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SYNTHESIS AND STUDY OF THE INFLUENCE OF ALKALI CONCENTRATION AND TEMPERATURE ON THE PHYSICOCHEMICAL PROPERTIES OF CRYOGELS BASED ON GELATIN AND CHITOSAN

G.K. Kudaibergen[1*], G.M. Zhumanazarova[2]

[1]National Center for Biotechnology, Astana, Kazakhstan
[2]Karaganda Buketov University, Karaganda, Kazakhstan
[*]E-mail: kudaibergen@biocenter.kz

Abstract. *Introduction.* Much of the polymer research is focused on improving existing polymers or developing new biomaterials with tunable properties. Natural polymers that have certain segments that contribute to an additional therapeutic effect are more often used as a biocompatible polymer. Polymers can be produced using a variety of methods, including covalent cross-linking, dynamic covalent cross-linking, physical cross-linking, cryopolymerization, 3D printing, electrospinning, etc. Cryopolymerization is a unique method and has several advantages over other methods. By cryofreezing it is possible to obtain a porous structure in which the size and volume of the pores can be controlled by changing the concentration of the initial monomers and temperature. *The goal of this study* is to determine the effect of alkali concentration and cryopolymerization temperature on the properties of cryogels. *The objects of study* in this work are cryogels based on gelatin and chitosan. *Research methods.* In this study, physicochemical research methods was used. *Results and discussion.* The functional groups of the cryogels were determined by IR spectroscopy. The results of a study of sorption and desorption of polymers are presented. The results of polymer degradation in phosphate-saline solution over 8 weeks are also shown. These studies were based on changes in mass, that is, they were carried out using the gravimetric method. *Conclusion.* The results of a study of the influence of alkali concentration and temperature on the physicochemical properties of cryogels based on gelatin and chitosan are presented. Cryogels based on gelatin and chitosan are synthesized without the use of harmful chemical cross-linking agents, which makes them attractive for tissue engineering.

Key words: cryogel, gelatin, chitosan, properties, polymer, biopolymers, cryopolymerization, temperature

Kudaibergen Gulshakhar Kudaibergenkyzy	*PhD, e-mail: kudaibergen@biocenter.kz*
Zhumanazarova Gaziza	*PhD candidate, e-mail: gaziza.zhumanazarova@mail.ru*

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1. Introduction

"Cryogels" are macroporous hydrogels that form at a negative temperature, providing their unique properties for bioengineering applications. The synthesis of these materials at negative temperatures provides cryogels with large pores (up to 200 μm), spongy and elastic morphology [1-3]. The advantage of using cryogels compared to conventional nano/mesoporous hydrogels lies in their well-developed three-dimensional interconnected 3-D porous structure, consisting mainly of open pores, which can be used as a framework and cell carrier [4-5]

Chitosan (Ch) is an unsulfated glucosaminoglycan (GAG) of natural origin, is a linear polysaccharide that is part of the ECM; consisting of β (1→4) bound residues of D-glucosamine with a variable number of randomly located N-acetyl-glucosamine groups [6]. Chitosan has excellent biocompatibility, biodegradability, non-toxicity, adsorption properties and the ability to be degraded by lysozyme, a natural enzyme [7-8].

Gelatin (Gel) is a biopolymer derived from animal collagen. It is cheaper, biocompatible, biodegradable, non-immunogenic and widely used in the clinic. In terms of chemical composition, gelatin is close to collagen, but in contrast, gelatin, whose macromolecules do not have an ordered structure, is soluble in water, which makes it convenient to prepare initial gel-forming systems for the formation of cryogels or cryostructures based on this biopolymer [9-10].

In the previous work, we considered the effect of temperature on cryogel properties, and in this work, the purpose of the study is to study the effect of alkali concentration and temperature on the physicochemical properties of cryogel [11]. In this study, the synthesis of gelatin and chitosan cryogels was carried out by cryopolymerization without the use of cross-linking agents and aggressive solvents, which in turn gives an advantage over other known methods for producing biopolymers.

2. The experimental part

400 mg gelatin (Sigma, USA) and 200 mg chitosan (Sigma, USA) were dissolved in 1% acetic acid solution. The mixture was stirred until the components dissolved completely at 37ºC. After complete dissolution, the monomer mixture was filtered to remove undissolved components and mechanical impurities. The solution is then basified 0,1M NaOH until a certain pH of the medium is obtained. Then the mixture is poured into a 2 ml polyethylene cylindrical syringe and centrifuged at 2000 rpm for 5 minutes 3 times to remove excess air. The mixture was placed in a cryostat at -12ºC, -30ºC and -70ºC for 48 hours. At the end of the time, remove the cryogel from the syringe and wash the cryogels with Milli-Q ultrapure water (3x50 mL).The resulting cryogel was then freeze-dried (-80ºC, MartinXTristBeta 2-8 LDplus, Germany) for 24 hours (Figure 1).

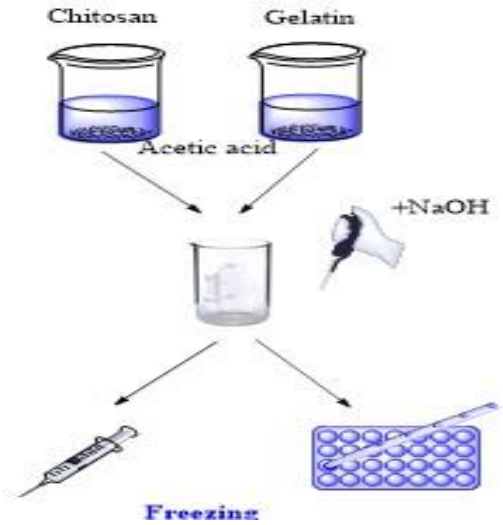

Figure 1 - Graphical illustration of cryogels synthesis

The degree of swelling of cryogels was determined gravimetrically. Freeze-dried cryogel samples (about 0.1 g) were accurately weighed (m1) and placed in a vial. Then, 10 ml of 0.1 M PBS (pH = 7.4) was added, applied over the hydrogels, and after a certain period of time, the polymers were weighed, removing the excess PBS solution (mi). The experiment was carried out at room temperature and continued until uniform sorption of the samples was achieved. The experiment was repeated three times. Sorption degree (α) was determined by formula 1:

$$\alpha = \frac{(m_i - m_1)}{m_1} \quad (1)$$

where m_1 – weight of dry polymer sample, g;
m_i – weight of polymer with solution, g

The desorption of the solution from the polymer was measured gravimetrically at room temperature. The swollen cryogel mass(m_0) was placed on a coverslip and weighed after a certain period of time (m_i). The experiment was repeated three times until constant weight. The degree of desorption was determined by formula 2:

$$\alpha = \frac{(m_0 - m_i)}{m_i} * 100\% \quad (2)$$

where m_0 – weight of the swollen polymer sample at 25ºC, g;
m_i – weight of dry polymer sample, g;

Biodegradation was also determined gravimetrically. Freeze-dried cryogel samples (about 0.1 g) were placed in a vial and accurately weighed (m_1), 10 ml of 0.1 M PBS (pH = 7.4) was added, and then incubated at 37 C for 8 weeks. The PBS solution was replaced twice a week and experiments were performed in triplicate. After 8 weeks, samples were removed from the solutions, washed with Milli-Q superfluous water, dried in an oven, and dry cryogels were weighed all night (m_i). Degradation rate (DM) was determined from the final dried residue using the following formula:

$$DD = \frac{(m_1 - m_i)}{m_1} * 100\% \quad (3)$$

где m_1 – weight of dry polymer sample, g;

m_i – polymer mass after drying, g

The identification of the samples was carried out with the help of IR-Fourier spectrometer Nicoleti S10 (Thermo Scientific). The transmission spectra of the studied powdery samples were recorded at a range of 400-4000 cm^{-1}.

3. Results and discussions

Cryopolymerization of polymers based on gelatin and chitosan was carried out at a temperature of -12, -30, -70ºC using 0.1 and 1M NaOH. The results are shown in Table 1.

Table 1 - Conditions for the synthesis of cryogels based on gelatin and chitosan

№	Sample name	C (NaOH), mol/l	Temperature, ºC	Yield, %
1	Gel:Ch (A-11)	0.1	-12	86,12
2	Gel:Ch (A-11-1M)	1	-12	88,36
3	Gel:Ch (A-18)	0.1	-30	82,19
4	Gel:Ch (A-18-1M)	1	-30	86,62
5	Gel:Ch (A-25)	0.1	-70	83,15
6	Gel:Ch (A-25-1M)	1	-70	85,12

The interaction between crosslinking agents occurs due to covalent and non-covalent bonds, in the case of using chemical crosslinking agents, the functional groups of the agent interact with monomers. Gel:Ch cryogels are synthesized without the use of chemical initiators, by the polyelectrolyte interaction of the carboxyl group (-COOH) of gelatin and the amine group ($-NH_2$) of chitosan, which form transverse bonds between the chains of macromolecules. As shown in Table 1, the addition of 0.1 and 1 M alkali solution in samples A-11, A-11-1M, A-18, A-18-1M, A-25 and A-25-1M did not significantly affect the polymer yield, which is up to 88%.

Chitosan (-NH_2) amines are known to protonate at low pH to (-NH_3^+) and cryogel has a high charge density and electrostatic repulsion between monomer units. At higher pH, amines become deprotonated and ionic repulsion decreases, allowing individual chains to degrade [45]. Studies have shown that when the pH of the medium increases, weak crosslinks and mechanical unstable compounds are formed, the yield of which was low. After a series of studies, it was established that the optimal samples of cryogels based on gelatin and chitosan for further research are: A-11, A-11-1M, A-18, A-18-1M, A-25, and A-25-1M, which have the appropriate shape and macroporous structure (Figure 2).

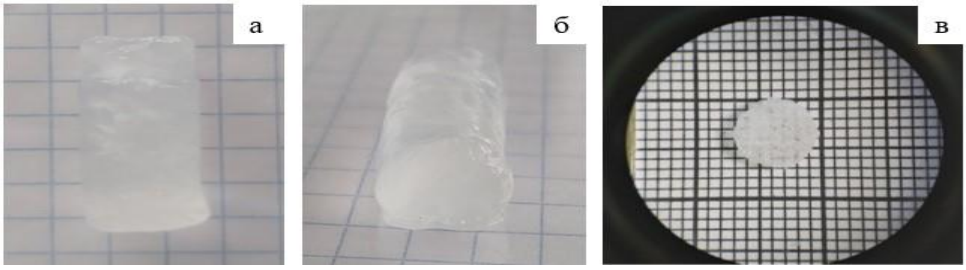

Figure 2 - Appearance of washed cryogel (a, b) and dried A-11 (c)

During cryopolymerization, phase separation occurs, which leads to the rearrangement of polymer chains during the construction of an interconnected network of pores and polymer substrate layers due to the formation of hydrogen bonds between polymer chains and water molecules. When the cryogel is thawed, macropores are formed between the polymer chains, thereby forming a large surface area for cell attachment and proliferation.

The presence of cryogel functional groups was identified by IR spectroscopy (Figure 3).

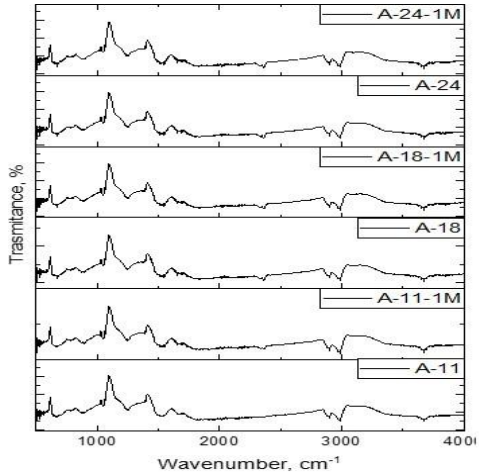

Figure 3 - IR spectra of cryogels based on Gel:Ch.

The spectrum of cryogels shows a band in the range 3000-3600 cm^{-1} relating to the O−H and N−H valence fluctuations of the functional group involved in the intramolecular hydrogen bond between chitosan and gelatin molecules. The bands at 2800-2900 cm^{-1} are due to several symmetric and asymmetric C−H valence fluctuations. Bands at 1650 and 1580 cm^{-1} are attributed to C=O amide I oscillations and N-H amide II bending oscillations. Absorption of the spectrum in the range of 1250 cm^{-1} refers to valence oscillations of the C-O group. The spectra at 1151 and 1046 cm^{-1} are due to asymmetric stretching of the C-O-C bridge, stretching vibration of C-O and -glucoside bonds, respectively. Bands at 893 and 650 cm^{-1} refer to oscillations of C-H and N-H groups. The spectra of cryogels are identical, since the temperature and concentration of alkali do not affect the functional groups, but contribute to the formation of a denser polymer network.

Cryogel swelling is the main parameter that determines its use as a scaffold for bioengineering. The swelling rate (sorption kinetics) directly depends on the porosity of the cryogel itself, the strength of the crosslinked bonds, the monomer ratio, the crosslinking density, the pore wall thickness, the temperature at which the gels are prepared, etc.

The swelling of the synthesized cryogels was investigated in PBS medium at room temperature for 24 hours, since further biodegradation of polymers occurs. The results of the swelling study are shown in Figure 4.

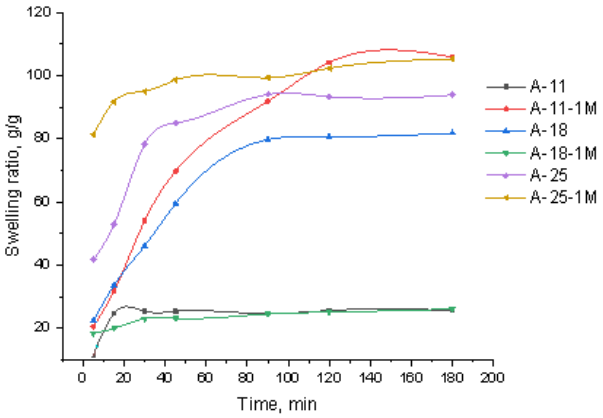

Figure 4 - Swelling kinetics of cryogels

As can be seen from the curves, the equilibrium swelling for many cryogels is reached within 90 minutes, and for A-11 sample 30 minutes, the maximum cryogel swelling is 25.3 g/g. When cryogels swell using 1M NaOH, the polymer increases in size and maximum swelling of the polymer network occurs compared to other samples, since the pores of the cryogel are quickly filled with a PBS solution, and a part of the solvent diffuses through the polymer walls. The swelling curves show that equilibrium swelling is reached after 120 minutes.

Thus, when swelling, two processes occur: filling the pores with a solvent and swelling of the polymer walls. Cryogels may have much greater swelling capacity than conventional hydrogels due to their large pores and high pore interconnectivity [12].

When calculating the degree of desorption by cryogels, the initial mass was taken as the mass of cryogel swollen after a day in a solution of PBS (pH = 7.4) at room temperature (Figure 5).

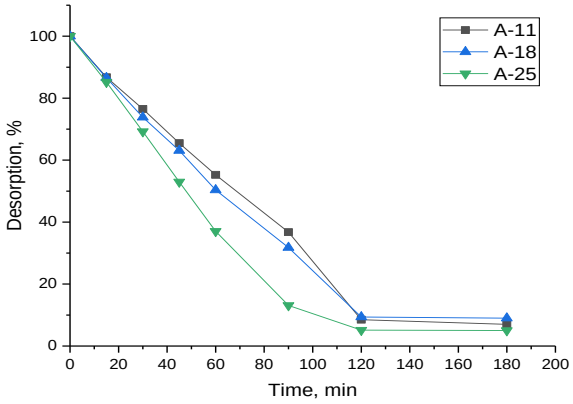

Figure 5 - Solvent desorption kinetics by cryogels of A-11, A-18 and A-25

As can be seen from the graph, all samples completely release the solvent molecules after 120 minutes. It should be noted that after complete drying, the sample A-11 retained its original shape, while the rest of the samples mechanically deformed after the test. This is justified by the compactness of the A-11 cryogel network, which does not provide spontaneous release of PBS, but retains a macroporous structure. When the cryogels swell with an aqueous medium, it causes a slight loosening of the chains under the influence of absorbed water (swelling) and spatial changes in the position of the chains. Perhaps changes in the structure lead to the rupture of some bonds, which makes the structure of cryogels more susceptible to elastic deformations.

Macroporous cryogels used in bioengineering should be biodegradable and maintain minimal resistance during use. This quality is advantageous in tissue engineering since it will not be necessary to further extract the polymer surgically. Biodegradation of cryogels was investigated for 8 weeks in PBS solution medium. Medium was changed 2 times a week. The results are shown in Figure 6.

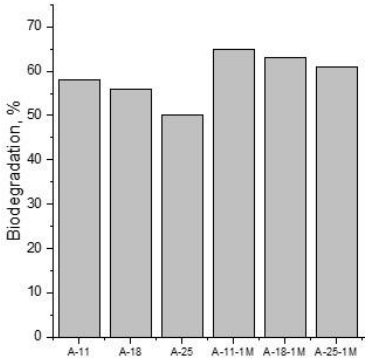

Figure 6 – Biodegradation of cryogels

As can be seen from the diagram, samples with a lower synthesis temperature (A-18, A-25) have a lower degree of biodegradation compared to other cryogels. This effect is explained by the fact that with a decrease in the temperature of cryopolymerization, compression of the polymer network occurs and denser polymer networks are formed, which are more resistant to degradation. Samples A-11-1M, A-18-1M and A-25-1M have a degree of degradation in the range of 60-65%.

4. Conclusion

Advances in modern polymer science highlight the importance of developing complex biomaterials with well-defined architectures and tunable properties for new biomedical materials. Physically cross-linked hydrogels can be prepared without chemical modification of polymers or the use of cross-linking agents, which in turn can lead to the formation of toxic polymers.

This paper discusses the effects of alkali concentration and temperature on the physicochemical properties of gelatin and chitosan cryogels. The polymers were synthesized by cryopolymerization without using any cross-linking agents. The functional groups of cryogels are confirmed by IR spectroscopy. The study showed that cryogels with 1M NaOH (A-11-1M, A-18-1M and A-24-1M) have a high degree of swelling due to the formation of additional polyelectrolyte bonds between polymer chains. The maximum cryogel swelling is 25.3 g/g for A-11 sample in 30 minutes. Biodegradability studies have shown that all polymers are degradable and biocompatible.

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ЖЕЛАТИН МЕН ХИТОЗАН НЕГІЗІНДЕГІ КРИОГЕЛЬДЕРДІ СИНТЕЗДЕУ ЖӘНЕ ФИЗИКА-ХИМИЯЛЫҚ ҚАСИЕТТЕРІНЕ СІЛТІНІҢ КОНЦЕНТРАЦИЯСЫ МЕН ТЕМПЕРАТУРАСЫНЫҢ ӘСЕРІН ЗЕРТТЕУ

Г.Қ. Құдайберген[1*], Г.М. Жуманазарова[2]
[1]Ұлттық биотехнология орталығы, Астана, Қазақстан
[2]Е.А. Букетов атындағы Қарағанды университеті, Қарағанды, Қазақстан
[*]E-mail: kudaibergen@biocenter.kz

Түйіндеме. *Кіріспе.* Полимерлі зерттеулердің көпшілігі бар полимерлерді жақсартуға немесе реттелетін қасиеттері бар жаңа биоматериалдарды жасауға бағытталған. Биологиялық үйлесімді полимер ретінде қосымша емдік әсерге ықпал ететін белгілі бір сегменттері бар табиғи полимерлер жиі қолданылады. Полимерлер әртүрлі әдістерді қолдана отырып өндірілуі мүмкін, соның ішінде коваленттi кросс-байланыс, динамикалық коваленттік кросс-байланыс, физикалық кросс-байланыс, криополимеризация, 3D басып шығару, электроспиннинг және т.б. Криополимерлеу бірегей әдіс болып табылады және басқа әдістерге қарағанда бірнеше артықшылығы бар. Криомұздату арқылы бастапқы мономерлердің концентрациясын және температураны өзгерту арқылы кеуектер мөлшері мен көлемін басқаруға болатын кеуекті құрылымды алуға болады. *Бұл зерттеудің мақсаты* сілтінің концентрациясы мен криополимерлену температурасының криогельдердің қасиеттеріне әсерін анықтау. *Бұл жұмыстың зерттеу объектілері* - желатин мен хитозан негізіндегі криогельдер. *Зерттеу әдістері.* Бұл зерттеуде физика-химиялық зерттеу әдістері қолданылды. *Нәтижелер мен талқылаулар.* Криогельдердің функционалды топтары ИҚ спектроскопиясы арқылы анықталды. Полимерлердің сорбциясы мен десорбциясын зерттеу нәтижелері берілген. Фосфат-тұзды ерітіндіде 8 апта ішінде полимер ыдырауының нәтижелері де көрсетілген. Бұл зерттеулер массаның өзгеруіне негізделген, яғни гравиметриялық әдіспен жүргізілді. *Қорытынды.* Желатин мен хитозан негізіндегі криогельдердің физика-химиялық қасиеттеріне сілтінің концентрациясы мен температурасының әсерін зерттеу нәтижелері берілген. Желатин мен хитозан негізіндегі криогельдер зиянды химиялық кросс-байланыстырушы агенттерді қолданбай синтезделеді, бұл оларды тіндік инженерия үшін тартымды етеді.

Түйіндеме сөздер: криогель, желатин, хитозан, қасиеттері, полимер, биополимерлер, криополимеризация, температура.

Құдайберген Гүлшахар Құдайбергенқызы	*PhD*
Жұманазарова Ғазиза Мұстафаевна	*докторант*

СИНТЕЗ И ИССЛЕДОВАНИЯ ВЛИЯНИЯ КОНЦЕНТРАЦИИ ЩЕЛОЧИ И ТЕМПЕРАТУР НА ФИЗИКО-ХИМИЧЕСКИЕ СВОЙСТВА КРИОГЕЛЕЙ НА ОСНОВЕ ЖЕЛАТИНА И ХИТОЗАНА

Г.Қ. Құдайберген[1*], Г.М. Жуманазарова[2]
[1]Национальный центр биотехнологии, Астана, Казахстан
[2]Карагандинский университет имени Е.А. Букетова, Караганда, Казахстан
[*]E-mail: kudaibergen@biocenter.kz

Резюме. *Введение.* Большая часть исследований в области полимеров сосредоточена на улучшение существующих полимеров или разработке новых биоматериалов с настраиваемыми свойствами. В качестве биосовместимого полимера чаще используются природные полимеры, которые имеют определённые сегменты, способствующие дополнительному лечебному эффекту. Полимеры могут быть получены с использованием различных методов, включая ковалентную сшивку, динамическую ковалентную сшивку, физическую сшивку, криополимеризацию, 3D печать, электроспининг и т.д. Криополимеризация является уникальным методом и имеет несколько преимуществ по сравнению с другими способами. Криозамораживанием можно получить пористую структуру, в котором размер и объем пор, возможно, регулировать, изменяя концентрацию исходных мономеров и температуру. *Цель данного исследования установить влияние концентрации щелочи и температуры криополимеризации на свойства криогелей.*

Объектами исследования данной работы являются криогели на основе желатина и хитозана. *Методы.* В данном исследовании были использованы физико-химические методы исследования. *Результаты и обсуждения.* Функциональные группы криогелей определены ИК-спектроскопией. Приведены результаты исследования сорбции и десорбции полимеров. Также показаны результаты деградации полимеров в фосфатно-солевом растворе за 8 недель. Данные исследования основывались на изменении масс, то есть были выполнены гравиметрическим методом. *Заключение.* Приведены результаты исследования влияния концентрации щелочи и температуры на физико-химические свойства криогелей на основе желатина и хитозана. Криогели на основе желатина и хитозана синтезированы без использования вредных химический сшивающих агентов, что делает их привлекательными для тканевой инженерии.

Ключевые слова: криогель, желатин, хитозан, свойства, полимер, биополимеры, криополимеризация, температура.

Кудайберген Гулшахар Кудайбергенкызы	*PhD*
Жуманазарова Газиза Мустафаевна	*докторант*

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