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PARAMETERS INFLUENCING THE PROPERTIES OF LAYERED SHAPED IONOTROPIC ALGINATE HYDROGELS DESIGNED FOR SOFT TISSUE ENGINEERING

D. DIMONIE[a], M. PETRACHE[a*], R. GABOR[a], I. TRANDAFIR[a], C. NICOLAE[a], M. DIMONIE[a], A. CIMPEAN[b], B. GALATEANU[b], E. VASILE[c], R. TRUSCA[c], G. JINESCU[d]

[a] *National Research and Development Institute for Chemistry and Petrochemistry, Bucharest, Romania*

[b] *University of Bucharest, Department of Biochemistry and Molecular Biology, Bucharest, Romania*

[c] *SC Metav-CD SA, Bucharest, Romania*

[d] *Spitalul Clinic de Urgenta Floreasca*

Considering the method of diffusive control migration of calcium ions, the alginate ionic cross-linking rate can be diminished till slow values (60 min. +) making possible the achieving of thin layer-shaped ionotropic hydrogels with controlled engineering and biological properties. According to this method, in order to control the cross-linker ions migration, the cross-linker salt solution is forced to pass, through a diffusion medium represented by a filter paper which is placed above the alginate solution. The obtained results prove that by choosing the pore size of the filter paper within 2.5 – 8 μm range and the calcium salt nature, the alginate can be cross-linked with slow rate till 150 min. – 300 min. The resulted hydrogels prove to have desired properties and enhanced biological performance as scaffolds for stem cell delivery and promising properties as scaffolds for adipose tissue engineering.

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1. Introduction

Successful biomaterials constructs for soft tissue engineering must support cellular adhesion, promote rapid vascular and tissue ingrowths throughout the implant site and induce a minimal fibrotic capsule and inflammatory response [1, 2].

In the presence of divalent cations (most often Ca^{2+}), sodium alginate can be ionically cross-linked through cations exchange with sodium ions on the G blocks binding together in this way adjacent chains which cause formation of an "egg" structure and polymer cross linking through a sol – gel transition [1, 2]. Because the resulted network is held together by secondary forces (ionic, hydrogen bonds or hydrophobic interactions) and/or molecular entanglements, the alginate hydrogel is a physical 3-D scaffolds [3, 4]. Since the feasibility of making ionotropic gels of alginate is simple and rapid, the alginate hydrogels have been extensively investigated as scaffolds for cartilage and bone regeneration [5-10]. The alginate hydrogel can be obtained by external [11 -14] and/or internal gelation [11, 12, 15-17]. The external gelation involves extrusion of droplets of sodium alginate-active agent solution into a calcium salt solution and in internal gelation, the calcium chloride solution is introduced into a sodium alginate one. Due to the very rapid and irreversible binding reaction between divalent cations and alginate, the direct mixing of the two components results in most often porous beads, rarely as homogeneous gels [18-22]. The only exception is if a low molecular weight alginate is mixed with low amounts of cross-linking ions at high shear, generally with the formation of weak hydrogels [16].

*Corresponding author: avy_marius2005@yahoo.com

According to [23] the gelling rate is fast if the cross-linking time is smaller than 5 min. If the gelling time is ranged between 20 and 60 min, then the cross-linking reaction develops with medium rate and if this time is by 60 min +, the crosslinking occurs with slow rate. Generally, ionotropic alginate hydrogels were obtained in 1 - 16 min at 20°C [25], 3 hours at 5°C [25] or very long time (76 h) at medium temperature [24].

In order to achieve layered shaped alginate hydrogels by controlling the cross-linking rate, the authors had proposed in [26 -29] a method to slow down the gelling rate through controlling the cross-linker ions migration. According to this method the calcium salt solution is forced to pass through a diffusion medium represented by a filter paper which is placed above the alginate solution (fig.1). The authors have demonstrated that by using this method, the alginate ionical cross-linking rate can be diminished till slow values (60 min.+) ensuring the achievement, under physiological conditions (37^0C, 3 h in oven), of homogeneous, elastic, manipulable, thin layer-shaped ionotropic hydrogels with interconnected pores, high porosity, and optimum pore size (fig.1) [26 -29]. The obtained hydrogels proved to have promising properties as scaffolds for adipose tissue engineering because of their enhanced biological performance as scaffolds for stem cell delivery in tissue engineering applications [28].

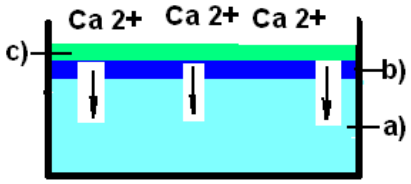


Fig.1 Schematic representation of diffusion controlled migration method for obtaining layer shaped alginate hydrogels (a – alginate solution; b – filter paper; c – salt solution which supplies the calcium ions)[26, 27].

This paper is devoted to studying the influence of the size of the diffusion medium pores on the engineering and biological properties of the alginate hydrogels obtained through diffusive control method.

2. Experimental

2.1 Hydrogel preparation

The hydrogels were achieved according to a previously developed method [26, 27, 29] by placing 1 ml aqueous solution of 1.5% alginate (Fluka –71240), in each of the 24 wells of culture plates. Then, rings of filter paper moistened with solution of cross-linker were layed over alginate solution. Subsequently, in each well was pipetted 1ml of calcium salt solution. The multi-well culture plate was kept, in oven, at 37°C, for 3hours. The calcium ions concentration was of 9.27g/l.

As diffusion medium, three filter papers with different pore sizes were used. As calcium ions supplier were used two calcium salts which have different dissociation constants [26, 27]: calcium gluconate (Zentiva) and calcium chloride (SIGMA Aldrich –C1016).

2.2 Hydrogel characterization

The obtained hydrogels were characterized as follows:

Cross linking time was estimated considering the time variation of stiffness, storage (G ') and loss (G ") module dependences[27] that were recorded on a DMA Q800 device, working in air at a frequency of 1.00 Hz and using cylindrical hydrogels of 2.3 – 4.3 mm thickness and: 23.55 mm diameter. Method: Frequency sweep; Procedure: Equilibrate at 37.00 °C; Isothermal for 300.00 min. Repeat segment 4 for 99 times. The averages and the standard deviations of triplicate values were reported.

Hydration degree was estimated considering the free water content revealed by the *heat to thaw* frozen hydrogels [31] (cooling / heating in the range -50 / + 70 ^{0}C in differential scanning calorimetry (DSC) measurements) and *to evaporate* the hydrogel water content - DSC

measurements: heating with 10^0 C/min. in the range 60^0C - 120^0C, air on a Mettler Toledo device, DSC8 23e type. Mean value of triplicate were reported

Porosity was expressed as the water loss at around 100 ºC measured by thermo-gravimetric analysis (TGA) [26, 29], performed on a Mettler Toledo device, TGA/SDTA851 type between 90 ^{0}C and 125 ^{0}C. Mean value of triplicate values were reported.

Pore micro – architecture The microstructure of hydrogels was analyzed considering the hydrogels diameter (D) and the aspect ratio (A) [33]. The pore diameter was calculated with the formula $D = \sqrt{l \times w}$, where *l* represents the longest distance across the pore in question (length) and *w* is the longest length across the pore perpendicular to *l* (width)[3] . The aspect ratio of pore was calculated with the relation $A = l/w^3$. Obviously, a value which is equals to 1 or very close to 1 would equate to a circular or square pore. The values lower or higher than 1 represent the pores of increasingly elliptical and greater values than one indicating cylindrical pores. The averages and standard deviations for 50 pore measurements in each case are reported. The statistical analysis was done with Origin Pro 8 software. For these measurements was used SEM micrographs registered on a Quanta INSPECT F device equipped with electron field emission gun - EFG with a resolution of 1.2 nm. The lyophilisation was performed in a CHRIST ALPHA 1-2 LD equipped within freezer, in the following conditions: main drying at -42.9 ° C and 0.091 mbar and finally drying at -43.2°C and 0.012 mbar.

Homogeneity represents the differences in the ratio of dry and wet weight of each subsequent slices in which the hydrogel with 3 mm thickness was transversally cut [30]. Each slice was weighed and dried at room temperature (about 22 ºC) till constant weight (W_{wet}) and then was immersed and maintained in water for 96 h when it was weighed again (W_{wet}). The dry weight was reported to wet weight. The equal values of the obtained ratios for each of the two slices cut from each hydrogel prove its structural homogeneity. At least three measurements were performed for each sample and the mean values and standard deviation (mean ± standard deviation) are reported.

Transport properties *The swelling degree* (SD) was calculated according to Eq. (1) where m_t is the weight of swollen hydrogel at a given time, m_o represents the hydrogel initial weight considering its time variation till swelling equilibrium represented by the plateau values of this dependence (constant m_t) [27, 32]. At least three swelling measurements were performed for each sample and the mean values are reported (mean ± standard deviation).

$$SD,[\%] = \frac{(m_t - m_0)}{m_0} \times 100 \qquad (1)$$

Elastic properties [26] were estimated based on the values of the storage modulus, loss modulus and stiffness which were recorded for 20 min. at 1 Hz, on a dynamo mechanical analysis (DMA) Q800 device in working conditions as in cross-linking time measurement.

Biological behavior: *3D cell culture model* - Murine preadipocyte cell line 3T3-L1 was provided by American Type Culture Collection and cultured in DMEM (Sigma Aldrich Co.) supplemented with 10% New Born Calf Serum (NBCS, Gibco) and 1% Antibiotic – Antimycotic (Sigma Aldrich Co) at 37°C in a humidified atmosphere and 5% CO2. To achieve the 3D culture, the cells were detached from the culture surface by treatment with trypsin-EDTA, centrifuged, counted and resuspended in 1.5% alginate solution at an initial density of 7x105 cells/ml. To produce alginate gel, two different gelling agents, 102mM calcium chloride and 95 mg/mL calcium gluconate, were used as follows: 1 ml of alginate cell suspension prepared as previously described was placed in each well of a 12-well culture plate and above it, a disc of Whatman filter paper with different porosities, soaked with gelling agents was added above it. After the addition of an equal volume of the respective gelling agent, the plates were incubated for 45-60 min in standard conditions of cultivation. The paper discs were removed and the resulted layer shaped constructs were washed with 0.9 % saline solution, covered with culture medium and subjected to incubation in standard conditions.

Investigation of cell viability and proliferation – The 3D culture systems were subjected to cell viability investigations after two and seven days of culture. In order to choose the proper porosity of the available Whatman filter papers used for the diffusion of the gelling agent (calcium gluconate) into the alginate cell suspension, fluorescent Live&Dead and spectrophotometric MTT assays were performed.

Fluorescent Live&Dead Assay is based on the simultaneous determination of alive and dead cells with two dyes which measure the recognized parameters of cell viability - intracellular esterase activity and plasma membrane integrity. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within alive cells, producing an intense uniform green fluorescence in these (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in the dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of alive cells. After two and seven days of culture 3D constructs were incubated for 10 min at dark with 2 μM calcein AM and 4 μM EthD-1solution and inspected using a fluorescence Olympus IX7 Microscope.

For MTT cell viability and proliferation assay, at two and seven days post-seeding the hydrogels were incubated for 24 hours in a 1 mg/ml MTT solution in culture medium. The second day, the purple formazan crystals were solubilised by incubating the hydrogels for two hours with isopropanol. The absorbance of the extracts was immediately measured at 550 nm on Thermo Scientific Multireader.

3. Results

3.1 Crosslinking time (induction period)

The alginate ionic cross-linking according to the diffusive control method is characterized by a systematic induction period which can be longer or shorter, depending on the calcium salt which supplies the cross-linker ions [26]. According to fig. 2, at gelling with calcium gluconate the cross-linking develops with slow rate (60 min. +) if the filter paper has pores of 2.5 μm (140 min.) or of 3.5 μm (145 min.) or with medium rate (20 – 60 min) if the filter paper has bigger pores of 8 μm (70 min.). At gelling with $CaCl_2$, the cross linking rate is always within the range of medium values regardless the nature of the calcium salt and the size of the filter paper pores (55 min. for 2.5 μm, 50 min. in case of 3.5 μm and 40 min. for 8 μm filter paper pores). In case of cross-linking with calcium chloride, the gelling rate is two – three times smaller than the values characterizing the cross-linking with calcium.

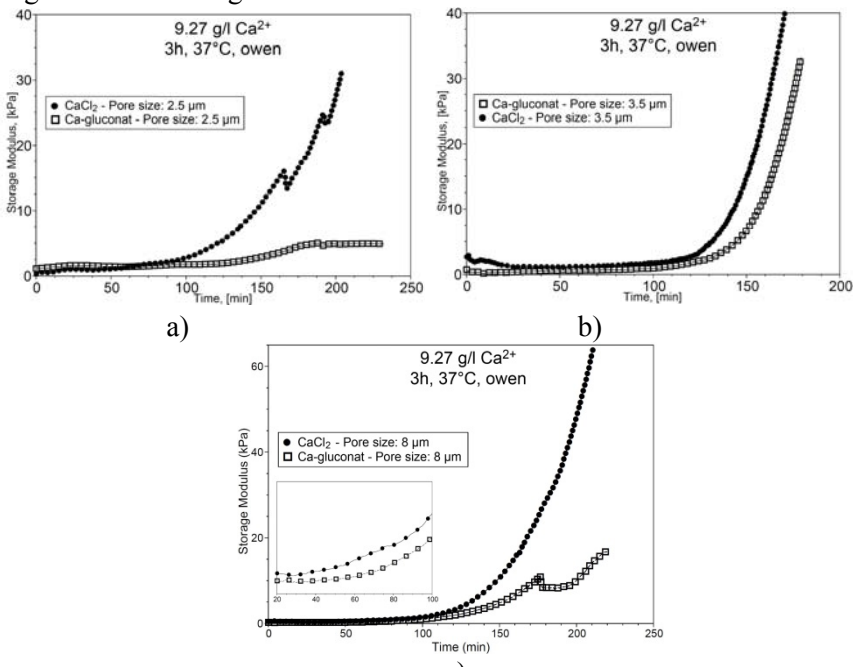


Fig. 2 Dependency of induction period on the size of the filter paper pores and the nature of the calcium salt(filter paper type: a-2.5 μm, b- 3.5 μm , c-8 μm pore sizes)

3.2 Hydrogel weight and diameter

The diameters and the weights of the hydrogels achieved with the three filter papers at cross-linking with calcium gluconate increase with decreasing the pore size of the diffusion medium (table 1). In all cases, the hydrogels diameters and weights are smaller if the cross-linker was calcium chloride.

Table 1 Dependency of the hydrogels weight and diameter on the pore size of the filter paper and calcium salt nature

Crosslinker \ Filter paper pore size	**8 µm**	**3,5 µm**	**2,5 µm**
$Ca(C_6H_{11}O_3)_2$	- Diameter[x]: 12.6 ± 0.5 - Weight[x] :1.21±0.17	- Diameter[x]: 13.4± 0.28 - Weight[x] : 1.23 ±0.17	-Diameter[x]: 13.5 ±0.36 - Weight[x] : 1.24 ±0.14
$CaCl_2$	- Diameter[x]: 8.78 ± 1.3 - Weight[x] :0.70 ±0.1	-Diameter[x]: 9.1±0.8 - Weight[x] :0.910±0.08	-Diameter[x]: 9.07±0.6 - Weight[x]: 0.920±0.1

[x] Mean value on three hydrogels (diameter - [mm], weight – [mg])

3.3 Hydration degree

If the filter paper has pores by 2.5 µm or by 3 µm, then the needed heat to thaw frozen hydrogels (thaw enthalpy) is higher than the thaw heat required for the hydrogels acquired with filter paper of 8 µm (red dot filter paper), especially at crosslinking with calcium gluconate (table 2).

Table 2 Dependency of thaw enthalpy and evaporation heat on pore's size of filter paper type and the cross-linker salt nature

Enthalpy \ Heat \ Filter paper pore size / Salt	**Thaw enthalpy, J/g (DSC Freeze – thaw method / -30 ^{0}C - + 50 ^{0}C)**			**Evaporation enthalpy, J/g (Heating with 10 ^{o}C / min / 20 ^{0}C - + 120 ^{0}C)**		
	2,5 µm	**3,5 µm**	**8 µm**	**2,5 µm**	**3,5 µm**	**8 µm**
$Ca(C_6H_{11}O_3)_2$	299,2±1,4	302,2±1,5	292,5±0,8	2153±1,2	2238±1,3	2047±1,2
$CaCl_2$	291,7±1,3	290,3±1,25	292,5±0,8	2074 ±1,7	2198± 1,8	1913±1,4

The heat required to evaporate the water content of the hydrogel depends both on the size of the filter paper pores and nature of the calcium salt (table 2). Thus, they have great and almost equal values in case of hydrogels achieved with filter papers with pores sized as 2.5µm or 3.5µm and calcium gluconate and smaller sizes at crosslinking with calcium chloride and filter paper with pores by 8µm.

These results show that the free water content of the hydrogels acquired with the filter paper having pores by 2.5 µm or by 3µm and calcium gluconate as ion cross-linker supplier is greater than the water content of hydrogels achieved with filter paper having 8 µm pores.

3.4 Porosity

The water loss around at around 100 ^{0}C (table 3) is almost the same in case of filter papers with pores sized as 2.5 µm or 3.5 µm and higher in case of filter paper with pores of 8 µm. If the cross-linker is calcium chloride then the water loss at around 100 ^{0}C is smaller than at cross-linking with calcium gluconate in all the three cases (2.5 µm, 3.5 µm and 8 µm). These results show that the greatest hydrogel porosity can be attained if the diffusion medium has small pores (2.5 µ – 3µ) and the cross-linker is calcium gluconate.

Table 3 Dependency of the thermo-gravimetric loss at around 100 ^{0}C on pore size of filter paper and calcium salt nature

Weight loss\ Salt \ Filter paper pore size	**Hydration degree % (Weight lost at around 100 ^{0}C, %)**		
	2,5 µm	**3,5 µm**	**8 µm**
$Ca(C_6H_{11}O_3)_2$	95,87±0,37	96.42± 0,4	93.58±0,34
$CaCl_2$	92±0,3	93.55± 0,33	89.02±0,27

3.5 Micro-architecture

The size and the shape of the pores of the hydrogels obtained with the three filter papers depend on their pore sizes (fig.3). If the diffusion medium has pore of 2.5 μm then 70% of the hydrogel pore population has the diameter ranged between 200 μm and 400 μm (fig.3d). If the filter paper has pores of 3.5 μm then 50 % of hydrogel pores have diameter within the range 100 – 200μm and 30% between 200μm and 300μm (fig.3e). In case of the filter paper with pores of 8 μm, 80% of the pores have the diameter ranged as 100–250 μm (fig.3f). The hydrogels pore diameter is greater as the pore size of the filter paper is lower. In case of the filter paper with pores of 2.5 μm the hydrogels medium diameter is around 285.68 ± 95.75μm, value which becomes 230.76 ± 86.6 μm for the filter paper with 3.5μm and 189.03 ± 57.46μm for filter paper with pores of 8 μm.

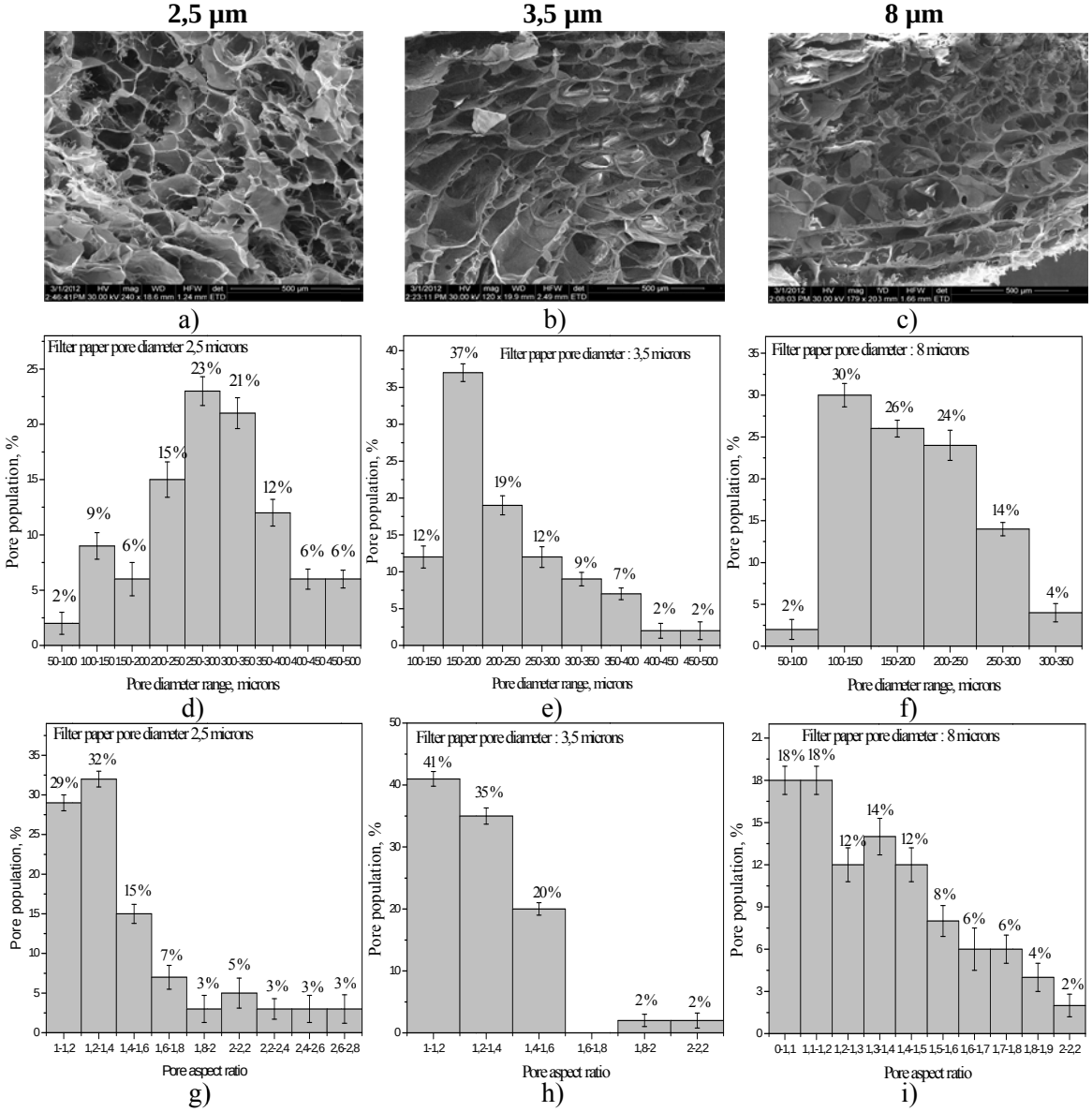


Fig.3 The pore micro-architecture of the alginate hydrogels obtained with the three filter papers and calcium gluconate (SEM morphologies a – 2.5 μm; b – 3.5 μm; c – 8 μm; Distribution of the pore population as a function of diameter d – 2.5 μm; e – 3.5 μm; f – 8 μm ; Distribution of the pore population as a function of aspect ratio values (g – 2.5 μm; h – 3.5 μm; i – 8 μm)

For the hydrogels achieved with calcium gluconate and the three filter papers, the aspect ratio (A) varies almost within same range: 1.01–2.66 for filter paper with pores of 2.5 μm , 1–2.2 in case of those with pores of 3.5 μm and 1.03 – 2.03 for filter paper with pores of 8 μm . The

hydrogels achieved with the three filter papers contain pores generally similar in shape, mainly spherical, but cylindrical too, especially for filter paper with pores of 8 μm. In this case more than 80 % from each of the three pore populations have A ranged between 1.01 and 1.8. The mean values of the aspect ratio of the hydrogels made with the three filter papers are almost around 1 (*2.5* μm - fig.3g, *3.5* μm – fig.3h, *8* μm – fig.3i)

2.6 Homogeneity

The differences between the homogeneity index in the top and the bottom part of each hydrogels acquired with the three filter papers and the two calcium salts are ranged between $2 \cdot 10^{-5}$ and almost $10 \cdot 10^{-4}$, values which demonstrates the homogeneity degree of the hydrogel morphology (fig.4).

The homogeneity index has roughly same values for the hydrogels achieved with the same cross-linker salt and the three filter papers but different values depending on the calcium salt type. The homogeneity indices of the hydrogels obtained with calcium chloride are by 12 - 16 % smaller than the values acquired when using the calcium gluconate as cross-linker ions supplier.

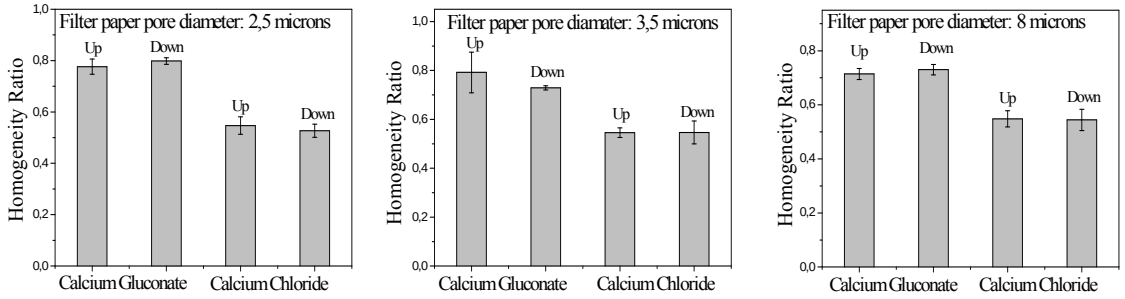
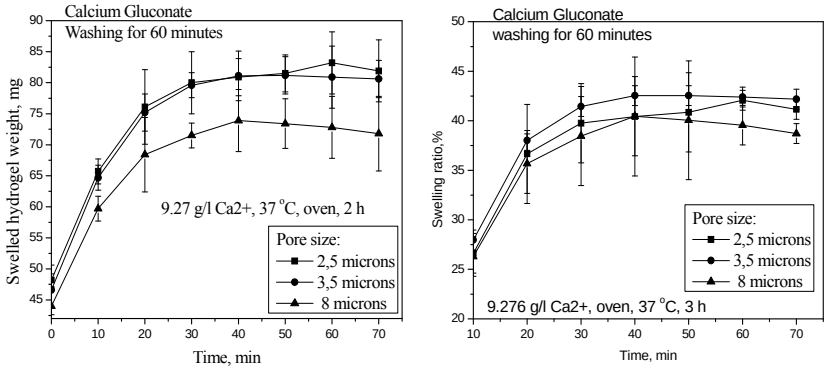


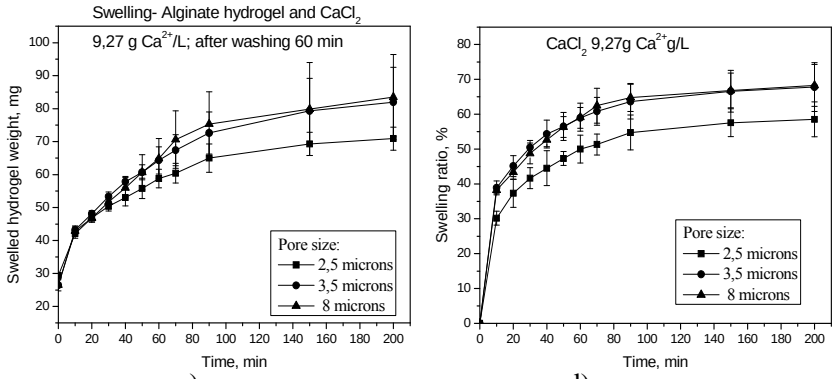
Fig. 4 The homogeneity of the hydrogel obtained with different filter papers and cross-linker salts

3.7 Transport properties

The time variation of the transport properties of the studied hydrogels are presented in (Fig.5 a-d). The weight at swelling equilibrium of the hydrogels obtained with calcium gluconate and filter papers with pores of 2.5 μm or 3.5 μm is reached in almost 25 min and is approximately 15 % greater than the equilibrium weight of the hydrogels achieved with filter paper with pores of 8 μm (fig.5 a). When $CaCl_2$ was used as cross-linker, the gelling runs with some differences as follows: the equilibrium is reached after 160 min., and the equilibrium weights of the hydrogels obtained with the three filter papers are almost half of the weights resulted at gelling with calcium gluconate (45 – 47 mg in case of $CaCl_2$ and 73 – 83 mg when calcium gluconate was used). At crosslinking with calcium chloride, the differences between weights of the hydrogels obtained with the three filter papers are not very evident (Fig.5c).



a) b)



c) d)

Fig.5 Transport properties dependency on the swelling time, filter paper type and cross-linker salt nature (calcium gluconate -a,b; calcium chloride - c,d ; hydrogel weight; b, d – swelling ratio)

3.5 Elastic properties (stability in dynamic conditions)

If the crosslinking is performed with calcium chloride then, not depending by the filter paper type, the deformation capacity of the new appeared network, are greater as in case of calcium gluconate (table 4). This property is reflected by the storage modulus values. If the hydrogels were obtained with filter paper having large pore size (8 μm), then the storage modulus at cross-linking with calcium chloride is almost twice higher than in case of using calcium gluconate ($CaCl_2$ - 13290± 411 Pa ; $Ca(C_6H_{11}O_3)_2$ - 7500± 200 Pa). Stiffness is not a propriety that depends very much on the obtaining conditions. The viscous dissipation reflected by loss modulus has greater values in case of the networks created with calcium chloride.

*Table 4 DMA for the hydrogels achieved through diffusion of **$CaCl_2$** or calcium **gluconate** $Ca(C_6H_{11}O_3)_2$ through filter papers with different pore size*

Property / Filter paper pore size	**$CaCl_2$ (calcium chloride)**			**$C_6H_{11}O_3$ (calcium gluconate)**		
	Storage modulus, Pa	**Loss modulus, Pa**	**Stiffness, Pa**	**Storage modulus, Pa**	**Loss modulus, Pa**	**Stiffness, Pa**
2,5 μm	6000± 234	500± 151	198± 25	4250± 120	738± 78	264± 15
3,5 μm	7000± 321	600± 75	200± 75	5570± 130	820± 67	390± 65
8 μm	13290± 411	2390± 321	440± 32	7500± 200	920± 101	450± 87

The obtained results demonstrate that the smaller the pore size of the filter paper, the smaller are the resulted dynamo - mechanical properties, especially in case of calcium gluconate. All the obtained hydrogels keep their shape in the studied dynamo –mechanical conditions (1 Hz, 20 min.)

3.6 Biological behaviour

Fluorescent Live&Dead labeling of murine 3T3-L1 preadipocytes embedded in the alginate based constructs prepared by the diffusion of calcium gluconate through filter paper with different porosities, revealed that the cellular viability increased with the decrease of the paper pores dimensions. As shown in fig. 6, the filter paper displaying pores of 2.5 μm is the most suitable for maintaining the preadipocyte viability.

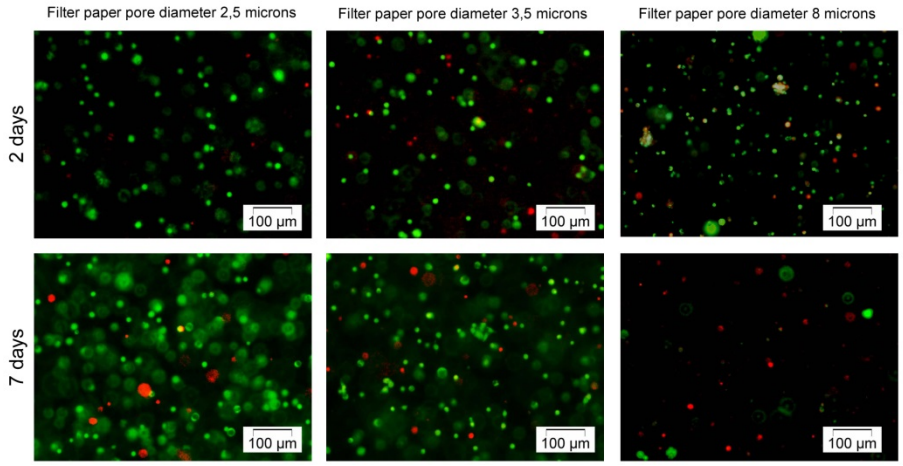


Fig.6 Fluorescence microscopy micrographs of embedded 3T3-L1 preadipocytes in alginate based hydrogels prepared by the diffusion of calcium gluconate through filter papers with different porosity, at two and seven days of culture

The spectrophotometric quantification of cell viability has confirmed the fluorescence microscopy observations and provided quantitative information (fig.7) about the behavior of the preadipocytes over the incubation time.

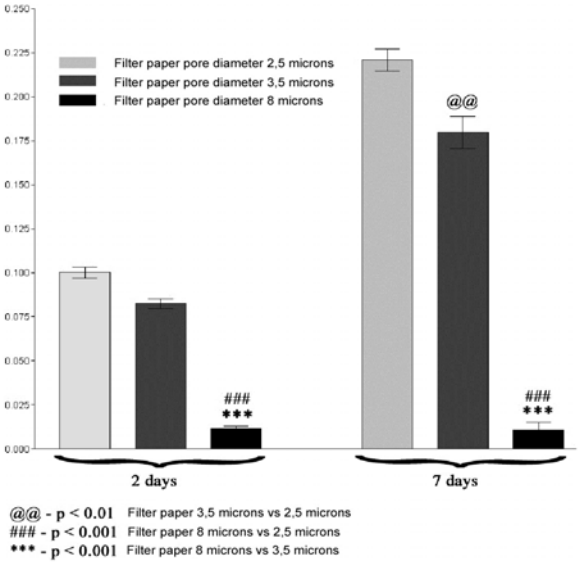


Fig. 7 Viability and proliferative potential of 3T3-L1 cells embedded in the layer shaped alginate hydrogels prepared with filter papers displaying pores of 2.5, 3.5 and 8 µm, at two and seven days of culture

After two days of culture, the cells embedded in the construct obtained by diffusion of the reticulating agent through filter paper with pores of 2.5 µm showed an increased viability by 18.32% and 88.44% in comparison with the ones prepared by using filter papers with pores of 3.5 mm and 8 µm, respectively. The highest level of viability was observed at seven days post-seeding in the same hydrogel, keeping almost the same proportion regarding the other two samples, namely 19.45% and 92.76%, respectively. The proliferation profile of 3T3-L1 cells embedded in the layer shaped alginate hydrogels was also evidenced. After seven days of culture, it was noticed an increase in the number of metabolically active cells by 54.57%, 53.9% and 27.5% for the cells embedded in hydrogels reticulated by diffusion of calcium gluconate through filter papers of 2.5, 3.5 and 8 µm hydrogels, respectively, as compared with two days-culture. Taken together, the above data show that the viability and cell proliferation in hydrogels obtained by diffusion of calcium gluconate through filter papers with pores of 2.5 µm and 3.5 µm is double compared with the situation of hydrogels obtained with filter paper pore size 8 µm. It might be estimated that the

main parameters which control the viability and cell proliferation in this type of hydrogel are the crosslinking density and the diffusion speed of the cross-linker salt.

4. Discussions

The main characteristics of the alginate gelling by diffusive control method are determined by the mechanisms which generate the calcium ions inside the reaction medium. The diffusion controlled migration of Ca ions by forcing the cross-linker ions to pass through a filter paper with controlled porosity corresponds to a time and space non-stationary process controlled by the type of the salt which supplies the cross-linker ions [26] and, according to the new results presented above, by the pore size of the diffusion medium.

The gelling performed by diffusion controlled migrations of calcium ions runs with an induction period which corresponds most likely to the time needed for the calcium ions to pass through the filter paper and other barriers formed during cross- linking [29]. The movement of the calcium ions towards inside of the sodium alginate solution is controlled by the concentration gradient between the upper and lower sides of the wells from culture plates in accordance with the Fick's first law. This gradient decreases in time according to the Fick's second law.

Depending on the pore size of the filter paper, the concentration of the calcium ions which passes toward the reaction medium can be greater or smaller. Considering the same initial calcium ions concentration, the local concentration in the reaction medium of calcium ions, at a given time, will be higher in case of the diffusion through filter paper with pores of 8 μm and smaller for filter paper with pores of 2.5 μm.

The calcium ions result from the dissociation of $CaCl_2$ and calcium gluconate. These two salts have different dissociation constants [26, 27], which further controls the concentration of calcium ions existing at a given time, in the reaction medium. The calcium gluconate has the structure of a chelate complex, being soluble in water and partially dissociated at equilibrium. The equilibrium is shifted to the left (non dissociated form). The CaCl2 salt is a strong electrolyte that totally dissociates, providing suddenly all the Ca^{2+} ions [26, 27]. For this reasons, considering the same filter paper as diffusion medium and the same initial concentration of calcium ions, at a given time, the number of calcium ions coming from the two salts is different, higher in case of calcium chloride and smaller for calcium gluconate.

These differences are reflected by the kinetics of gel formation which was studied considering the time variation of dynamic-mechanical properties between the starting point of cross-linking and its stabilization. When the calcium ions quantity is smaller, as in case of filter paper with pores of 2.5 μm and calcium gluconate, then the induction period will be longer compared with the situation of usage of filter paper of 8 μm and calcium chloride when this quantity will be greater. The reticulation degree (RD) of the hydrogels obtained with a small quantity of calcium ions will be smaller than the RD of the hydrogels achieved with a higher quantity of calcium ions. Therefore, the final RD of the hydrogels will depends on the used filter paper and calcium salt nature.

The micro – architecture is also controlled by the hydrogel RD, a high RD meaning pores of small sizes and small RD denoting pores of large size. The differences between the weights and diameters of the hydrogels obtained with different filter papers and the two calcium salts have the same explanation. At low RD, the hydrogel diameter and weight will be greater because of the higher hydration degree comparatively with the hydrogels with high RD which will have a smaller hydration degree.

The hydration degree of hydrogels is a function of many parameters such as polymer composition, pH, ionic strength, but also the cross-linking density. A high hydration degree means high hydrogel porosity [34–39]. Increased porosity has a beneficial effect on the diffusion of nutrients and oxygen [40]. Increased porosity and great pore interconnectivity have a beneficial effect on vascularisation and on the diffusion of nutrients and oxygen required for the survival of cells. The different porosity which characterizes the hydrogels obtained with the three filter papers and the two salts is determined also by the different values of the RD as a consequence of the dependence of the calcium ions concentration from the reaction medium on the filter paper type and the calcium salt nature.

The elastic properties of the hydrogels obtained with filter paper with pores of 8 μm and calcium chloride are higher than those of the hydrogels achieved with filter paper with pores of 2.5 μm and calcium gluconate also due to the control of the cross-linking degree by the cross-linker ions quantity from the reaction medium. The transport properties will be more favourable to the cells growth if the hydrogel RD will be smaller as in case of hydrogels obtained with filter paper with pores of 2.5 μm and calcium gluconate.

The slowly reaching of the swelling equilibrium in case of $CaCl_2$ is probably a consequence of the differences in micro-architecture in comparison with the hydrogels prepared in the same conditions but with calcium gluconate (homogeneity index for calcium chloride is smaller than that of calcium gluconate).

The viability and cell proliferation into the hydrogels obtained by diffusion of calcium gluconate through filter paper with pores of 2.5 μm / 3.5 μm is double compared with the situation in which the hydrogels were made with filter paper with pores of 8 μm. The reason for this differential cell response could be the difference in the reticulation degrees of these hydrogels. The observed variations between the engineering and biological properties can be large if the differences between the pores sizes of the diffusion media would be greater.

5. Conclusions

The presented results are new evidences that, based on the controlled diffusion method, the gelling rate of alginate with divalent cations can be diminished till slow values. Depending on the pore size of the diffusion medium and the nature of the calcium salts which supply the cross-linker ions, the gelling rate could be diminish till 150 min. – 300 min. if the size of the filter paper pores is ranged between 2.5 μm and 8 μm.

By controlling the diffusion medium porosity, the engineering and biological properties of the alginate hydrogels can be designed for being used as 3D scaffolds in adipose tissue engineering.

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