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## Study of the effect of temperature on the properties of gelatin-chitosan cryogels

Cryopolymers are a class of 3D structural polymers, which are widely used in tissue engineering. Using cryopolymerization technology, physical cross-linked macroporous cryogels based on gelatin and chitosan were synthesized at  $-12\text{ }^{\circ}\text{C}$ ,  $-30\text{ }^{\circ}\text{C}$  and  $-70\text{ }^{\circ}\text{C}$  for application as carriers for cell cultures. The presence of functional groups was investigated by IR spectroscopy. The effect of temperature on physicochemical properties such as pore volume, density, gel fraction and biodegradation of cryogels was studied. The results obtained showed that the pore volume (up to 87.6 %) and the gel fraction (up to 80 %) increased, and the density (0.078 %) and pore sizes of cryogels decreased as the temperature decreased from  $-12\text{ }^{\circ}\text{C}$  to  $-70\text{ }^{\circ}\text{C}$ . The study of biodegradation showed that, polymers had a more degradable property in relation to saline solution with an increase in the cryopolymerization temperature. The results of electron microscopy showed the porous morphology of the surfaces of the synthesized cryogels. The average pore size varied from 150 to 300  $\mu\text{m}$ . The toxicity test showed that aqueous extracts from cryogels did not have a highly toxic effect on MSCs in the adipose rats tissue, since the cell viability was 55–75 %.

**Keywords:** cryogel, gelatin, chitosan, biopolymer, non-toxic, biocompatible, porosity, tissue engineering.

### Introduction

Cryogels obtained by the cryotropic gelation process/cryopolymerization are macroporous hydrogels with a well-developed system of interconnected pores, high swelling capacities and large surface areas [1–5]. Professor V.I. Lozinsky made a huge contribution to the development of the cryopolymerization concept [6–10]. Cryogels can be used in controlled drug delivery, carriers for cell immobilization, sensors, bioseparation, purification, and tissue engineering [11–12]. Common cryogel compositions include natural polymers such as gelatin, chitosan, alginate, hyaluronic acid and synthetic acrylamide-based polymers and poly (lactic acid) (PLA), poly (lactic-co-glycolide) (PLGA), poly( $\epsilon$ -caprolactone) (PCL) [13–14].

Chemical and physical crosslinked cryogels are known to exist. Crosslinkers such as glutaraldehyde, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide are often used in the creation of chemically crosslinked cryogels. Physically cross-linked cryogels are formed through the interaction of intermolecular subchains in macromolecules [15–17].

To date, there are many studies on the synthesis of polymers based on gelatin and/or chitosan using chemical crosslinking agents [18–25]. There are also published studies on the synthesis of polyelectrolyte polymers of gelatin and chitosan [26–30]. However, there are no data on the synthesis of physical crosslinked Gel:Ch scaffolds by cryopolymerization without the participation of crosslinking agents. The synthesis of such polymers is advantageous since no chemical crosslinking agents are used that can be toxic to cells or tissues.

Pinto Ramos derived biopolymer films from chitosan, gelatin and Ch/Gel mixture in salt solutions ( $\text{NaCl}$ ,  $\text{CaCl}_2$  and  $\text{Na}_2\text{SO}_4$ ) with varying concentrations and ion charges. The authors investigated the polyelectrolyte and polyampholytic properties of the films, so that in the future it would be possible to create biopolymer films using them in ionic media [31]. A team of scientists synthesized 3D chitosan scaffolds combined with bFGF facilitated the neural differentiation of dental pulp stem cells (DPSCs). As shown by the results DPSCs adhered successfully and grew well on the surface of chitosan-scaffolds. According to the authors, the transplantation of DPSCs/chitosan-scaffold+bFGF might be a secure and effective method of treating spinal cord injury and other neuronal diseases [32–33].

The main goal of this work is to obtain macroporous scaffolds as a base and carriers for mesenchymal stem cells (MSC). To achieve this goal, we first synthesized and characterized novel physical cross-linked scaffolds based on gelatin (Gel) and chitosan (Ch) by cryopolymerization. This research is of great importance in tissue engineering, as it provides a new understanding of effective and new ways of obtaining

biopolymers without the use of chemicals toxic to cells and tissues. Thus, the obtained cryogels can be used as carriers of stem cells and can be used in the treatment of bone damage.

### Experimental

#### Preparation and characterization of cryogels

The GelCh cryogels were prepared by dissolving a gelatin (0.4 % w/v) and a chitosan (0.2 % w/v) in 1 % acetic acid solution. The acidity of the solution was then adjusted using 1 M of NaOH to pH = 5 to protonate amine groups of Ch. After the solution was transferred in syringes and incubated for  $-12\text{ }^{\circ}\text{C}$  (GelCh12),  $-30\text{ }^{\circ}\text{C}$  (GelCh30) and  $-70\text{ }^{\circ}\text{C}$  (GelCh70) for 24 h. After thawing at room temperature, the physically crosslinked polymers thus formed were washed with MilliQ water and PBS (pH = 7.4) and lyophilized using Martin Christ Beta 2-8 LDplus freeze dryer. GelCh cryogels were stored in a dark place at room temperature for further use.

Gel fractions, the degree of degradation and the density of cryogels were determined according to a well-known method [15].

The gel percent was calculated by the formula [34]:

$$Gel(\%) = \frac{W_w}{W_i} \times 100, \quad (1)$$

where  $W_w$  and  $W_i$  are weight of the swollen dry gel and a sample, which was not immersed in water but directly freeze-dried.

Cryogels were weighed ( $W_1$ ) and transferred to 50 ml tubes filled with sterile 0.1M PBS (pH 7.4). The tubes were incubated at  $37\text{ }^{\circ}\text{C}$  for 8 weeks, during which the solution was refreshed twice in a week. At pre-determined times, cryogel samples were taken from the solution and washed with deionized water. After freeze-drying overnight and weighing ( $W_2$ ) the degree of degradation was determined by the following formula [15, 18]:

$$DD(\%) = \frac{W_1 - W_2}{W_1} \times 100. \quad (2)$$

The density of cryogels was evaluated from the mass-to-volume ratio of dry cryogels. The apparent density ( $\rho$ ) was obtained from the equation [15, 35]:

$$\rho = \frac{W}{\pi \times (D/2)^2 \times H}, \quad (3)$$

where,  $W$  is the weight of the cryogel,  $D$  is the diameter and  $H$  is the thickness of polymers.

The pore volume of the cryogels was estimated from the uptake of ethanol into the pores. Ethanol is a non-solvent for the cryogels and it easily penetrates into the pores. The measurements were carried out by immersing dry cryogel specimens with a mass of  $W_D$  into absolute ethanol for 1 h and then recording their final mass  $W_S$ . The pore volume (PV) was calculated by the following formula [15, 36]:

$$PV(\%) = \frac{(W_S - W_D)}{W_S} \times 100. \quad (4)$$

The measurements were performed in triplicate and the average value was found.

The cryogels were lyophilized and the Fourier transform infrared (FTIR) spectra (Nicolet iS 10, Thermo Fisher Scientific) of these lyophilized samples were recorded in the wavelength range of  $4000\text{--}400\text{ cm}^{-1}$ .

The morphology of dried cryogels was observed using a scanning electron microscope (SEM, Auriga Crossbeam 540, Carl Zeiss) after coating with gold 5 nm.

#### MTT assay

The cultivation of rat adipose-derived MSC (ADMSC) and MTT assay were obtained as previously described [15]. In this study outbred male Wistar rats weighing 280–330 grams were used. The study was carried out according to the guidelines of the Declaration of Helsinki and approved by the Local Ethics Committee of the National Center for Biotechnology (number NCB-04-2020).

For the statistical analysis, all the physical, chemical and biological experiments on cryogel samples were performed in triplicate. All the experimental values were expressed in the form of mean  $\pm$  standard error and the limit of experimental error of each test was  $\pm 5\%$ , which had been considered as statistically significant.

### Results and Discussion

Ch contains amine groups and exhibits the properties of polyelectrolytes [37]. Gel, consisting of negative carboxyl and positive amine groups, shows the properties of polyampholytes [38]. The formation of the 3D macroporous structure of GelCh cryogels occurs due to the interaction of negatively charged carboxyl groups ( $-\text{COOH}$ ) of gelatin and positively charged amino groups ( $-\text{NH}_2$ ) of chitosan, which form a polyelectrolyte interaction between the macromolecules chains (Fig. 1).

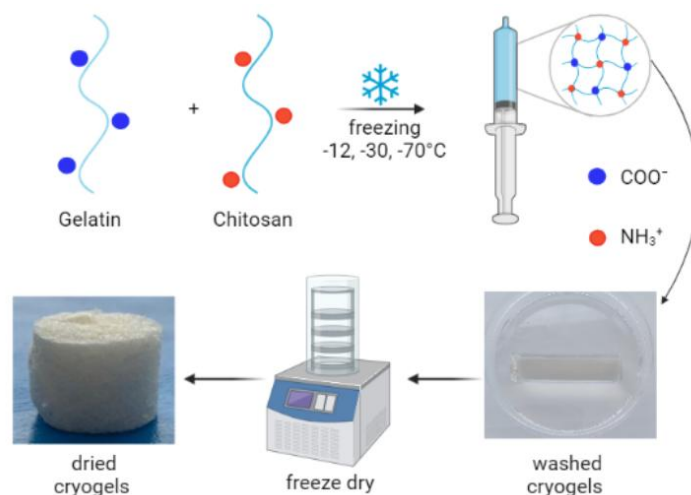


Figure 1. Scheme showing the cryogels formation

The spectrum of cryogels shows a band in the range of  $3000\text{--}3600\text{ cm}^{-1}$ , which belongs to the stretching vibrations of the O–H and N–H functional group (amide A) that is involved in the intramolecular hydrogen bond between chitosan and gelatin molecules. The bands at  $2800\text{--}2900\text{ cm}^{-1}$  are due to several symmetric and asymmetric stretching vibrations of C–H. The bands at  $1650\text{ cm}^{-1}$  refer to CO and CN amide I. The spectra at  $1535\text{ cm}^{-1}$  refer to bending vibrations of NH groups and stretching vibrations of CN groups (amide II). The absorption of the spectrum in the  $1243\text{ cm}^{-1}$  range belongs to stretching vibrations of CN-groups (amide III). The troughs at  $1065\text{ cm}^{-1}$  are due to stretching vibrations of C–O groups. The bands at  $887$  and  $675\text{ cm}^{-1}$  are related to the vibrations of C–H and N–H groups (Fig. 2).

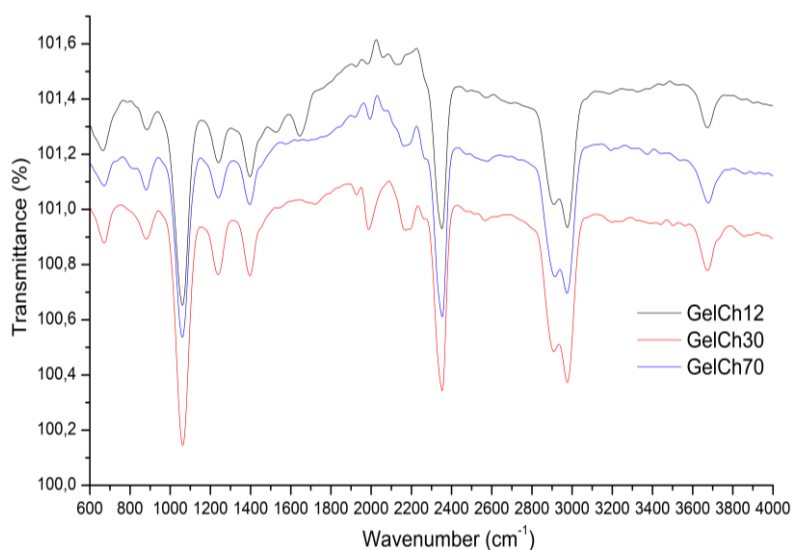


Figure 2. FTIR spectra of GelChCS cryogels

GelCh cryogels were synthesized by dissolving gelatin and chitosan in acetic acid using cryogelation technology at  $-12\text{ }^{\circ}\text{C}$ ,  $-30\text{ }^{\circ}\text{C}$  and  $-70\text{ }^{\circ}\text{C}$  without using any chemical crosslinkers (Table 1).

Summary properties of GelCh cryogels

Samples	T, °C	Gel percent (%)	Density (g/ml)	Pore volume (%)
GelCh12	-12	73±1	0.095±0.005	84.6±3
GelCh30	-30	79±3	0.085±0.006	86.5±4
GelCh70	-70	80±1	0.078±0.005	87.6±2

As can be seen from Table 1, the yield of the gelfraction for cryogels is maximum one with decreasing temperature. This is due to the fact that at a lower temperature tightly crosslinked polymer networks are formed, in which the sol (soluble part) fraction is present to a lesser extent. Compared to the GelCh70 sample in the GelCh12 sample, the yield of the gel and sol fractions is 80 and 73 %, respectively. This may also be due to the hydrophilic properties of gelatin and the looser structure of the polymer. As the results show, the porosity of scaffolds decreases with an increase in the their density. The synthesized cryogels have a high pore volume of 85-88 %. The more porous the polymer, the better it is for the penetration of fluids and cells. Biodegradation in PBS solution over 8 weeks was studied to confirm the cryogels biocompatibility (Fig. 3).

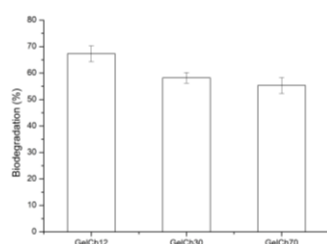
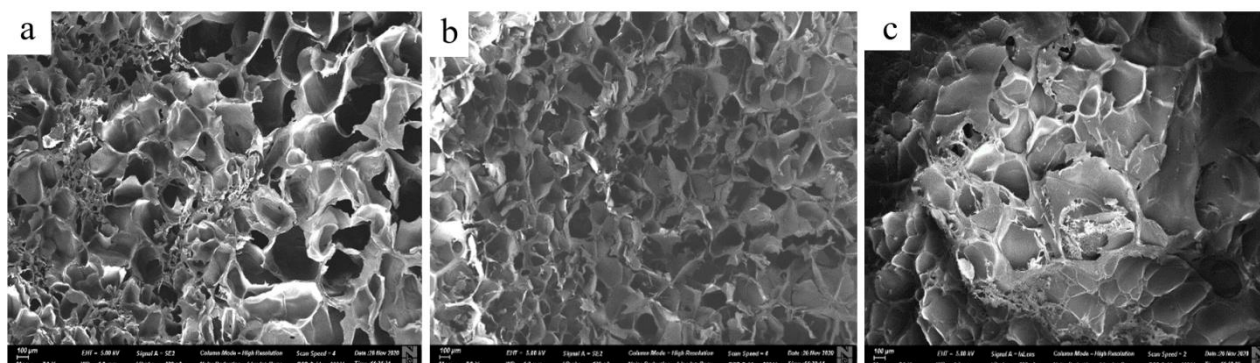


Figure 3. Biodegradation behavior of the cryogels in PBS at 37 °C for 8 weeks

The *in vitro* decomposition rate of cryogels was 67 % (GelCh12), 58 % (GelCh30) and 55 % (GelCh70). Compared to covalently bonded cryogels, the synthesized cryogels showed a high percentage of degradation of the polymer matrix. Upon biodegradation, cryogel macromolecules (long polymer chains) break down into low molecules (oligomeric units), which dissolve in the solvent and lead to weight loss. The presence of water-soluble gelatin gives additional hydrophilicity to polymers, in which monomer chains are rapidly hydrolyzed. Interestingly, the degree of biodegradation decreases with decreasing temperature, since cryogels are not linked by a covalent bond. All cryogels exhibit a high degree of decomposition. The degradation of cryogels is influenced by its surface morphology and pore size (Fig. 4).



a — GelCh12; b — GelCh30; c — GelCh70

Figure 4. SEM of cryogels

The surface morphology of the synthesized polymers was investigated by the SEM method. As can be seen from Figure 4, the surface of the synthesized cryogels is changed significantly under the influence of temperature. The surface of cryogels has a porous structure with unevenly distributed pores. As the temperature decreases, the formation of more closed pores is observed. The pore size of cryogels varies from 150 to 300  $\mu\text{m}$ . Comparing cryogels, one can notice a tendency that GelCh30 has a more uniform pore distribution, while GelCh12 has a random distribution. This is possibly due to self-assembly between gelatin and chitosan. The resulting pore sizes can be sufficient for cell cultivation, since the biggest pore size accommodates more cells that can agglomerate. Thus, the pores provide intercellular contact between cells, showing higher markers of chondrogenesis, and can be used for the treatment of bone regeneration and in tissue engineering in general.

To determine the cytotoxicity of cryogels, an MTT assay was conducted using primary culture of rat ADMSC (Fig. 5).

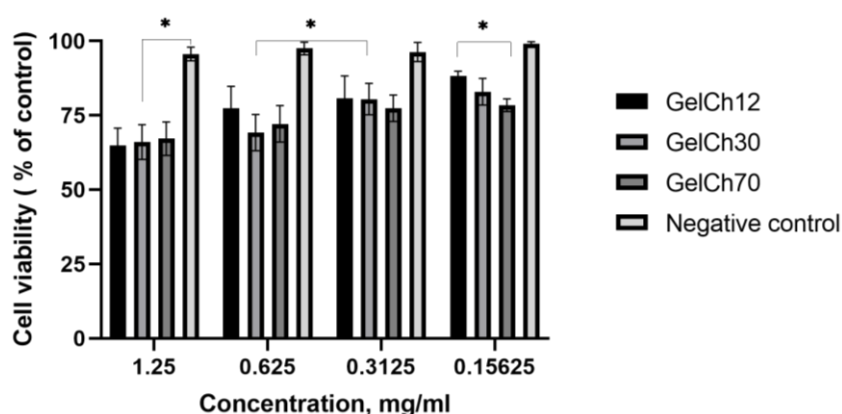


Figure 5. Effects of extracts of cryogels on cell viability of rat ADMSC presented as percentage of cell viability versus concentration of the extracts (\* $p$  value  $\leq 0.05$ )

The extracts from cryogels exhibit a weak toxic effect on rat ADMSC and display more than 55 % of cell viability after treatment with concentration of 1.25 mg/ml. The viability of rat ADMSC cells at a concentration of 0.156 mg/ml with cryogel extracts showed the highest cell viability (up to 75 %). Thus, MTT assay revealed that GelCh12, GelCh30 and GelCh70 cryogels were biocompatible and suitable for further applications in *in vivo* studies.

### Conclusions

Macroporous cryogels based on gelatin and chitosan were synthesized using the cryopolymerization method at various temperatures ( $-12$ ,  $-30$  and  $-70$   $^{\circ}\text{C}$ ) without chemical crosslinking agents. The formation of the 3D macroporous structure of GelCh cryogels occurs due to the interaction of negatively charged carboxyl groups ( $-\text{COOH}$ ) of gelatin and positively charged amino groups ( $-\text{NH}_2$ ) of chitosan, which form a polyelectrolyte interaction between the macromolecules chains. The functional groups of cryogels were identified by IR spectroscopy. The effect of temperature on physicochemical properties of cryogels, namely GelCh12, GelCh30 and GelCh70 was studied. Thus,, the pore volume (up to 87.6 %) and the gel fraction (up to 80 %) increase and the density (0.078 %) and the pore size of cryogels decrease with decreasing temperature. SEM results showed a macroporous surface of the cryogels. Comparing cryogels, one can notice a tendency that GelCh30 has a more uniform pore distribution, while GelCh12 has a random distribution. Since cryogels are composed of natural polymers and obtained without the use of chemical crosslinking agents, degradation products are expected not to cause immune rejection problems during implantation, which in turn makes such materials potentially useful for tissue engineering.

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## Криогельдердің қасиеттеріне температураның әсерін зерттеу

Криополимерлер тіндік инженерияда кеңінен қолданылатын 3D құрылымдық полимерлер класы болып табылады. Криополимеризация технологиясын қолдана отырып, дінгек жасушалар үшін негіз ретінде пайдалану үшін желатин мен хитозан негізіндегі физикалық байланыстырылған торланған макрокеуекті криогельдер  $-12\text{ }^{\circ}\text{C}$ ,  $-30\text{ }^{\circ}\text{C}$  және  $-70\text{ }^{\circ}\text{C}$  температурада синтезделді. Функционалдық

топтардың болуы ИК спектроскопиясы арқылы зерттелінді. Температураның криогельдердің физико-химиялық қасиеттерге әсері зерттелді, мысалы, кеуектер көлемі, тығыздық, гель фракциясы және биодеградациясы. Алынған нәтижелер температура  $-12\text{ }^{\circ}\text{C}$ -тан  $-70\text{ }^{\circ}\text{C}$ -қа дейін төмендеген сайын криогельдердің кеуектер көлемі (87,6 %-ға дейін) және геледік фракция (80 %-ға дейін) артып, криогельдердің тығыздығы (0,078 %-ге дейін) мен кеуектерінің кішірейетінін көрсетеді. Биодеградацияны зерттеу криополимерлену температурасының жоғарылауымен полимерлердің тұз ерітіндісіне қатысты ыдырайтын қасиеті жоғарылайтынын көрсетті. Электрондық микроскопияның нәтижелері синтезделген криогельдердің беттерінің кеуекті морфологиясын көрсетті. Кеуектің орташа мөлшері 150-ден 300 мкм-ге дейін өзгерді. Уыттылық сынағы криогельдерден алынған сулы сығындылардың егеуқұйрықтардың майлы тіндеріндегі МДЖ-ге жоғары уытты әсер етпейтінін көрсетті, өйткені жасуша өміршеңдігі 55–75 % құрады.

*Кілт сөздері:* криогель, желатин, хитозан, биополимер, уытты емес, биоүйлесімді, кеуекті, тін инженериясы.

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## Исследование влияния температуры на свойства криогелей

Криополимеры — это класс 3D структурных полимеров, которые широко используются в тканевой инженерии. Используя технологию криополимеризации, синтезированы физически сшитые макропористые криогели на основе желатина и хитозана при  $-12\text{ }^{\circ}\text{C}$ ,  $-30\text{ }^{\circ}\text{C}$  и  $-70\text{ }^{\circ}\text{C}$  для применения в качестве основы клеточных культур. Наличие функциональных групп исследовано методом ИК-спектроскопии. Изучено влияние температуры на физико-химические свойства, такие как объем пор, плотность, гелевая фракция и биодеградации криогелей. Полученные результаты показывают, что при понижении температуры от  $-12\text{ }^{\circ}\text{C}$  до  $-70\text{ }^{\circ}\text{C}$  увеличивается объем пор (до 87,6 %) и гелевой фракции (до 80 %), и уменьшается плотность (0,078 %) и размер пор криогелей. Исследование биодеградации показало, что при повышении температуры криополимеризации, полимеры обладают более деградируемым свойством по отношению к солевому раствору. Результаты электронной микроскопией показали пористую морфологию поверхностей синтезированных криогелей. Средний размер пор варьировался от 150 до 300 мкм. Тест на токсичность показал, что водные вытяжки из криогелей не оказывают высоко токсического действия на МСК жировой ткани крысы, поскольку жизнеспособность клеток составляла 55–75 %.

*Ключевые слова:* криогель, желатин, хитозан, биополимер, не токсичный, биосовместимый, пористость, тканевая инженерия.

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