during the initial stages of freezing. In the case of cryogel crosslinked by covalent bonding the degree of supercooling and rate of cooling affects the final microstructure of the resulting cryogel. Hwang et al. demonstrated that PEG cryogels formed at colder temperatures using a rapid cooling rate, and accelerated rate of ice nucleation, resulting in the quicker initiation of ice crystals following supercooling and creation of homogenous macroporous structure [144]. If the precursor solutions remain in a supercooled nonfrozen state for too long the resultant cryogels are heterogenous and non-porous in structure. Krisebom et al 2009 [33] compared the cryogelation of dimethylacrylamide (DMAAm) cross-linked with PEG diacrylate in a semi-frozen and a supercooled system using proton nuclear magnetic resonance spectroscopy (^{1}H NMR). The two systems exhibited significantly different concentrations of the monomer in the liquid phase when synthesized using the same initial monomer concentration. Specifically, the supercooled system had a 6% w/v monomer concentration, whereas the semi-frozen system due to the cryoconcentration effect had a concentration of 33% w/v monomer in NFLMPs. As expected, under supercooling conditions the cryogels had non-homogenous, non-porous structures and high structural gradients in the resulting material (Figure 2A), while a spongy, macroporous, elastic and opaque cryogel was formed under semi-frozen state (Figure 2B).

Similar observations about the impact of modulating ice nucleation and the degree of supercooling have also been made in physically crosslinked cryogels, especially PVA cryogels. Zhang et al generated ultrasoft PVA cryogels by modulating the ice crystal size using calcium chloride as the antifreeze agent. The addition of calcium chloride to the PVA solution suppressed ice growth and led to a depression in freezing point upon (0 to -58 °C) freezing in a concentration-dependent manner, leading the solution to be supercooled liquid phase for a longer time [145]. The dynamics and balance of free and hydrogen-bonded -OH groups in the PVA polymer allowed assembly of soft, uniform and tunable cross-linked network. Compared to traditional PVA cryogels, these exhibited tissue-like properties such as a Young's modulus of 4–10 kPa, high stretchability (~600%), transparency, and self-healing. Consequently, researchers identified potential applications in pressure sensors and artificial nerve fibers. [145]

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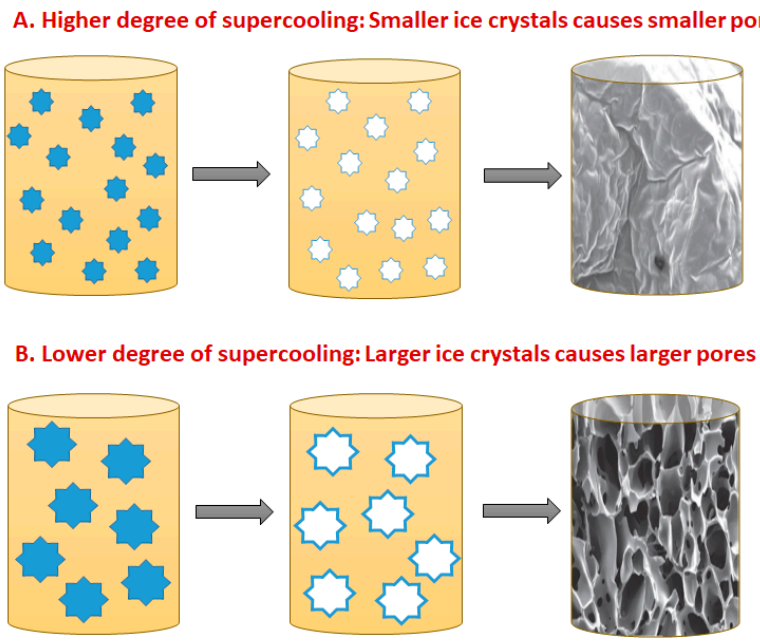

Figure 2. Schematic description of ice nucleation starting temperatures for DMAAm-co-PEG diacrylate cryogel synthesis. (A) At lower negative temperatures, due to the higher degree of supercooling a larger fraction of ice freezes forming smaller and numerous ice crystals. (B) Ice nucleation at −5 °C generates a larger but fewer ice crystals.

4.2. Effect of Solvent

Cryotropic gelation may take place either in aqueous or organic solvents by choosing a suitable temperature regime under which the solvent crystallizes and does not solidify [146]. The choice of solvent system is restricted by the solubility of the precursor and the intended application of the cryogels. For example, cryogels intended for biomedical applications are preferably made in aqueous solution due to the toxicity issues associated with organic solvents. Some of the examples of the restriction imposed by the solubility of the precursors are a) formation of chitosan cryogel in dilute aqueous solutions of acetic acid, and b) cross-linked polystyrene cryogels formed by the catalytic activity of tin (IV) chloride in nitrobenzene as solvent. Some other commonly used solvents for cryogel synthesis included cyclohexane [147] and dimethylsulphoxide (DMSO) [148,149]. The type of solvent used for the formation of the cryogels influences the pore structure, strength, and other physical characteristics of the cryogel since the solvent plays a vital role in pore formation wherein solvent crystals are the porogens. For instance, cryogels of regular pore arrangement may be formed by use of appropriate solvent, as in the case of polyacrylamide (PAAm) cryogels containing *N, N′* methylene bis acrylamide (MBAAm) as cross-linker prepared in aqueous solutions have an oval pore shape (Figure 3 A-D). Formamide solvent produces prolate pores aligned in the direction of the temperature gradient in PAAm cryogels. PAAm cryogels made with 1,4 dioxane have bimodal pores due to solvent-induced phase separation. Poly(NiPAAm) cryogels prepared in DMSO have different pore structures and swelling properties than those made in aqueous solvents [150] (Figure 3 E-H). Furthermore, a mixture of solvents can also be used to obtain cryogels of hierarchal porosity [151]. This may be possible by deriving the ternary phase diagram of the system to optimize the best temperature for synthesis. Tripathi et al used water and acetic acid containing aqueous solution for synthesis of agarose and gelatin cryogels. The cryogels synthesized in water showed a gradient of pores from top to bottom (187 to 76 μm) while the cryogels made in 0.1% acetic acid had uniform pore size throughout the cryogel column [151]. Moreover, the use of a miscible/immiscible solvent system allows for the incorporation of mesopores along with macropores. Such biporous systems are well desired for various applications which require simultaneous mass transport and catalysis or high surface area [151]. Thus, the choice of appropriate solvent system can be used to control pore

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shape and size and, in some cases, the mechanical stability of cryogels based on the type of solvent. Apart from being just a porogen, the solvent can also act as an initiator for gelation for example, in silk cryogels, where ethanol or other organic solvents induce the gelation of silk [130,152]. Although solvents can lead to different microstructures and physical properties of the resulting cryogels, this is a relatively underexplored area for shaping the cryogel properties and merits further investigation.

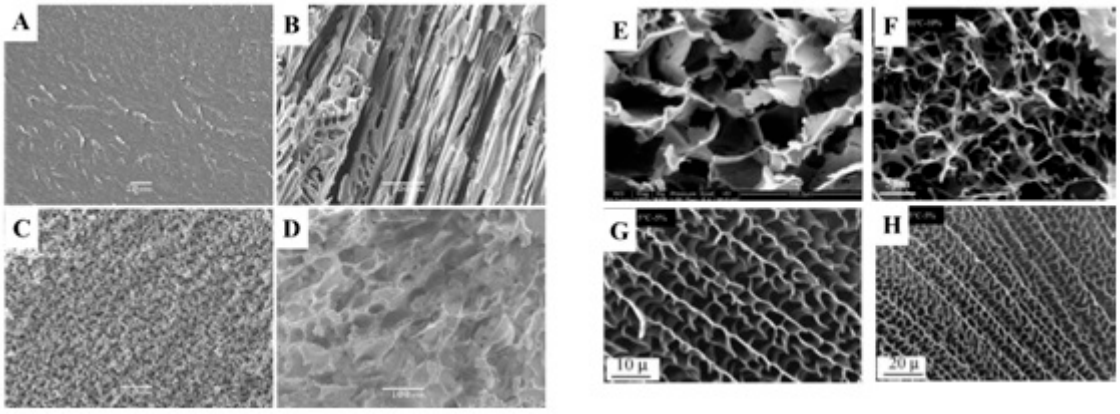

Figure 3. Effect of solvent on cryogel pore morphology. SEM images of PAAm cryogel prepared: (A) at 22 °C, (B) at −20 °C, (C) in 95 % formamide at −20 °C and (D) in 95 % dioxane at −20 °C Reproduced from cited reference with permission. [178] PNiPAAm cryogel prepared: (E) in aqueous medium at −12 °C Reproduced from cited reference with permission [193] Copyrights [2007/Elsevier] and (F–H) in DMSO at different temperatures (−20 °C, 0.5 °C). Reproduced from cited reference copyrights [1991/Royal Society of Chemistry]. [194].

4.3. Effect of Temperature

Temperature regime is one of the most important parameters that has been demonstrated to influence both the synthesis and properties of cryogels. Typically, the production of cryogels involves optimization of the temperature during synthesis and the thawing temperature as well as the thawing rate in specific instances [22,37,43,60,67,153]. It has been established that the temperature dependence of cryogelation has an optimum point, as observed in cryogels formed either by polymerizing systems/covalent cross-linking of polymers, or gelation by physical cross-linking of polymeric networks [154,155]. This is irrespective of the solvent system being used for the synthesis of the cryogels [39,156]. The temperature used for cryogel synthesis should be such that while it allows the solvent system to freeze and form crystals, it should not be so low that the whole process becomes sluggish.

The cryogelation temperature affects cryogel synthesis in several ways. Incubation temperature and cooling rate affect the reaction kinetics and pore structure by altering the extent of NFLMP and the magnitude of ice crystals formed during freezing. [33,157] Reducing the freezing temperature enhances the apparent concentration of solutes in the NFLMP, consequently reducing its volume. This could potentially result in thicker pores and greater mechanical strength. However, contrary to expectations, studies indicate otherwise. Various methods, including NMR and solute diffusion studies in NFLMP, confirm that diffusion within the NFLMP is further hindered by decreasing temperatures and the apparent increase in reactant concentration cannot appropriately offset the sluggish reaction rates at much lower temperatures. Additionally, prolonged reaction times diminish reaction efficiency, yielding smaller gel fractions and consequently weaker cryogels [33,157].

Secondly, in polymerizing systems initiated from monomeric precursors like AAm, when starting with equivalent precursor concentrations, lower the temperature smaller the pore sizes and reduced porosities compared to higher temperatures. This can be comprehended logically by considering ice nucleation: as temperatures deviate further from the freezing point, a greater number of smaller ice nuclei are generated. Consequently, this results in smaller ice crystals leading to smaller pore sizes in cryogels formed at lower negative temperatures [37,157]. For example, while the total

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pore volume of the PAAm cryogels prepared at –12 °C and –18 °C exceeded 90%, there was considerable difference in the distributions of large and small pores between these cryogels, resulting in variations in flow resistance [136].

This can be further explained based on the degree of supercooling of the precursor solution at a given negative temperature. Supercooling, happens when water is chilled below its freezing point without formation of the ice crystals. This unstable state of water is influenced by the temperature and rate of freezing used to create cryogels. Higher degrees of supercooling at lower temperatures promote faster gelation and the formation of heterogeneous hydrogel-like networks (Figure 2). Conversely, higher negative temperatures and less supercooling lead to uniform macroporous networks due to quicker nucleation. [33] Additionally, conditions promoting a faster nucleation rate also promote larger pore sizes while the faster rate of gelation promotes smaller pore sizes. This can be understood based on the size of the ice crystals formed as a result of faster nucleation which grows to form bigger size crystals that lead to bigger pore sizes in these ice-templated polymeric networks upon thawing [82,135]. Thus, a balance of the freezing temperature which promotes both freezing and formation of adequate ice crystals along with realistic gelation rates is required. It can be derived from these observations that increasing the freezing rate or lowering the freezing temperature leads to the formation of more nucleation sites and smaller ice crystals.

The type of polymeric or monomeric precursor used for cryogel formation also regulates how the rate of freezing will affect cryogel formation. Experiments exploring the cryopolymerization mechanism of monomeric systems, especially employing PAAm as a model for cryogels, have observed that the process of forming PAAm cryogels is influenced by the method of freezing. The cryogels prepared with and without pre-cooling in liquid nitrogen to -196 °C differ significantly in pore morphology and physical properties. Cryogels prepared using pre-cooling have collapsed and smaller pores while cryogels prepared without pre-cooling have open polyhedral pores [158]. On the contrary, in the case of physically crosslinked systems, the final incubation temperature plays a significant role in the cryo-structuration rather than the rate of freezing. Freezing-induced cryo-concentration in NFLMP promotes physical crosslinks to form between the suspended polymer chains merely due to the increased proximity of the functional groups involved in forming the hydrogen bonds. Therefore, covalently and physically crosslinked cryogel systems also have an optimum temperature, above and below which the cryogels obtained have inferior physical properties [153].

A common limitation with ice-templated materials like cryogels is their sensitivity to temperature variations. The achievable temperature homogeneity in such systems is restricted. In larger systems, there can be significant temperature gradients, which may result in property gradients even at lower cooling rates, potentially making them unsuitable for fast-gelling systems. Thus, the accurately controlled temperature of synthesis and incubation is critical to generating homogenous cryogels. Although systematic studies comparing the reproducibility of cryogels formed under slightly different synthesis temperatures are limited, existing reports suggest that maintaining the freezing temperature within a narrow window of 0.5–1 °C significantly enhances reproducibility. This level of control is typically achieved using refrigerated circulating baths, which offer stable and uniform cooling conditions. Others have shown that controlling the freezing or thawing rate at precisely 1 °C/min results in more uniform and reproducible gel architectures. Additional evidence for the importance of tight thermal control is provided by studies fabricating cryogels with asymmetric or hierarchical porosity through the application of temperature gradients across the sample.[159–162] This highlights the need to minimize temperature gradients during cryogelation to avoid spatial variations in pore size.

The combined effects of solvent choice and temperature on cryogel properties are complex and closely interconnected. Key factors such as the solvent's type, freezing point, and behavior during freezing—particularly ice crystal formation—play crucial roles in determining the final cryogel structure. Generally, lower freezing temperatures and solvents that encourage phase separation tend to produce smaller, more numerous pores. In contrast, higher temperatures and solvents that are

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more compatible with the polymer typically lead to larger, fewer pores. These structural differences have a direct influence on mechanical properties; smaller pores are often associated with greater compressive strength and modulus. In butyl rubber cryogels, using benzene as the solvent results in larger pore volumes compared to cyclohexane. This occurs because phase separation of butyl rubber from benzene is more pronounced at low temperatures, leading to increased pore formation. [163] Changing the cryogelation temperature from -18°C to -2°C results in organized and aligned pores and increases the material's stretchability. Similarly, for silk fibroin cryogels, lower gelation temperatures typically lead to smaller pore diameters[152].

4.4. Rate of Thawing

The cryostructuring of gels can occur at various stages of synthesis, and this process is also influenced by the specific type of polymeric system under investigation. Cryostructuration may take place while freezing, for instance, in the case of an aqueous solution of locust bean gum wherein the formation of thermoreversible physical cryogels depends on the freezing temperature and rate [59,164]. Alternatively, cryostructuration may happen in the frozen state, particularly in covalently cross-linked cryogels such as PAAm, poly(NiPAAm), or poly(vinylcaprolactam) (poly(VCL)) [68,86,165]. In the case of, physically cross-linked cryogels, the cryogelation also takes place while thawing, and thus the gel properties depend greatly on the rate of thawing and the temperature history (# freeze-thaw cycles) of the sample [153,166]. The slower the thawing rate, the greater the time for reorientation of intermolecular bonds and formation of physical bonds like hydrogen bonds, leading to greater mechanical strength of the gels [59]. The sol-gel transition in a system takes time, progressing slowly in a highly viscous NFLMP medium. This phenomenon is most notable in cryogels, where hydrogen bonding stabilizes the polymer network junctions. [59] Cryogels synthesized using PVA [154], locust bean gum [59], starch/polysaccharides, maltodextrin [60], amylopectin [32], and agar-agar [32], are examples of such systems. Later studies showed that the gel yield increases for a high initial polymer/monomer concentration and a decrease in defrosting rate but it is the thawing rate or defrosting rate that has the greatest influence [154,167]. Furthermore, multiple freeze-thaw cycles in PVA and other physically crosslinked cryogels increase the mechanical strength by 10 to 20 times while total porosity increases by a factor of 1.5 to 2 depending upon the initial concentration of the polymer. One to five cycles of freeze-thaw are optimum but maximum changes in gel strength and mechanical properties are observed with a freeze-thaw cycle of 2 [59,153].

Recently cryogels of linear polyethyleneimine (L-PEI) were fabricated by the process of repeated freeze-thaw leading to physical crosslinking between the polymer chains [61]. The gel's mechanical strength correlated to the freezing temperature and was highest for a freezing temperature of −196 °C. The cryogels produced at this temperature exhibited the maximum crystallinity within the gels and the highest enthalpy of fusion. Further, these cryogels exhibited thermoreversibility. The authors hypothesize that the hydrogen bonding among primary amines and hydroxyl groups leads to the formation of the physically crosslinked cryogel.

4.5. Effects of Added Solutes

Another factor that might influence the cryo-structuration of the polymers is the presence of solute particles. The type and concentration of these low molecular weight particles modify the formation of cryogels, greatly by affecting the size of NFLMP or sometimes even disrupting the crosslinking mechanism. Moreover, physicochemical properties of solute particles such as particle size, shape, mechanical properties, and chemical structure all influence the final cryogel properties. Particles like salts, sugars, dextran beads, microorganisms (bacteria, fungi, yeast) [168–171], ionic particles (anionic or cationic)[41], surfactants which cause foaming [172] hydrophilicity or hydrophobicity of particles [173], *etc.*, all affect the mechanical and physical properties of cryogel [174,175].

More pronounced effects of added solute have been observed in the case of physically cross-linked cryogels. Most of these studies have been done in PVA cryogel [176]. However, such studies

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have far-reaching consequences for cryogels such as proteins where crosslinking is also mediated by physical interaction between the amino acid chains and may be disrupted by salt type and concentration [177]. The presence of low molecular weight solutes capable of interfering with the hydrogen bonds also interferes with cryotropic gelation. In a study in PVA cryogels, chaotropic ions (LiCl, NaBr, NaSCN) which are capable of interfering with hydrogen bonding, disturb the cryotropic gel-formation process and facilitate swelling, whereas antichaotropic ions (CsCl, NaOAc, NaF) which promote the hydrogen bonding, reinforces cryogel formation, mechanical strength and shrinking of cryogels when compared to PVA cryogel prepared in pure water without salt additives. The ability to reinforce or disrupt is proportional to the order of the anion or cation in the Hoffmeister series. Both anion and cation show the phenomena with the anion showing stronger effects than the cation [41]. Moreover, a stronger salting out effect is seen with stronger antichaotropic anions like NaF and water-soluble zwitterion forming hydrophilic amino acids like glycine, lysine, and aspartic acid. All of these factors reinforce cryogel formation and strength [178].

More pronounced effects are observed for anions as compared to cations [41,55]. By the same phenomena, the addition of anion exchange resin (Amberlite) loaded with either OH^- or Cl^- as the exchange ion reinforces the PVA cryogel strength. The extent of the effect of resin on cryogel is determined by the ion providing the negative charge for instance OH^- has a greater reinforcing strength than the Cl^- [176]. The greater effect of the OH^- containing Amerblite resin can be attributed to cryoconcentration of the OH^- in the NFLMP phase which causes a localized rise in pH leading to the deprotonation of PVA chains forming O^- charge on the chains which then strongly interacts with basic quaternary tetraalkylammonium groups (+) ions on the resin in these cryoconcentrated NFLMP. As the polymerization proceeds the interaction between the dispersed resin phase and PVA chains is strengthened due to immobilization of the PVA chains. This effect is not seen when Cl^- containing Amberlite resin is added. This gave a way to control the strength of cryogel by modulating the ionic interaction of the dispersed phase[176]. Moreover, the stronger the ion less the decrease in mechanical strength. For instance, strong ions like OH^- and H^+ make PVA cryogels of higher strength compared to weak ions like Na^+, and Br^- [175]. Similarly, the addition of ionic and non-ionic surfactants induces foaming in PVA and PVA-like polymeric solutions and affects the mechanical stability and pore structure. In general, the addition of surfactants induces foaming and results in larger pore formation of up to 180 μm formation along with the regular pore size of 1 μm commonly found in PVA cryogels. This can be due to the ionic and surface characteristics of the surfactant. For instance, the addition of non-ionic surfactants like decaoxyethylene cetyl ether increases the mechanical strength of the PVA cryogels compared to cryogels formed with ionic surfactants like sodium dodecyl sulfate and cetyltrimethylammonium bromide [172].

The addition of solutes not only influences the crosslinking in cryogels but also the first step of cryogel preparation which is *freezing and phase separation*. This is mainly a function of cryo-concentration of added solutes in the NFLMP regions leading to apparently very high concentrations of dissolved solutes in these regions. Although the dissolved solutes do not take part in the reaction or crosslinking, they interfere with the composition and properties of NFLMP considerably. An NMR study of such phenomena in polyacrylamide cryogels formed by free radical polymerization was done by Kirsebom et al [38]. The study showed that the presence of water-miscible polar (methanol) and non-polar solvent (acetone) in small amounts caused a phase separation in NFLMP as water freezes at the incubation temperature while the non-polar solvent remains unfrozen. Simultaneously the unfrozen solvent acts as a poor solvent for the formed polymer-phase separation giving rise to bimodal pore sizes of 10-80 μm due to cryogelation while 1 μm in the polymer walls forming the pore (Figure 4A-B). Moreover, the addition of solvent causes freezing point depression which increases the size of NFLMP. Similar, observations have been made by Kumar and coworkers [67,179] when cryogel of sparingly water-soluble monomeric precursors like acrylonitrile was made. The pores were formed due to both cryogelation as well as phase separation of the acrylonitrile in NFLMP (Figure 4C-D). In comparison to the organic solvents addition of salts does not cause phase separation in NFLMP but leads to higher cryo-concentration of the solutes in NFLMP leading to thicker non-

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porous wall formation and reduction in porosity of the cryogels as demonstrated in a polyacrylamide cryogel system using NMR [38]. Similar observations have also been made for PVA cryogels upon the addition of 2-11% methanol in NFLMP. The presence of methanol causes the polymeric walls to have the fibrous appearance and lose the polymeric network leading to a ~ 30% decrease in mechanical strength in a concentration-dependent manner [180]. Thus, the addition of solutes of different physiochemical properties gives a way of modulating the NFLMP and affects the final properties of the cryogels.

Additional inert solutes can also induce pore formation in the polymeric walls of the cryogel. The properties in such cases can be modulated by the molecular weight of the inert solute, e.g., PEG polymer of different molecular weights [109].

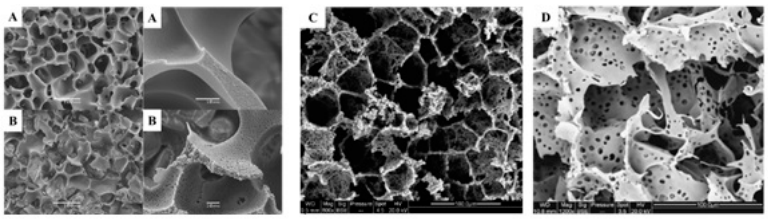

Figure 4. Effect of solute on cryogel synthesis. SEM images of PAAm cryogels at low and high magnification **A)** 0.3M NaCl added, **B)** 0.6M acetone added. SEM image of **C)** polyacrylonitrile cryogel made at -12°C **D)** polyacrylonitrile-gelatin (2:1) interpenetrating network cryogel. Reproduced from cited references with permission copyrights [2010/American Chemical Society]; copyrights [2012/Taylor&Francis]; copyrights [2008/Springer Nature]. [38,67,176].

4.6. Precursor Composition

The type of gel-forming agent, its initial concentration, and the type of bonding also determine the properties of the resulting cryogels. Covalently cross-linked cryogels by polymerization of low molecular weight precursors characteristically differ from cryogels synthesized by cross-linking of high molecular weight precursors. These rather have different pore morphology and properties than cryogels formed by physical cross-linking. Covalently crosslinked cryogels obtained using natural polymers are found to have larger pore sizes than the cryogels of synthetic low molecular weight precursors, which might be due to the efficiency of polymerization under frozen conditions affecting the molecular weight of the generated polymeric chains [42,43,86,135]. As expected, the initial concentration of gel-forming precursors has been found to be inversely related to porosity (Figure 5) while it relates directly to mechanical strength [86,135]. However, the upper limit of initial precursor concentration is limited by their solubility in the solvent used for synthesis, beyond which the precursor concentrations may become too high and precipitate out immediately on freezing, leading to the formation of poor gel structure [181]. Specifically, in the case of polymeric precursors (both for covalently or physically cross-linked cryogels), very high initial concentration may lead to very high viscosities in NFLMP which might sterically and physically hinder the bond formation processes [33]. Moreover, high viscosities are found to affect the crystallization of solvent into ice crystals which are porogen for the formation of cryogel upon thawing [33]. With more polymer, less water turns to ice at the same low temperature.

In cryogels formed by cross-linking of macromolecules by physical bonds such as hydrogen bonds, van der Waals interaction, or hydrophobic interactions [60,154], the nature of the polymer and its molecular weight influences the strength of bond formation at the cross-links [154]. As guided by the type and starting concentration of the polymer and the cycles of freezing treatment, this gives either spongy cryogels with moderate strength or elastic non-spongy cryogels which are exemplified by PVA cryogels [182]. Such cryogels are formed by repeated freeze-thawing and are thermally reversible [182]. The morphology and physicochemical properties of non-covalent cryogels significantly depend on the type of polymer , which do not matter in the case where cryogels are

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made via covalent cross-linking of macromolecules eg., molecular weight (MW), chain tacticity, functional groups on the polymer [182]. When physically crosslinked cryogels that are formed through hydrophobic interactions, the exposure of hydrophobic regions for intermolecular interactions is a critical factor. [32,183]

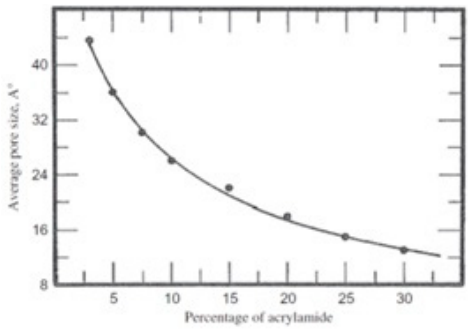

Figure 5. Influence of precursor concertation on cryogel porosity. PAAm cryogel was made at different starting concentrations and the porosity was measured. Reproduced with permission from cited reference copyrights [2013/Taylor and Francis]. [195] .

5. Conclusions and Future Directions

Cryogels are synthesized at subzero temperatures and have features that make them superior to regular hydrogels for tissue engineering applications. They have a three-dimensional, highly interconnected macroporous structure that allows for the interchange of nutrients and waste products, creating an ideal milieu for cell adherence. In comparison to other methods that may involve the use of inorganic/organic solvents (salt leaching, hydrogenation), probability of toxic intermediate formation during synthesis procedure (thermally induced phase separation), complex and expensive procedures (bioprinting, 3D printing); cryogelation is a relatively simpler, environment-friendly, and cost-effective procedure that can produce desired biomaterials in large scale under mild synthesis conditions.

Cryogel is advantageous for tissue engineering applications in comparison to hydrogels due to its enhanced porosity, mechanical stability, elasticity, and injectability. This review summarizes and discusses the cryogel synthesis methods, crosslinking mechanisms, and, factors affecting the cryogelation process. A thorough discussion of these features, as well as their pros and downsides, could assist researchers in comprehending the exact requirements for the development of cryogels as tissue-engineered scaffolds. Methods for making macroporous cryogels have improved, highlighting cryogelation's importance in creating tissue-engineered scaffolds. Nonetheless, methods to control ice nucleation and shape will provide novel ways of synthesizing and controlling pore formation and architecture, an area that remains underexplored and needs further investigation.

Improving the overall properties of cryogel will require a deeper comprehension of this intricate gel-forming system. Cryogel synthesis lacks control over pore uniformity and size at the micrometer level. In contrast, three-dimensional printing can customize scaffold size and shape with micron precision. Hence, the integration of two methods, i.e., three-dimensional printing technology and cryogelation is emerging as an advanced technology wherein the benefit of both the fabrication methods can be harnessed to obtained well-defined macroporous cryogels of desired pore characteristics.[50,184] Therefore, this review can provide a one-platform, comprehensive information about the cryogelation phenomenon which in turn can help the readers to inculcate a deeper understanding of this topic in order to develop advanced cryogenic biomaterials. Given their substantial benefits for tissue engineering, recent trends indicate that cryogels hold great promise as scaffolding materials for biomedical applications.

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