Digest Journal of Nanomaterials and Biostructures Vol. 8, No. 1, January - March 2013, p. 11 - 24

MODULATION OF IONOTROPIC ALGINATE HYDROGEL MICROARCHITECTURE BY CONTROLLING THE CROSSLINKER IONS MIGRATION

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Among the various basic requirements for scaffold manufacturing, controlled 3D pore architecture is the critical manufacturing criterion. Traditional hydrogel micro-architecture is characterized in terms of bulk porosity and bulk porosity homogeneity, pore size, poreshape, pore interconnectivity and alignment. These parameters play a significant role in cell survival, proliferation and migration to fabricate functional hydrogel, and secrete ECM with needed strength and structure. The article analyzes the possibility to control the pore architecture of ionotropic alginate hydrogels that were designed for adipose tissue regeneration by controlling the crosslinking rate in two different ways.The new hydrogels were obtained by crosslinking in physiological conditions, in oven, for 3 hours, at 37 ^{0}C. The obtained results based on the statistical analysis of the pore micro-architectureof the hydrogels thus obtained,prove that controlling the alginate crosslinking rate is an important route that enables the obtaining of ionotropic alginate hydrogels for adipose tissue engineering with tunable morphology and desired pore micro-architecture.

(Received December 11, 2012; Accepted December 18, 2012)

Keywords: Ionotropicalginate hydrogel, Soft tissue, Crosslinking rate, Ions migration, In situ chemical generation, Diffusion controlled method, Internal gelling

1. Introduction

Highly hydrated hydrogels based on natural and / or synthetic polymers with physical and chemical properties similar to those of natural extra cellular matrix (ECM), are ideal 3-D supports for cells survival, proliferation and differentiation in tissue engineering [1-7]. Properties that make possible the use of hydrogels as 3-D scaffolds in tissue engineering are biocompatibility, biodegradability, high porosity, mechanical and transport properties similar to living tissue, high water content, minimal invasive effects on natural tissue during in vivo testing [5 – 7].

Sodium alginate is of interest for use as 3-D scaffolds for cell survival, proliferation and differentiation due to its material properties like: biocompatibility, lack of toxicity, easy accessibility, and lower price compared to most natural biodegradable polymers[8]. The alginate hydrogels are also easy wettable, allowing more efficient cells penetration into the polymeric matrix [9]. In addition, potential disadvantage of low cellular adherence to the alginate 3-D sscaffold can be very much diminished by grafting or by incorporating bioactive species. For this reason the studyof alginatebased hydrogelsstill presents scientific and practical interest.

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Sodium alginate hydrogels are widely used as 3-D scaffolds for bone and cartilage tissue engineering[10], drug delivery [11], vehicles for biologically active molecules [12], cell encapsulation for immunoprotections, gene delivery, wound dressing, dental impression materials and so forth. Several therapeutic agents including antibiotics, enzymes, growth factors, DNA, etc have already been successfully incorporated in alginate hydrogels [13-15]. The mechanical strength of ionicallycrosslinked alginate hydrogels increases when the ion concentration is enhanced and when divalent ions that have a higher affinity for alginate are used for crosslinking [3]. Thus the crosslinking density, mechanical properties and pore size of the ionically crosslinked hydrogels can be manipulate.

Among various basic requirements for scaffold manufacturing, controlled 3D pore architecture is the critical manufacturing criterion. Traditional micro-architecture has been characterized in termsof bulk porosity and homogeneity of the bulk porosity, pore size, pore shape,and poreinterconnectivity and alignment [16-19]. These parameters play a main role in cell survival, proliferation and migration to fabricate functional hydrogel, and secrete ECM with needed strength and structure [20-22].

An increased porosity and great pore interconnectivity have a beneficial effect on vascularisation and on the diffusion of nutrients and oxygen for cells survival [19, 23]. Higher gel porosity can facilitate cell migration and population of the 3 - D matrices and hence it is a desirable feature for tissue engineering applications. The porosity degree has substantial effect on the mechanical properties with the stiffness of the scaffold decreasing as porosity increases [24]. The average pore size greatly affects the growth and penetration of cells into the hydrogel 3D structure. By controlling pore sizes it is possible to regulate the exchange of nutrients and waste products of cells [25]. The bulk porosity of the scaffolds plays an important role in directing tissue formation and function [26]. Homogeneous scaffolds porosity is necessary to allow for homogeneous cell distribution and interconnection throughout engineered tissues [27, 28]. Hydrogels homogeneity ensures structural integrity, uniform porosity throughout the scaffolds and thus uniform cells distribution.

The article analyzes the possibility to control the pore architecture of ionotropic alginate hydrogels that were designed for adipose tissue regeneration by controlling the crosslinking rate in two different ways. The article examines the dependency of the hydrogels micro - architecture with the obtaining method and with the reaction conditions. The results are compared to those that characterize the micro-architecture of hydrogels achieved by internal gelling, a classical well known method of sodium alginate crosslinking with bivalent cations.

2. Experimental

The ionotrope alginate hydrogels were obtained in physiological conditions, in oven, for 3 hours at 37 ^{o}C, using the following methods to control the crosslinking rate:

*In situ chemical generation, in simultaneous reactions method*was realised by crosslinking the sodium alginate (Fluka – 7120) with the $CaCO_3$(Scharlow) – Glucono δ lactone (DGL) (Merk). Well-defined amouns of reactans were mixed together. The reaction product, with homogeneous appearance, was pipetted into wells of a 24 Well Multiwell culture plate. The plate was maintained for 3 hours at 37 ^{o}C, in oven

*Diffusion controlled migration of calcium ionsmethod*was realisedby crosslinking of sodium alginate with calcium gluconate (Zentiva).In order to avoid uncontrolled mixing and to ensure the slow flow of the calcium ions towards the reaction medium from the multi-well culture plates, the crosslinker was passed through a filter paper with selected porosity of 2-3 μm, before reaching the alginate solution. Thus the hydrogels were obtained by placing 1 ml aqueous solution of 1.5 % sodium alginate into each well of the24-well Multiwell culture plates. After that blue band ring-shaped filter papers moistened with cross-linker solution were placed in between the cross-linker and alginate solution. Subsequently, in each well 1 ml calcium gluconate solution was pipette (Fig.1) and then the plate was maintained for 3 hours at 37 ^{o}C, in oven.

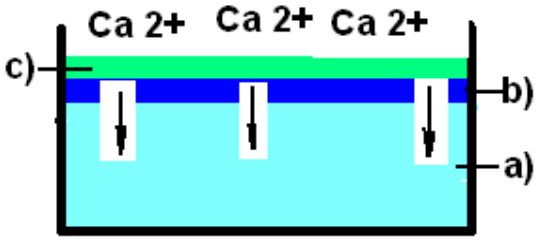


Fig.1 The schematic representation of the diffusion controlled migration method for obtaining layer shaped alginate hydrogels (a – alginate solution ; b – filter paper; c – supplier of calcium ions).

Internal gelling method was realised by dropping an aqueous solution of 0.1 % $CaCl_2$ (Scharlaw) solution in aqueous solution of 1.5 % sodium alginate (classical method).

The experiments were accomplished at similar alginate and calcium ion concentrations, respectively. In all experiments, the same alginate type and the same cross-linking conditions were used. For all obtained hydrogels, the freeze-drying conditions during lyophilisation were identical. In other words, all parameters which, according to the literature, might have affected the results were held constant. A special attentionhas been paid to the reproducibility of results and their statistical analysis.

Pore architecture was studied by comparative analysis of pore shape, connectivity degree, pore diameter range, mean diameter values, pore aspect ratio range and aspect ratio mean values. Hydrogels micro-architecturewas analyzed using a scanning electron microscope Quanta INSPECT F equipped with electron field emission gun - EFG with a resolution of 1.2 nm was used. Lyophilisation was performed in a CHRIST ALPHA 1-2 LD plus 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. It was consideredthe hydrogels diameter (D) and the aspect ratio (A). The pore diameter was calculated by using 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] [16]. The aspect ratio of pore was calculated by means of the relationship $A = l/w^3$ [16]. Obviously, a value which equals to 1 or very close to 1 would equate to a circular or square pore. Values lower or higher than 1 represent the pores of increasingly elliptical (oval) nature (rectangular pores) [16]. For each analysed hydrogel 50 pore measurement are reported. The statistical analysis was done with Origin Pro 8 software.

3. Results

3.1 Influence of the obtaining method

In all three cases the hydrogels obtained have open-cell architecture with interconnected pores (Fig.2). The main pore population has different diameter range depending on the obtaining methods. 76 % from the pore population of the hydrogels obtained by in situ chemical generation method have diameter ranged between 20 μm and 120 μm (Fig.2b). In case of the hydrogels obtained by diffusion controlled method 71 % from pore population have diameter ranged between 200 μm and 400 μm (Fig.2e). If the hydrogels were obtained by internal gelling method, 80 % from pore population have diameter ranged between 20 μm and 60 μm (Fig.2h).

The same significant differences in terms of pore shape dependence on the used crosslinking method were recorded. For in situ chemical generation method, the mean aspect ratio is 1.3654 ± 0.32 with a variation range of 1.03 - 2.91. 89% of these pores have aspect ratio ranging between 1.03 and 1.6 which means that these pores are almost spherical in shape (Fig.2c). For hydrogels obtained by physical controlled method, the mean aspect ratio is 1.41 ± 0.37. 76% of

these pores have aspect ratio between 1.01 and 1.8meaning that these pores are spherical and rectangular in shape (Fig.2f).The mean value of the pore aspect ratio of the hydrogels made by internal gelling is 1.05 ± 0.3 (0.78 - 1.05 variation range) meaning that these hydrogels are predominantly circular in shape (Fig.2 i).

If the range and mean diameter values for the hydrogels obtained by the 3 methods are compared, major differences can be observed (Fig. 3a). The diameter range is between 14 μm and 140 μm for hydrogels obtained by in situ chemical generation of calcium ions method, by 63 μm to 500 μm for hydrogel obtained by physical diffusion controlledmethod and between 3 μm and 75 μm for the hydrogels obtained through internal gelling. If the mean pore diameter obtained by internal gelling is 35 ± 7 μ, those of the hydrogels obtained by in situ chemical generation method is 77 ± 54 μ and those of the hydrogels achieved by diffusive controlled method is 296 ± 96 μm. This means that by physical control method, pores with mean diameter eight times higher that the pores diameter achieved in case of internal gelling and approximately 4 times greater if in situ chemical generation method is used as hydrogels obtaining method.

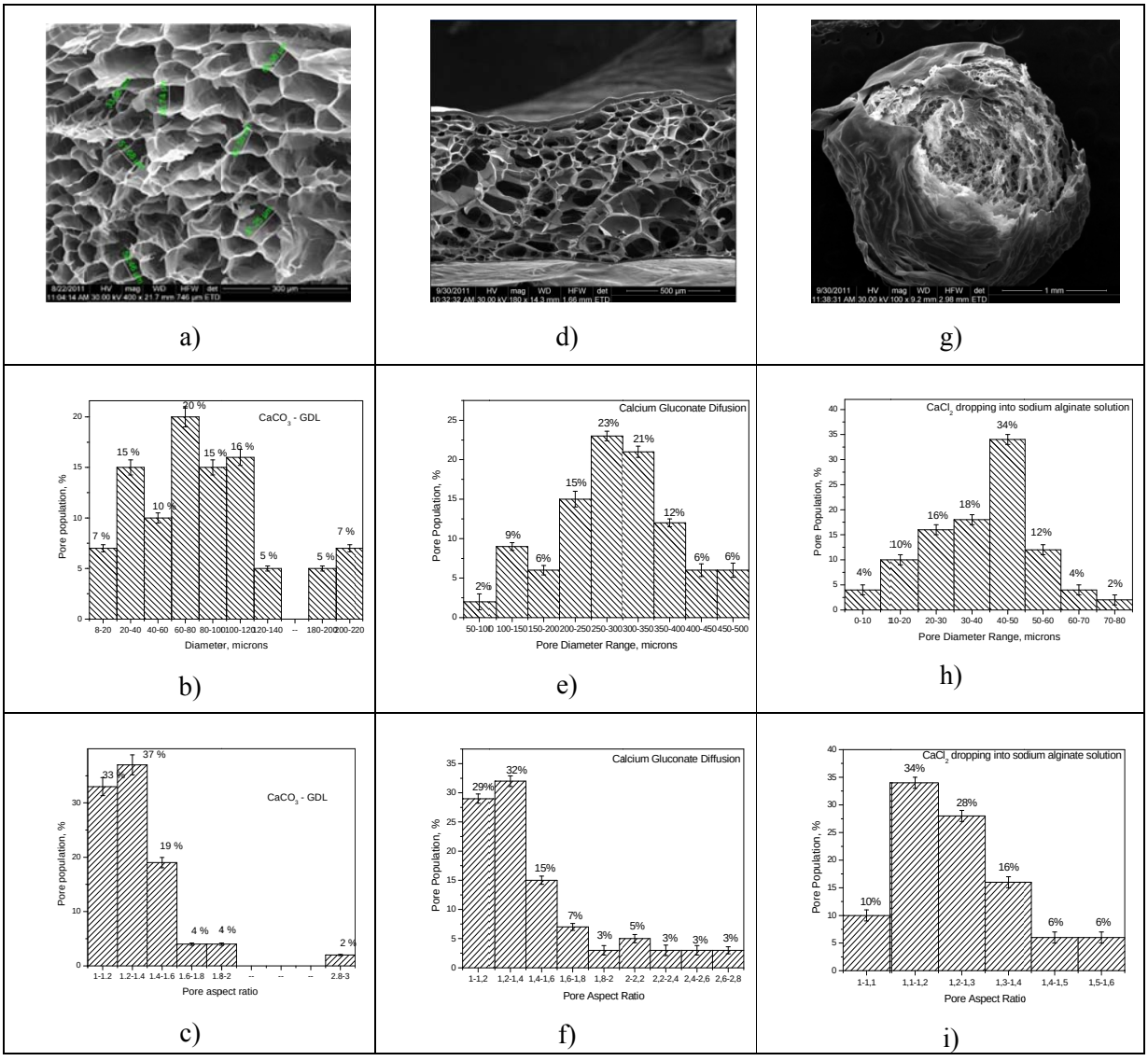
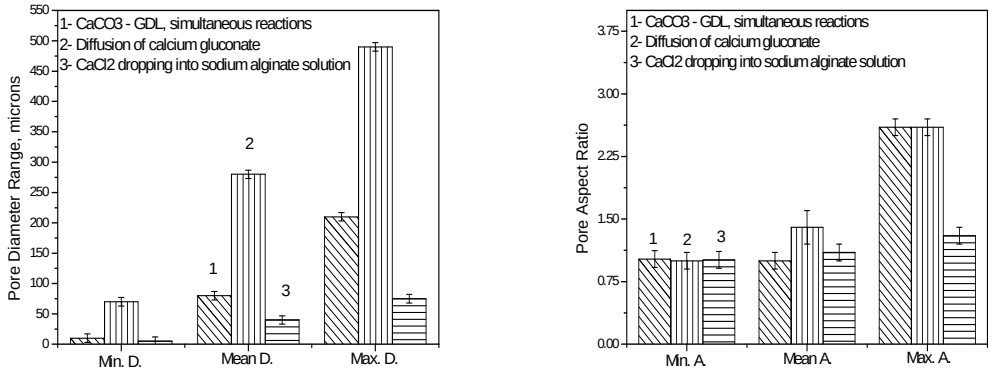


Fig. 2 Dependence of the hydrogels micro-architecture by the obtaining method: In situ chemical generation method (a – c), Diffusion controlled method (d – f), Internal gelling (g – i). Pore population distribution versus diameter (b, e, h) and aspect ratio (c, f, i) range (Pore measuring direction: Length: Left to right; Width: Up to down)



a) b)

Fig. 3 Dependence of the range and mean value of diameter (a) and aspect ratio (b) by the hydrogel obtaining method.

3.2 Influence of the hydrogel heightobtained by diffusive control method

3.2.1 Hydrogels with intermediate height (aprox.4 mm)

The cross section of this hydrogel is presented in fig.4a. The upper part of the hydrogel that has been in contact with filter paper (Fig.4b) contains pores with maximum diameter of 200 μm, from which approx.48% are below 100 μm (Fig.4c). Approx.48% of these pores have length 2-4 times greater than width and approx. 16% length greater from 4 to 14 than width (aspect ratio ranged between 4 to 14) (Fig.4d). In the bottom part the hydrogel (fig.4e) contains approx. 56% pores with diameters between 100 and 200 μm and approx. 34% pores with very large diameter ranged between 200 and 400 μm (Fig. 4f). 24 % of the pores in this area are spherical in shape because the aspect ratio is ranged between 1 and 1.4. There are also rectangular pores with 1.4 – 4 times length greater than width in a proportion of about 61%. It can be concluded that this type of hydrogels contains elongated and narrow pores in the upper area and pore with big diameter up to 400 μm in the lower area.

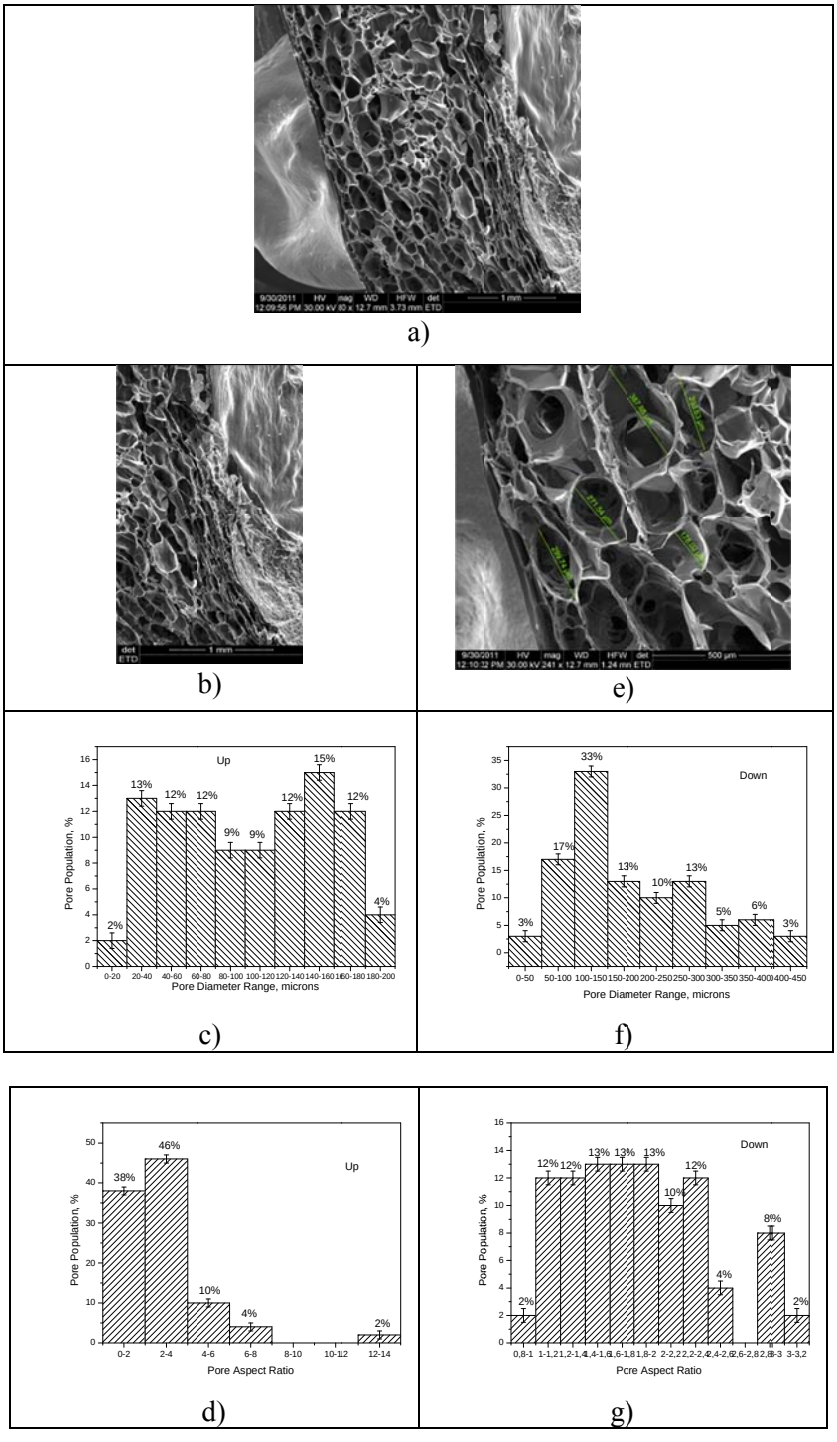
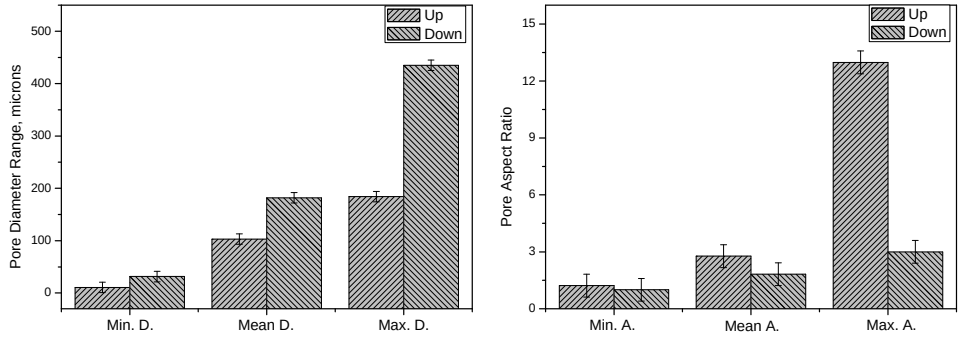


Fig. 4 General cross section (a) and cross section of top (b) and bottom (b) part of the hydrogel with medium height (4 mm) Pore population distribution against diameter (c – top, f – bottom) and aspect ratio (d – top, g – bottom)(Pore measuring direction: Length: Up – down; Width: Left – Right)

There are obvious differences between the diameter and aspect ratio range in the two significant areas of the hydrogels (Fig.5). The mean diameter in the upper area of hydrogel is 200 µm and at the bottom of hydrogel the mean diameter is 100 µm. Also the maximum diameter is double in the upper area as compared to the lower one (approx.400 µm against 200 µm). Differences in aspect ratio values are mainly related to maximum values in the lower hydrogel area (14 in the hydrogel bottom against 3 in the top area).



a) b)

Fig. 5 Pore population distribution versus diameter (a) and aspect ratio range (b) in the top (up) and bottom (down) area of the hydrogel with medium height

Although these hydrogels have interconnected pores withinteresting shape and size for tissue engineering we still cannot make a recommendation for their use for cell culture. Due to the differences between pore size diameter and aspect ratio from upper and lower area of the hydrogels it can be concluded that the bulk porosity of these hydrogels is strongly irregular. The reaction conditions which have led to such hydrogels present no interest for tissue engineering.

3.2.2 Thin hydrogels (2.5 mm – fig.6)

This hydrogel (Fig.6 a) contains pores with diameter up to 200 μm (from which 34% are ranged between 100 and 200 μm (Fig.6 c)) at its upper part which was in contact with the diffusion medium (Fig.6 b). Because the aspect ratio that characterizes the pores from this part is ranged between 1 and 1.5 it can be said that 50% of the pores in this area are almost spherical (Fig.6d). In the lower part pores have also diameters up to 200 μm of which 66% ranged between 100 and 200 μm (fig. 6f). 52% of the pores in this area are almost spherical because the aspect ratio is between 1 and 1.5 (Fig. 6g). Another percentage of about 42% are elliptical pores having almost twice the length greater than the width. There is a small percentage of pores of approx. 6% with length 2 – 3.5 times greater than width (fig.4 g). From table 1 and fig.7 can be observed that the minimum, maximum and mean values of diameter (Fig. 7a) and aspect ratio (Fig.7b) have almost the same values in the hydrogel upper and lower areas. Since micro-architecture features of these hydrogels have approximately similar characteristics in all areas it can be concluded that these hydrogels have homogeneous bulk porosity.

Table 1 The range and mean values for pore diameter and aspect ratio of the hydrogel with thin height

Zone \ D, A	Values							
	Diameter, microns				Aspect ratio			
	Min	Max	Mean	DS	Min	Max	Mean	DS
Up	38.48	187.65	90.61	40.33	1	2.53	1.39	0.31
Down	41.39	213.9	123.54	48.27	1.02	3.42	1.53	0.41

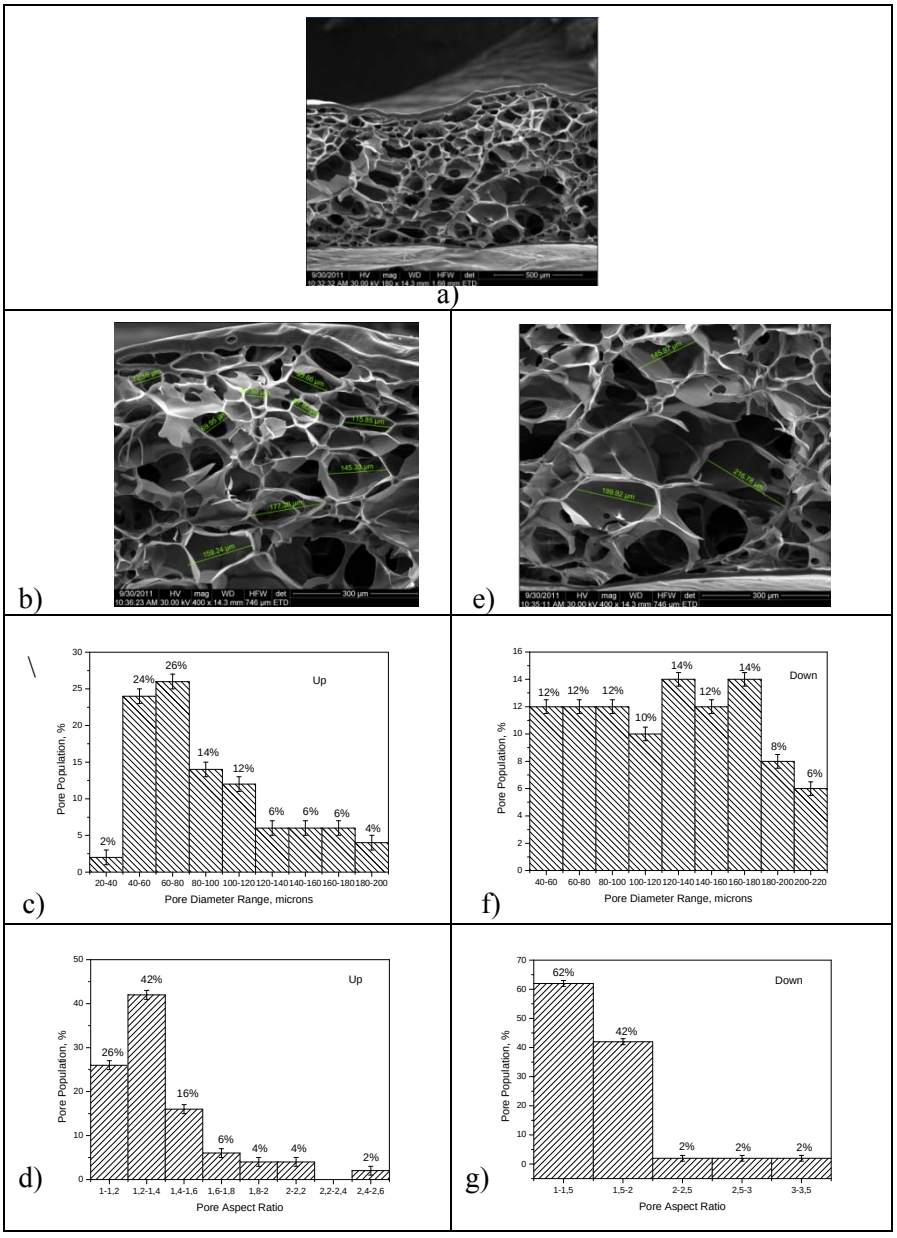
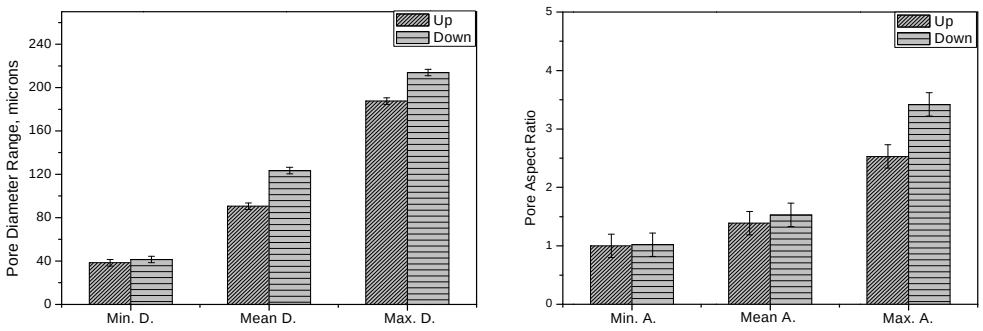


Fig. 6 General cross section (a) and cross section of top (b) and bottom (b) areas of the hydrogel with thin height (2.5 mm). Pore population distribution versus diameter (c – top, f – bottom) and aspect ratio (d – top, g – bottom)(Pore measuring direction: Length: Up – down; Width: Left – Right)



a) b)

Fig. 7 Pore population distribution versus diameter (a) and aspect ratio range (b) in the top (up) and bottom (down) part of the hydrogel with thin height

3.2.3 Hydrogels with high height(8 mm)

The micro-architecture of the hydrogel achieved in a culture plate with 24 wells each of them with 10 mm diameter and 10 mm height varies across the entire structure (Figs.8, 9) At the top of the hydrogel (Fig.9 Ia), which was in contact with the diffusion medium (filter paper), 84% from pore population has diameter from 50 to 200 μm (Fig.9Ib). 61% of pores that are found in this area have aspect ratio between 1.2 and 1.6 which means rectangular pores with length greater than width by approx. 2 times (Fig. 9Ic).

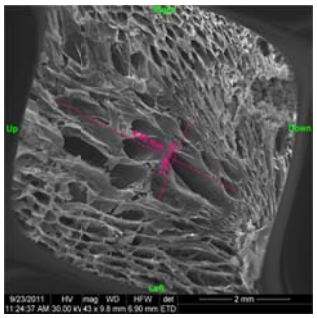
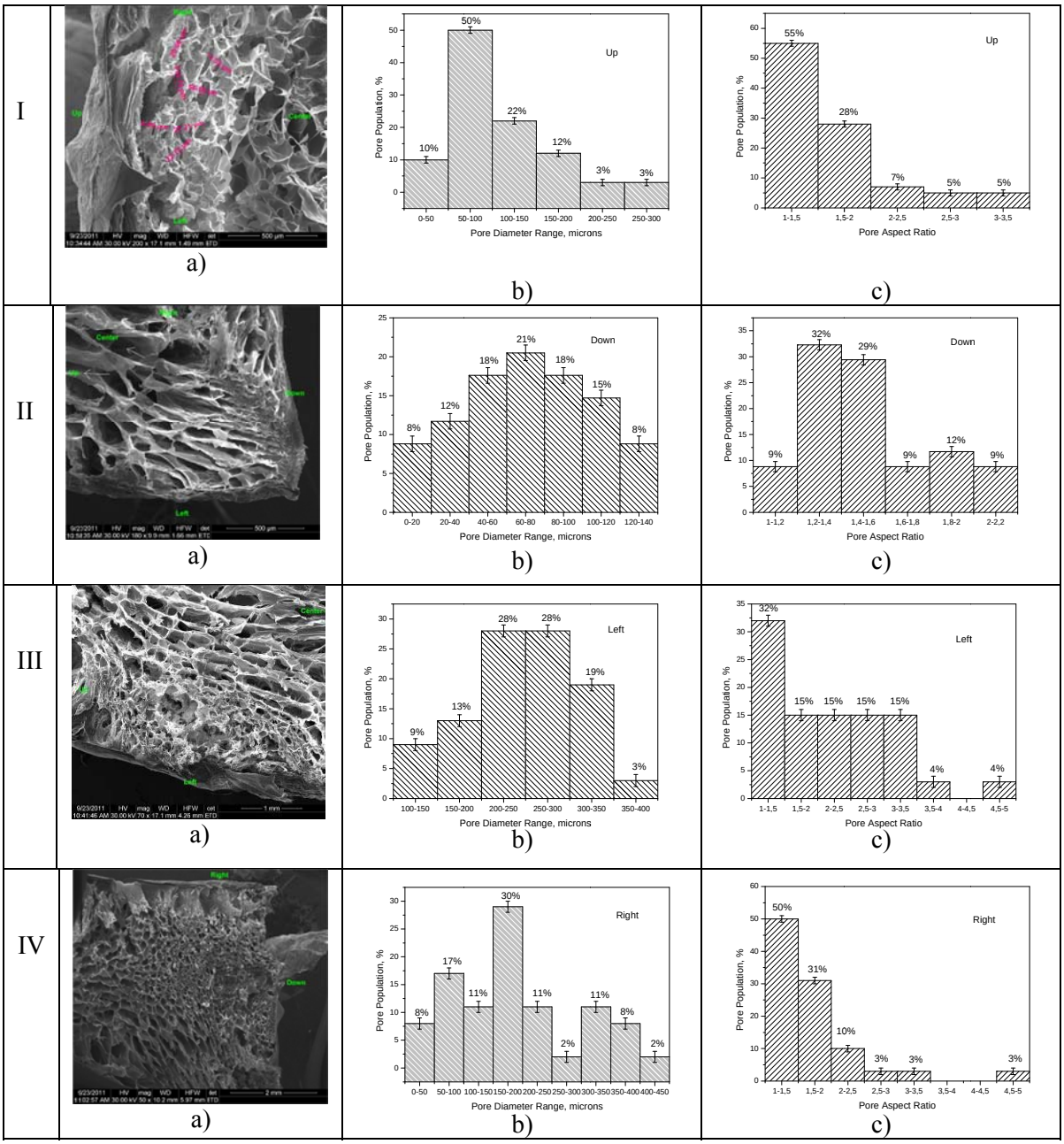


Fig. 8.Hydrogel obtained by diffusive controlmethod and areas with specific micro-architecture (pore measuring direction: Length left – right,width up – down)

At the bottom of the hydrogel (Fig. 9 II a), which that was in contact with the bottom of the well are found 82% pores with diameter from 20 to 120 μm (Fig.9 IIb). They have a rectangular shape because specific pore aspect ratio is 1.2 to 1.6 (Figure 9IIc). On the left side of the hydrogel (Fig.9 IIIa) are found a proportion of 46% pores with diameters of 200-300 μm, 22% of them having diameter of 100-200 μm and 21% with diameter of 300-400 μm (Fig. 9 III b). In this area, the highest proportion of 60% is represented by pores elliptical in shape because the aspect varies between 1.5 and 3.5 (Figure 9 III c). On the right side of the hydrogel (Fig.9 IVa) thepores have micro-architecture similar to those on the left part. In this area, a percentage of approx. 30% of pores have diameter between 150 and 200 μm, approx. 30% have diameter ranged between 30 and 150 μm and aprox.30% between 200 and 400 μm (Fig.9 IV b). About the pore shape, apercent of about 50% are almost spherical in shape because the aspect ratio isranged between 1 and 1.5, another percentby 41% are rectangular pores with length 1.5 – 2.6 greater then width (Figure 9 IVc). In the centrearea the pore diameter is very high, a percentage of 52% pores have diameter ranged between 100 and 300 μm, 34% between 300 and 500 μm. 14% are very large pores with a diameter between 500 and 900 μm (Fig. 9Vb). 91% of these pores are rectangularwith length1.5 – 3 greater than the width (Fig. 9Vc). The obtained results show that the largest pore diameter range is specific to the central area, where the diameter is ranged from 141.22 μm to 859.19 μm (Fig. 10 a). The pores have the lowest average diameter in the upper area

(101.36 ± 55.6) µm and lower those (71.47 ± 33.55). The mean diameter has high values in the side zones (248.54 ± 61.65 µm left and right 184.7 ± 105.22). The pores have the largest diameter in the centre area where the average value is 343.14 ± 185.65 µm. In the top and bottom zones, the pores are circular to elliptical in shape with length slightly larger than the width (mean aspect ratio: up: 1.62 ± 0.61, down 1.49 ± 0.28). The shape of the pores in the left and right areas is highly elongated similarly with the pores from the central zone (mean aspect ratio: left: 2.21 ± 0.91, right 1.67 ± 0.71, centre 2.33 ± aspect ratio 0.48) (fig .10 b) . These hydrogels cannot be use in tissue engineering due to the very pronounced inhomogeneity of porosity.



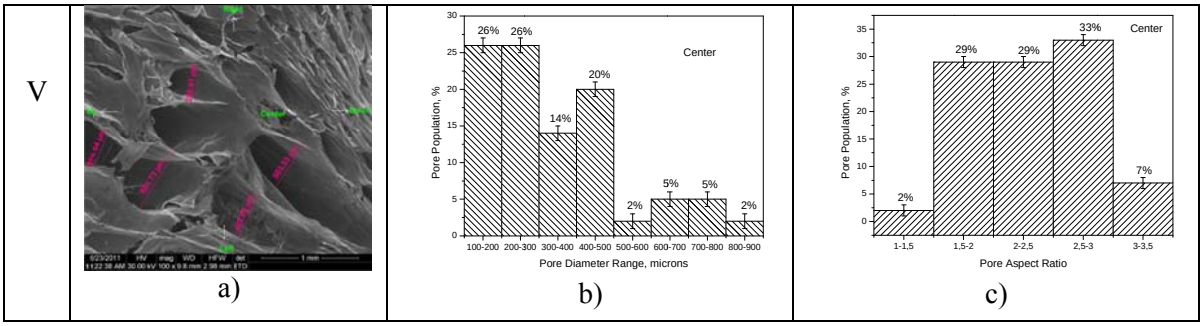


Fig. 9 Generalcross section(a) and cross section in the following areas of the hydrogel with high height (10 mm): top (I), bottom (II), left (III), right (IV), centre (V). Pore population distributionversus diameter (b) and aspect ratio (c) range (Pore measuring direction: Length: Up – down; Width: Left – Right)

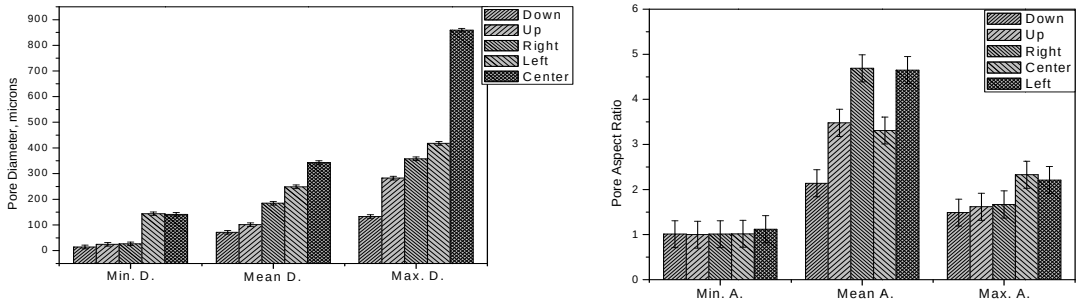


Fig. 10 Pore population distribution versus diameter (a) and aspect ratio(b) range and mean values in the representative zones of the hydrogel with high height

4. Discussions

The ionically crosslinking of sodium alginate with CaCO3 – GDL system in order to decrease the reaction rate for control the properties of hydrogels designed for bone and cartilaginous tissue regeneration is well known [25, 29, 30]. In our previos work [29] the possibilities to use this system in order to obtain hydrogels for adipose tissue engineering based on a crosslinking process of 3 hours in physiological conditions (37 ºC) was studied. The characterization of the obtained hydrogels proved that from the point of view of the elastic properties, the new hydrogels present similar elastic behaviour with following adipose tissues types: mammary gland (160Pa), lymph node (120 Pa), brain (260-490 Pa), liver (640 Pa) [29, 31]. *In situ chemical generation method* consists in fact in chemically controlling the crosslinking ions existing, at a time, in the reaction medium because these ions results from a complex and simultaneously chemical reactions occurring between the reaction medium components. GDL reacts with water and results in gluconic acid, acid which in turn reacts with $CaCO_3$ to form calcium gluconate. Calcium ions resulting from the dissociation of $CaCO_3$ and calcium gluconate appeared in the reaction medium by mentioned reactions [29]. These two salts have low dissociation constant [29], matter which further reduces the concentration of calcium ions existing at a given time, in the reaction medium.

By the diffusion control method of the calcium ions designed by the authors [32], calcium ions concentration at a given time is controlled by diffusion of calcium ions through the filter paper. Method and properties of the obtained hydrogels are presented in detail in other papers [33-

35]. In this method, the movement of the calcium ions towards and inside the sodium alginate solution are controlled by the concentration gradient between the upper and lower sides of the wells from culture plates (Fick's first law), which decreases in time (Fick's second law). During their movement, the calcium ions meet the following barriers: the filter paper fibrils covered with perforated calcium alginate films, the perforated membrane appeared at the interface between the filter paper and the hydrogel, the reaction mass in which the access to the alginate macromolecules can be hindered by their entanglement degree, the three-dimensional network formed in the early stages of the crosslinking. All these barriers explain the smaller calcium ions concentration and the induction period existence [34, 35].

In classical well known internal gelling method**,** the crosslinking begins at the outer surface of the calcium chloride solution and then proceeds into the sodium alginate outer body. By dropping a solution of $CaCl_2$ in a solution of sodium alginate hydrogels like rigid beads with uneven structure result. Since crosslinker ions come from a salt with quick disassociation, they instantly appear in the reaction medium, immediately after dropping of the calcium salt in the reaction medium, after which diffuses rapidly between and within alginate molecules [36-38].

At the same initial calcium ions concentration, after the same reaction time, the momentary concentration of the calcium ions in the reaction medium decreases in the order:

Internal gelling >>>In situ chemical generation method >>Diffusion controlled method

Different calcium ions concentration means different crosslinking rate, which for the three crosslinking methods decrease in the same order. Therefore at the same initial concentration of calcium ions, after the same reaction time, based on the three methods hydrogels with different degrees of crosslinking will be obtain. This means that totally different morphologies and micro – architecture can result. The lower the concentration of calcium ions is, the smaller is the degree of crosslinking and larger the pore size. To explain the hydrogels' pore size dependence on the obtaining procedure, it should be also recalled that before lyophilisation, the hydrogels are suddenly cooled. The rapid cooling causes a thermodynamic instability in the system which, in its turn, results in a phase separation. During the cooling, the water is separated as micro drips placed into a polymeric network. After the removal of water by sublimation, in the place of the micro-drips, pores will be formed. The size of water micro-drips depends on several parameters including the cooling temperature where lyophilisation procedure is performed. On the other hand, the pore formation and the behaviour of polymeric network at phase separation depend on the mechanical characteristics of network, respectively on the hydrogel cross-linking density [39-40]. In other words, phase separation is related to a process of dehydration of the polymer chains within the hydrogel network. Clearly, the development of this process, namely pores formation, depends both on the cooling temperature and on the polymeric network cross-linking density. High cross-linking density results in faster polymer chain dehydration and water separation and, consequently, the size of water dropletsare bigger [29].

The variation of the micro-architecture of the pores that characterizes the hydrogel obtained by diffusion control method can be explained by the decrease in concentration of the crosslinker ions from the well surface to its bottom. As the hydrogel formation height increases so the difference between crosslinker ions in the top and bottom area of the well is more pronounced. In the area where the crosslinker ions is higher the hydrogel pores will be smaller and the one in which the crosslinker ion concentration is smaller the pores will be larger.

If the hydrogel formation height is very higher and the filter paper is not perfectly horizontal with the well bottom placed thendrains form for the crosslinked solution which increases the heterogeneity of hydrogels porosity.

The obtained results proves that the controlling of the alginate crosslinking rate is an important route that enable the obtaining of alginate hydrogels for adipose tissue engineering with tunable morphology and desired pore micro-architecture.

The hydrogels optimum pore micro-architecture depends by the type of tissue that will be engineered [17, 41].

5. Conclusions

The obtained results based on the statistical analysis of the pore micro-architecture proves that the controlling of the alginate crosslinking rate is an important route that enable the obtaining ionotropic alginate hydrogels for adipose tissue regeneration with tunable morphology with desired pore micro architecture.

The variation of the micro-architecture of the pores that characterizes the hydrogel obtained by diffusion control method can be explained by the decrease in concentration of the crosslinker ions from the well surface to its bottom. As the hydrogel formation height is greater with so the difference between crosslinker ions in the top and bottom area of the well is more pronounced. In the area where the crosslinker ions is higher the hydrogel pores will be smaller and the one in which the crosslinker ion concentration is smaller the pores will be larger.If the hydrogel formation height is very higher and the filter paper is not perfectly horizontal with the well bottomplaced then drains form for the crosslinker solution which increases the heterogeneity of hydrogels porosity.

Acknowledgements

The authors gratefully acknowledge the financial support from CNCSIS – UEFISCDI ROMANIA by Complex Exploratory Research Project no. PCCE 248/2010.

References

[1] G.D. Nicodemus, SJ. Bryant, Tissue Eng., **14**, 149 (2008)
[2] K.Y. Lee, D.J. Mooney, Chem. Rev., **101**, 1869 (2001).
[3] J.L. Drury, D.J. Mooney, Biomaterials, **24** (24), 4337 (2003)
[4] Q.P. Hou, P.A. De Bank, K.M. Shakesheff, J. Mater.Chem., **14**, 1915 (2004)
[5] C.R. Nuttelman, M.A. Rice, A.E. Rydholm, C.N. Salinas, D.N. Shah, K.S. Anseth, Prog.Polym.Sci., **33**, 167 (2008).
[6] H. Tan, Y. Gong, L. Lao, Z. Mao, C. Gao, J. Mater. Sci.:Mater. Med., **18**, 1961 (2007).
[7] H.A. Awad, M.Q. Wickham, H.A. Leddy, J.M. Gimble, F. Guilak, Biomaterials, **25**, 3211 (2004).
[8] M.M. Stevens, H.F. Qanadilo, R. Langer, V.P. Shastri, Biomaterials, **25**, 887 (2004).
[9] N.Mohan, D.P. D Nair, Novel Porous, Trends Biomater. Artif.Organs, **18**(2) (2005)
[10] A. Gutowska, B. Jeong, M. Jasionowski, The Anatomical Record, **263**(4), 342 (2001)
[11] T.R. Hoare, D.S. Kohane, Hydrogels in drug delivery: Progress and challenges, Polymer **49** 1993 (2008)
[12] M.A. Barbosa,P.L. Granja, C.C. Barrias, I.F. Amaral, ITBM-RBM **26** 212 (2005)
[13]O. Jeon, K.H. Bouhadir, J.M. Mansour, E. Alsberg, Biomaterials, **30**(14), 1, (2009)
[14]J.F. Mano, G.A. Silva, H.S. Azevedo, P.B. Malafaya, R.A. Sousa, S.S. Silva, L.F. Boesel, J.M. Oliveira, T.C. Santos, A.P. Marques, N.M. Neves, R.L. Reis, J R SocInterface., **4**(17), 999 (2007)
[15]H. Zimmermann, F. Wählisch, C. Baier, M. Westhoff, R. Reuss, D. Zimmermann, M. Behringer, F. Ehrhart, A. Katsen-Globa, C. Giese, U. Marx, V.L. Sukhorukov, J.A. Vásquez, P. Jakob, S.G. Shirley, U. Zimmermann, Biomaterials **28,** 1327 (2007)
[16] W.M.Parks, Y.B.Guo, Materials Science and Engineering C **28**, 1435 (2008)
[17] K. Whang, K.E. Healy, D.R. Elenz, E.K. Nam, D.C. Tsai, C.H. Thomas, G. Nuber, R. Glorieux, R. Travers, S.M. Sprague, Tissue Eng**5**, 35 (1999)
[18] S.M. Lien, L.Y. Ko, T.J. Huang, ActaBiomater**5**, 670 (2009).
[19] D.J. Griffon, M.R. Sedighi, D.V. Scaeffer, A. Eurell Jo, L. Johnson Ann, ActaBiomater **2**, 313 (2006).
[20] I. Martin, B. Obradovic, S. Treppo, A.J. Grodzinsky, R. Langer, L.E. Freed, G. Vunjak-Novakovic, Biorheology**37**, 141 (2000)
[21] M.S. Stoichet, R.H. Li, M.L. White, S.R. Winn, Biotechnol. Bioeng.,**50**, 374 (1996)

[22] H. Aubin, J.W. Nichol, C.B. Hutson, H. Bae, A.L. Sieminski, D..M. Cropek, P. Akhyari, A. Khademhosseini, Biomaterials, **31**(27), 6941 (2010)
[23] A. Khademhosseini, R. Langer, Biomaterials, **28**, 5087 (2007)
[24] S. Gerecht, S.A. Townsend, H. Pessler, H. Zhu, C.L. Nijst, J.P. Bruggerman, J.W. Nichol, R.A. Langer, Biomaterials, **28**, 4826 (2007)
[25] K. Kuo, P.K. Ma, MRS Proceedings, **662 (**2000)
[26] N. Annabi, J. W Nichol, X. Zhong, C. Ji, S. Koshy, A. Khademhosseini, F. Dehghani, Controlling the Porosity and Microarchitecture of Hydrogels for Tissue Engineering, Tissue Eng., Part B, **16**(4) (2010)
[27] D.L. Cohen, E. Malone, H. Lipson, L.J. Bonassar, Direct Freeform Fabrication of Seeded Hydrogels in Arbitrary Geometries, Tissue Eng., **12**(5), 1325 (2006)
[28] C. Gao, M. Liu, J. Chen, X. Zhang, Preparation and controlled degradation of oxidized sodium alginate hydrogel, Polym. Degrad. Stabil.**94,** 1405 (2009)
[29] D. Dimonie, I.Trandafir, A. Cimpean, et all. Hydrogels for regeneration adipose tissue realized by chemical control of the ionic crosslinking rate, 1st International Conference on Analytical Chemistry, Valahia University of Targoviste, September 18 – 21 2012, Romania
[30] United States Patent 6497902
[31] I. Levental, P.G. Georges, P.A. Janmes, Soft biological materials and their impact on cell function, Soft Matter, **2,** 1 (2006)
[32] D. Dimonie, I. Trandafir, C. Nicolae, et all. "Composition and process for production of some hydrogels for tissue regeneration", Patent Request No. 01263/29.11.2011
[33] D. Dimonie, M. Petrache, R. Fierascu, et all , Influence of the diffusion medium on the migration of the cross-linkers and on the properties of the hydrogels obtained through diffusive control, ROCAM 2012, 28 – 31 august 2012, Brasov, Romania
[34] D. Dimonie, M. Petrache, R. Fierascu, et all New Method to Control the Morphology of IonicallyCrosslinked Alginate Hydrogels for Soft Tissue Regeneration, The biennial international conference "Materials Science Engineering - MSE 2012" Darmstadt, Germania, 25 – 27.09.2012
[35] D. Dimonie, I. Trandafir, M. Petrache, et all, The Mechanism of the Calcium Ions Migration in the Ionotropic Alginate Hydrogels Obtained by Controlled Fickian Diffusion Method, International Symposium Chemistry Priorities for a Sustainable Development - PRIOCHEM - 8th edition
[36] X. Liu, W. Xu, Q. Liu, W. Yu, Y. Fu, X. Xiong, X. Ma, Q. Yuan, Swelling behaviour of alginate-chitosane microcapsules prepared by external gellation or internal gellation techniques, Carbohidrate Polymers, **56**, 454 (2004)
[37] G.O. Phillips, P.A. Williams, Handbook of hydrocolloids, Woodhead Publishing Amazon.com, **22** Alginates, 379 (2000)
[38] M. Davidovici – Pinhas, H. Bianco – Peled, A quantitative analysis of alginate swelling, Carbohidrate Polymers, **79,** 1020 (2010)
[39] L. Li, Y. Fang, R. Vreeker, I. Appelqvis, Reexamining the Egg-Box model in Calcium-Alginate Gels with X –ray Diffraction, Biomacromolecules, **8**, 464 (2007)
[40] S. Ramachandran, P. Fontanille, A. Pandey, C. Larroche, Gluconic Acid: Properties, Aplications and Microbial Production, Food Technol. Biotechnol. **44** (2), 185 (2006)
[41] C. Fidkowski, M.R. KaaempurMofrad, J. Borenstein, J. Vacanti, R. Langer, Y. Wang, Endothelialized microvasculature based on a biodegradable elastomer, Tissue eng. **11**, 302 (2005)