Digest Journal of Nanomaterials and Biostructures Vol. 6, No 4, October-December 2011, p. 1867-1875

THE INFLUENCE OF GLYCOSAMINOGLYCAN TYPE ON THE COLLAGEN-GLYCOSAMINOGLYCAN POROUS SCAFFOLDS

A. LUNGU[a], M. G. ALBU[b], N. M. FLOREA[a], I. C. STANCU[a], E. VASILE[a*], H. IOVU[a]

[a] *Advanced Polymer Materials Group, University Politehnica of Bucharest, Romania*

[b]*INCDTP - Leather and Footwear Research Institute Division, Collagen Department, Bucharest, Romania*

This report describes the preparation and characterization of collagen-glycosaminoglycan (Coll-GAG) porous scaffolds with potential uses in tissue engineering. The combination of collagen and GAG has shown advantages over the use of either the material alone. Specific interactions in these scaffolds could exist due to the hydrogen bonding between O-H of GAG and C=O of collagen or between C=O of GAG and N-H of collagen. Scaffolds microstructure (porosity, mean pore size, interconnectivity) was evaluated using SEM. Also the addition of GAGs not only improved the water uptake significantly but also extended the degradation time of Coll-GAG scaffolds in comparison with Coll. The results suggest that the obtained Coll-GAG matrices as an analog of the natural three-dimensional extracellular matrix may be useful for *in vitro* investigation to support the attachment, proliferation and migration of a variety of cells.

(Received November 28, 2011; accepted December 5, 2011)

1. Introduction

Three-dimensional (3-D) matrices serve as analogue of extracellular matrix (ECM) acting as physical structural support which simulates favorable physiological environment for regeneration of affected tissues stimulating new tissue development, cell adhesion and wound bed granulation [1-3]. In order to create 3-D matrices able to support *in vitro* tissue formation, several key features have to be taken into account. Thus they have to be highly porous, with an interconnected architecture, to exhibit predictable and controllable biodegradability and chemical composition similar with extracellular matrix [4].

Collagen and glycosaminoglycans (GAGs) are the major components of the ECM that are commonly used for tissue engineering. Type I collagen is the major protein of the ECM in many tissues (skin, bone, cartilage, tendon, blood vessel, teeth) where it provides the principal structural and mechanical support. Also, collagen represents one of the mostly used biomaterials due to its low antigenicity, its excellent biocompatibility and biodegradability properties, haemostatic and cell-binding properties [5-7].

Attachment of GAGs may ofer the opportunity to exploit the biocharacteristics of these polysaccharides and to valorize collagen as a biomaterial. Chondroitin sulfate (ChS) belongs to the GAGs family and is a major component of extracellular matrix and important in maintaining the structural integrity of the tissue. Hyaluronic acid (HyA) is unique among the GAGs in that it does not contain any sulfate and is not found covalently attached to proteins as a proteoglycan. However it is a component of non-covalently formed complexes with proteoglycans in the ECM.

* Corresponding author: eugeniuvasile@yahoo.com

HyA has a high capacity for water absorption, water retention and influences several function such adhesion, migration and proliferation [8-14].

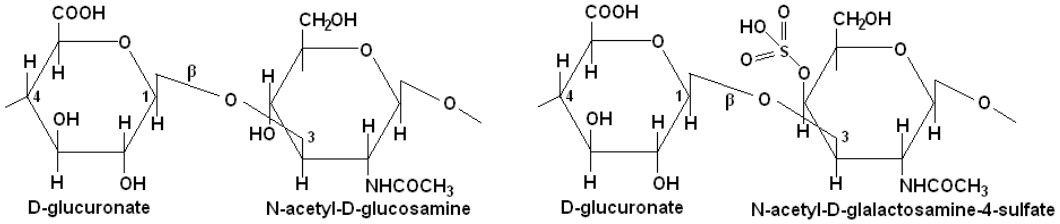
Because of the good biological activities of the collagen and GAGs their combination may have beneficial effects as scaffolds for tissue regeneration. Scaffolds fabricated from type I collagen and GAG have been utilized as ECM analogs for the regeneration of skin and are currently being considered for the regeneration of nerve and conjunctiva [15-21].

Although a number of investigations on collagen-GAG scaffold materials have been done to characterize their biochemical and biological properties, the effect of GAG on the conformational and physico-chemical characteristics of collagen is not clearly elucidated. Our group recently reported that the addition of HyA and human bone morphogenetic protein onto collagen scaffolds promoted adhesion, maintain viability and sustain the migration of osteoblast-like cells into the scaffolds [22]. The major goal of the present research was to study extensively the effect of HyA or ChS incorporation on the final features of the collagen scaffolds with potential uses in tissue engineering. The morphology and thermal features of the GAG loaded collagen scaffolds were investigated. We also evaluated the water-binding capacity as well as *in vitro* enzymatic degradation of the scaffolds.

2. Materials and methods

Materials

Type I fibrillar collagen (Coll) was extracted from calf hide in gel form with an initial concentration of 1.83% (w/w) by acid and alkaline treatments as previously described [23]. Chondroitin 6-sulfate sodium salt from shark cartilage (ChS) and hyaluronic acid potassium salt from Human Umbilical Cord (HyA) were purchased from Sigma (Germany). Glutaraldehyde (GA) was received from Merck (Germany). The chemical structures of the raw substances are shown in Fig. 1.



a) b)

Fig. 1. Chemical structure of the used glycosaminoglycans a) HyA and b) ChS

Collagen–GAG scaffolds preparation

Hyaluronic acid (HyA) or chondroitin sulfate (ChS) was added to collagen gel (Coll) keeping collagen concentration constant (1.2%), Coll:HyA = 10:1 or Coll:CS = 10:1 respectively. The pH was maintained at 7.4 during sample preparations. In order to stabilize the structure of these natural polymers all the gels were cross-linked with 0.25% GA (reported to the weight of dry collagen), then cast in disposable polystyrene dishes and kept at 4°C for 24 hours. After cross-linking the samples were frozen using a constant cooling rate technique that has been previously described by our group [24]. A constant starting temperature of 20°C paired with final freezing temperatures of -40°C were used to produce scaffold. Temperatures are increased gradually during the lyophilization process, reaching 10°C in 10 hours, 20°C in 8 hours and 35°C in 12 hours. Sublimation of the ice crystals produces the porous scaffold structure

Methods

ATR-FTIR spectra were registered on a VERTEX 70 BRUCKER FT-IR spectrometer equipped with an attenuated total reflectance (ATR) accessory. All FTIR measurements were

performed in the ATR-FTIR cell on Ge crystal, at room temperature. The FTIR spectra were recorded using 32 scans in 600-4000 cm^{-1} wavenumber region.

In order to detect the denaturation temperature (Td) of matrices and to assess its thermal stability the differential scanning calorimetry (DSC) supplemented by thermogravimetric (TGA) measurements was used. The denaturation temperature (Td) of matrices was determined by DSC using a Netzsch DSC 204 F1 Phoenix equipment. Samples of about 2 mg were heated from 20 to 300°C under a constant nitrogen flow rate (20 mL/min). A heating rate of 10°C/min was applied and the endothermic peak of the curve was monitored. The TGA results were achieved on a Q500 TA instrument. A typical sample was non-isothermal heated from 20 to 700°C at a heating rate of 10°C/min under a constant nitrogen flow rate (balance flow 10 mL/min, oven flow 90 mL/min). All samples were run in duplicate.

Morphological structure was investigated by scanning electron microscopy (SEM) after the scaffolds samples were sputtered with an ultrathin layer of gold for enhanced surface conductivity. The analysis was performed using a QUANTA INSPECT F SEM device equipped with a field emission gun (FEG) with a resolution of 1.2 nm.

In order to estimate the water-binding capacity matrix samples of about 5 mg dry weight were incubated in 3 ml PBS (pH 7.4) at 37°C. At scheduled time intervals, the samples were withdrawn, wiped (to remove the surface water) and weighed in the swollen state. The experiments were repeated five times under the same conditions and average values were reported. The swelling degree of the scaffolds at equilibrium was calculated using equation (1):

$$\% \ swelling = (W_t - W_d)/W_d \times 100 \quad (1),$$

where W_t denotes the weight of the swollen samples at immersion time t, and W_d denotes the weight of the dry samples.

In vitro enzymatic degradation of collagen scaffolds by collagenase was also investigated by monitoring the mass loss of scaffolds as function of exposure time to a collagenase solution according to a procedure described in the literature [23, 24]. Pieces of collagen scaffolds (1 cm in diameter) were accurately weighed (wet weight without excess of water), placed in a solution of PBS and collagenase (1μg/mL) at pH 7.4, and incubated at 37°C. At regular intervals, the swollen scaffolds were removed from the collagenase solution, wiped and weight. The percent of degradation of matrices was determined using equation (2):

$$\% \ weight \ loss = (W_i - W_t)/W_i \times 100 \quad (2),$$

where W_i is the initial weight and W_t is the weight after time t. Each biodegradation experiment was repeated 5 times. The final percentage of biodegradation was calculated as the average values.

3. Results and discussion

The 3-D matrices obtained by freeze-drying in form of porous structure were characterized by FT-IR, TGA and DSC, SEM, water uptake and enzymatic degradation.

Because the crosslinking agent used (GA) is a catalyst only of collagen-collagen crosslinks we presume that the GAG (HyA or ChS) is not covalently attached to collagen. However specific interactions in these blends could exist due to the hydrogen bonding between O-H of GAG and C=O of collagen or between C=O of GAG and N-H of collagen. Similar reactions may occur between collagen and HyA because the chemical structure of HyA is similar to ChS except that it does not include sulfate groups. A schematic view of the possible interactions between GAG and Coll molecules is drawn in Fig. 2.

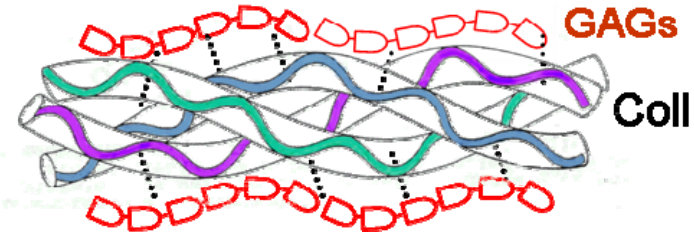


Fig. 2. Schematic representation for the interactions between Coll and GAG moieties

The FT-IR spectra successfully confirmed the presence of all the functional groups characteristic to the components of the obtained scaffold. Control Coll, HyA and ChS were used to identify the main specific vibrations for each polymer. Fig. 3 shows the FTIR spectrum of Coll scaffold in comparison with IR spectra of Coll-ChS and Coll-HyA scaffolds.

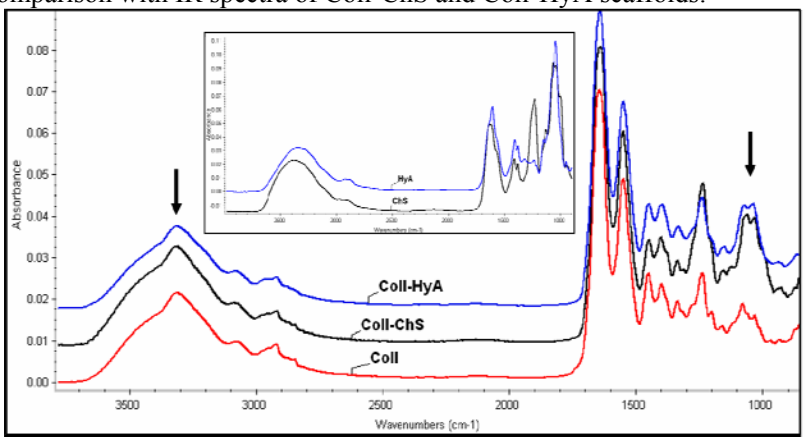


Fig. 3. FT-IR spectra for Coll, Coll-ChS and Coll-HyA; inset FTIR spectra for ChS and HyA.

The characteristic features for collagen were compared with peak assignments derived from literature [25]. The collagen spectrum exhibits typical features of a protein spectrum with absorbance bands at 1646 cm^{-1} (amide I), 1553 cm^{-1} (amide II) and at 1240 cm^{-1} (amide III).

The assignment of the different infrared bands for HyA and ChS was performed as follows: the broad band in the region 3600-3000 cm^{-1} is due to the υ(O-H) stretching mode (water, polysaccharides) and partially to the υ(N-H) stretching vibration of the N-acetyl side chain. The band at 1624 cm^{-1} is assigned to the amide I mode (mainly C-O stretching coupled with N-H bending) in ChS while the peak at 1608 cm^{-1} in HyA is assigned to the antisymmetrical stretching mode of the carboxylate groups, $\upsilon_{as}(COO^-)$. The shoulder at 1550 cm^{-1} can be assigned to the amide II band and the feature at 1408 cm^{-1} originates from the symmetrical COO^- vibration, $\upsilon_s(COO^-)$, respectively. The absorbance in the region 1100-950 cm^{-1} mainly results from the C-O stretching vibration of the uronic acids residues. Additional bands in the spectrum of ChS can be attributed to vibrations of the C-O-SO_3^-: the intense feature centered at 1238 cm^{-1} is due to the υ (SO_3^-); the peak at 1125 cm^{-1} is probably due to the antisymmetrical C-O-S stretching. These data are consistent with prior published spectra for HyA and ChS [26, 27].

Since Coll is the main component of the materials all the spectra display its characteristic vibrations. However in the Coll-HyA and Coll-ChS spectra one can notice a slight increase in the intensity of ester band (1082 cm^{-1}) due to the carboxylic groups of the uronic acid moiety of GAGs. Also the Coll-GAG scaffolds show a very broad band in the region 3500-3000 cm^{-1},

centered at about 3315 cm^{-1}. These data support previous hypothesis that new hydrogen bonds could occur between O-H of GAG and C=O of Coll or C=O of GAG and N-H of Coll.

Besides hydrogen bonds, electrostatic interactions could also appear to form polyelectrolyte complexes due to the opposite electrostatic charges when Coll and GAG molecules are mixed in aqueous solution (positively charged collagen and anionic polysaccharide).

The typical DSC and TGA curves of the obtained matrices Coll and Coll in the presence of HyA and ChS respectively are shown in Fig. 4.

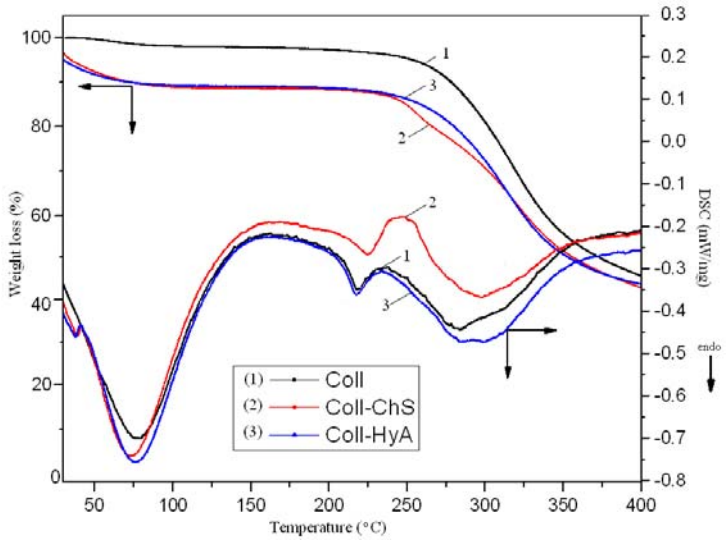


Fig. 4. The DSC/TGA curves of the (1) Coll; (2) Coll-ChS and (3) Coll-HyA scaffolds.

The thermograms obtained by DSC show three different endothermal peaks: the first one is related to temperature of thermal denaturation (Td) of collagen and the second and the third one were connected with a complex phenomenon of thermal modification, which finally leads to the destruction of materials.

As can be seen from Fig. 4, Td is a broad endothermal peak which appears in each sample. It is well known that Td strongly depends on the water content in collagen and its degree of cross-linking between the chains. Td for collagen modified with HyA or ChS compared to unmodified collagen appear to be slightly shifted to lower temperatures. This peak is connected with the transition from the triple helix to a randomly coiled conformation, taking place in the domains between the cross-links. Within the temperature range from 30 to about 50^{o}C, a small endothermal peak for Col-HyA and Col-ChS supplementary appears assigned to the water removal from HyA and ChS respectively prior to collagen denaturation.

The second weak endothermic peak on the DSC curves with maximum around 219^{o}C for both collagen and Coll-HyA and around 225^{o}C for Coll-ChS is associated with the evaporation of residual, strongly bound water and continued conformational changes of superhelix.

The DSC peaks are in good corellation with the characteristic TGA curve, which suggests the decomposition of the matrix. Td peaks noticed in DSC curves are simultaneously accompanied by gradual mass decrement. At a temperature of 130^{o}C weight loss is about 2% for Coll scaffold, 12% both for Coll-ChS and Coll-HyA, respectively. These significant differences between the studied samples indicate that the thermal denaturation strongly depends on the scaffold degree of cross-linking.

It is known that intra- and inter-molecular hydrogen bonds as well as hydrogen-bound water are responsible for the stability of the triple helix conformation of collagen macromolecules. Thereby, the obtained results indicate that partial changes in the secondary structure of collagen macromolecule are preceded by the breakage of inter- and intra-molecular hydrogen bonds on heating and the release of loosely bound water [8].

The third endothermic DSC peak is related with a significant weight loss in TGA curves probably due to the release of the low molecular products during thermal degradation of the

scaffold. The experimental results indicate that if the temperature increases above 300°C the destruction of materials occurs more quickly and easily. For unmodified collagen the major weight loss (50%) occurred at temperatures from 265 to 400°C, but the major decomposition temperature for Coll-HyA and Coll-ChS was from 250 to 400°C and the majorweight loss was about 55%.
SEM observations were carried out further to confirm the three-dimensional porous structures of the synthesized scaffolds. The scaffolds used for tissue engineering must have high porosity with a large surface area/volume ratio and interconnected pores to provide a greater space for cells and to allow the production of new ECM.
The SEM micrographs presented in Fig. 5 (a-c) showed highly porous structures with interconnected pores to facilitate not only cellular ingrowth but also the diffusion of oxygen and other nutrients from culture medium into the matrices.

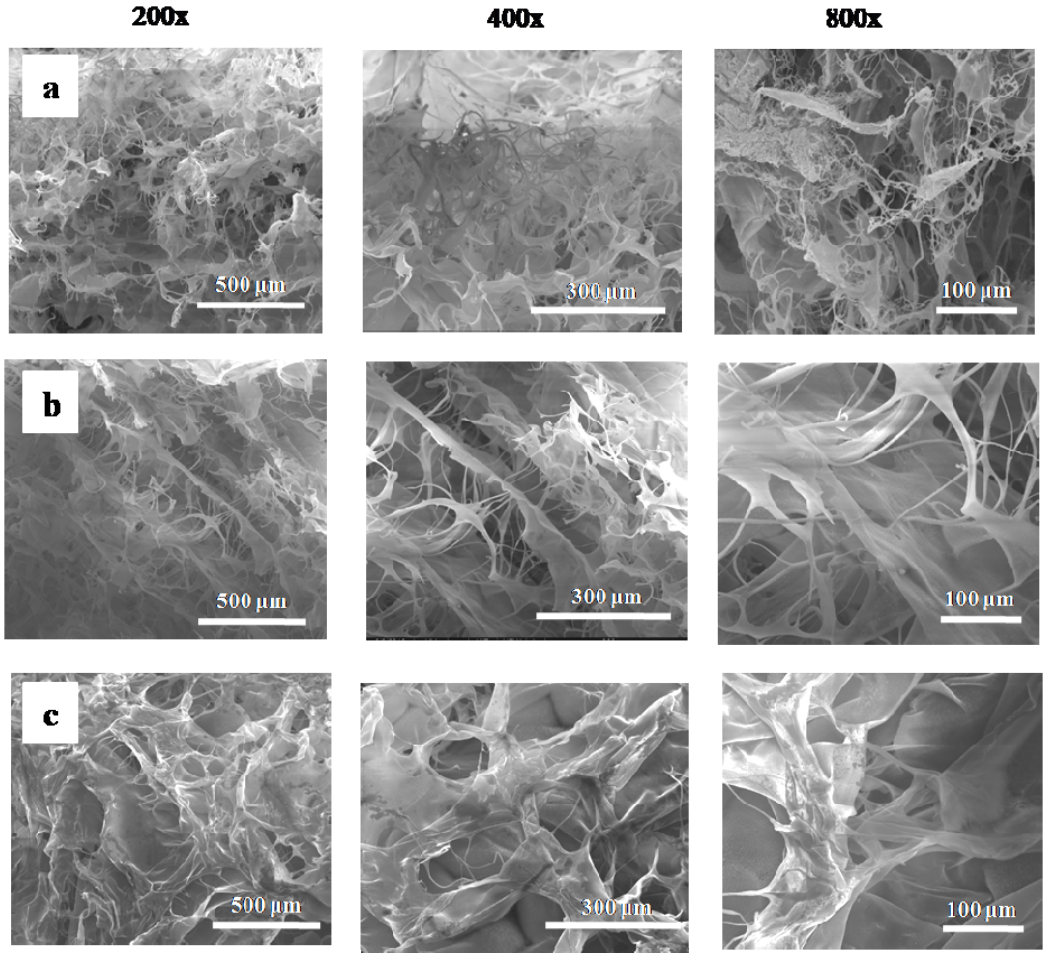


Fig. 5. Top-view SEM images (at three different magnifications) for collagen scaffolds without or with GAGs augmentation (a) Coll-only scaffold; (b) Coll-HyA scaffold; c) Coll-ChS scaffold.

The Coll scaffold microstructure was made up of a foam-like interconnected network of thin struts (Fig. 5 a). Introducing GAG in the scaffold modifies the porosity of the materials as shown in Figs 5 b) and c). Coll-HyA presents morphology similar to a honeycomb structure with parallel distributed pores while Coll-ChS is much denser.

The pore dimensions estimated from SEM micrographs by manual measurement ranged from 20-100 µm for Coll, 100-200 µm for Coll-HyA and 50-150 µm for Coll-ChS.

Water uptake

HyA and ChS belong to the most important GAG representing the main source of high water binding capacity of cartilage. The swelling of cartilage is based on the binding of water to polar groups of GAG (carboxylate, sulfate), on the electrostatic repulsion between GAG molecules and entropic contributions resulting from the mixing of water and counterions [20].
The chemical composition, porosity and three-dimensional structure of the scaffold influence the swelling behavior of the scaffold. Swelling was assessed in PBS pH 7.4 at 37^{o}C to mimic physiological conditions. It was observed that all the materials reached the maximum swelling degree (MSD) after 1h.

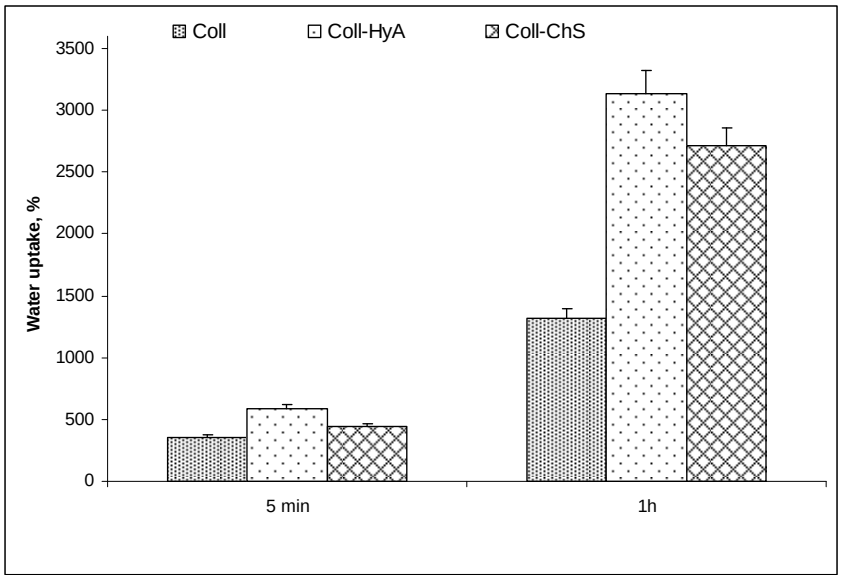


Fig. 6. Water uptake of scaffolds after 5 min and 1h

The experimental results indicate that the lowest water uptake was recorded for Coll scaffolds. Comparing water absorption values for Coll with or without GAG loaded, it was found that the scaffolds containing HyA or ChS respectively absorbed more water than the reference (Coll) due to the carboxyl and hydroxyl groups. The Coll-ChS scaffold exhibits lower swelling degrees in comparison with Coll-HyA scaffold. Hence, the results of the swelling tests are in good agreement with the morphology assessed by SEM, Coll-ChS scaffold being much denser and more tightly packed. To conclude, the swelling of the porous scaffolds can be finely-tuned by modifications of the ratio between the initial polymers solutions Coll and GAG.

***In vitro* degradation by collagenase**

Usually a scaffold material used for tissue engineering should provide an appropriate environment for cell proliferation and function and, at the same time, should be biodegradable. Collagenase digestion can represent an *in vitro* measure of degradation rate for a biological implant. The enzymatic degradation of the scaffolds was investigated as a function of exposure time to a collagenase solution. Fig. 7 shows the dependence of percentage weight loss (%) on the degradation time (4, 8, 24, 48 and 72 hours) for all the studied scaffolds.

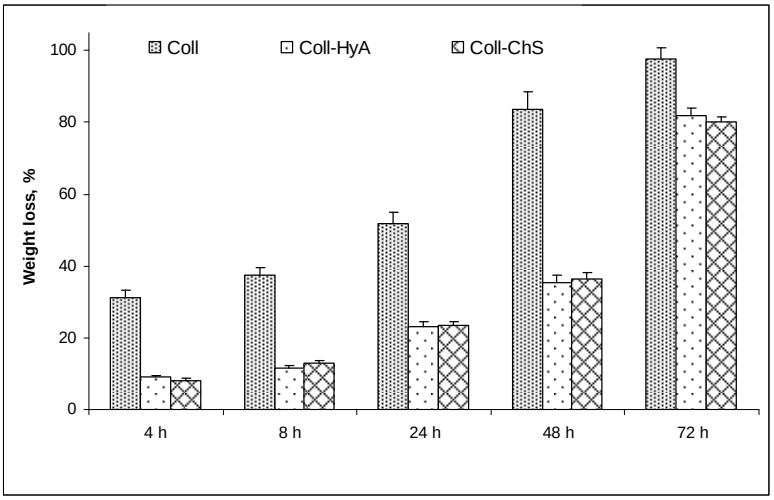


Fig. 7. In vitro enzymatic degradation of the obtained scaffolds

The results revealed that the reference scaffold (Coll) was digested faster than GAG loaded scaffolds. During the *in vitro* degradation Coll-HyA and Coll-ChS had a similar behavior due to the specific activity of the collagenase, an endopeptidase, which breaks down only the triple helix region from the collagen.

4. Conclusions

The aim of this study was to develop collagen scaffolds with two distinct types of GAGs: HyA or ChS. Similar reactions may occur between collagen - HyA and collagen - ChS because the chemical structure of HyA is similar to ChS except that is an unsulfated disaccharide.

Scaffolds microstructure (porosity, mean pore size, interconnectivity) was evaluated using SEM. The results suggest that this collagen-GAG matrix as an analog of the natural three-dimensional extracellular matrix may be useful for *in vitro* investigation to support the attachment, proliferation and migration of a variety of cells. The addition of HyA or ChS not only improved the water uptake significantly but also extended the degradation time of Coll-GAG scaffolds in comparison with Coll alone.

The morphology of the scaffolds could significantly affect the bioactivity of scaffolds used for in vivo tissue regeneration applications. Further investigations such as the colonizing cell capacity using different cell lines will be performed to confirm the performances of the collagen scaffolds with GAG loaded. Additionally, future work will be done upon chemical cross-linking by covalent binding via an amide bond using water soluble carbodiimides acting as a mediating agent between Coll and GAG.

Acknowledgements

This work was supported by CNCSIS – UEFISCSU, project number PNII – IDEI code 248/2010.

References

[1] F. J. O'Brien, B. A. Harley, I. V. Yannas, L. Gibson, Biomaterials **25**, 1077 (2004)
[2] P. B. Malafaya, G. A. Silva, R. L. Reis, Adv Drug Deliv Rev **59**, 207 (2007)
[3] P. Flocea, L. Verestiuc, M. Popa, V. Sunel, A. Lungu, J Macromol Sci Part A-Pure Appl Chem **47**, 616 (2010)

[4] N. Davidenko, J.J. Campbell, E.S. Thian, C.J. Watson, R.E. Cameron, Acta Biomater **6**, 3957 (2010)
[5] I. Titorencu, V. Jinga, A. Lungu, V. Trandafir, M. G. Albu, I. Rau, H. Iovu, Mol Cryst Liq Cryst **486**, 1189 (2008)
[6] M. Dumitrascu, V. Meltzer, E. Sima, M. Virgolici, M. G. Albu, A. Ficai, V. Moise, R. Minea, C. Vancea, A. Scarisoreanu, F. Scarlat, Dig J Nanomater Bios **6**, 1793 (2011)
[7] T.-W. Wang, M. Spector, Acta Biomater **5,** 2371 (2009)
[8] K. Pietrucha, Int J Biol Macromol **36**, 299 (2005)
[9] Ö. Malay, D. Yalcin, A. Batigün, O. Bayraktar, J Therm Anal Calorim **94**, 749 (2008)
[10] B. A. Harley, J. H. Leung, E. C.C.M. Silva, L. J. Gibson, Acta Biomater **3**, 463 (2007)
[11] B. A.C. Harley, L. J. Gibson, Chem Engin J **137**, 102 (2008)
[12] H. Liu, Y. Yin, K. Yao, D. Ma, L. Cui, Y. Cao, Biomaterials **25**, 3523 (2004)
[13] R. M. Lauder, Complement Ther Med **17**, 56 (2009)
[14] Y. L. Chen, H. P. Lee, H. Y. Chan, L. Y. Sung, H. C. Chen, Y. C. Hu, Biomaterials **28**, 2294 (2007)
[15] C. Peniche, M. Fernandez, G. Rodrıguez, J. Parra, J. Jimenez, A. Lopez Bravo, D. Gomez, J. San Roman, J Mater Sci: Mater Med **18**, 1719 (2007)
[16] J.S. Pieper, A. Oosterhof, P.J. Dijkstra, J.H. Veerkamp, T.H. van Kuppevelt**,** Biomaterials **20**, 847 (1999)
[17] C. M. Murphy, M. G. Haugh, F. J. O'Brien, Biomaterials **31**, 461 (2010)
[18] Y.S. Pek, M. Spector, I.V. Yannas, L.J. Gibson, Biomaterials **25**, 473 (2004)
[19] A. Surazynski,W. Miltyk, R. Czarnomysy, J. Grabowska, J. Palka, Pharmacol Res **60**, 46 (2009)
[20] S. J. Lee, S. Y. Kim, Y. M. Lee, Inc. J Biomed Mater Res Part B: Appl Biomater **82B**, 506, (2007)
[21] C. S. Ko, J. P. Huang, C. W. Huang, I. M. Chu, J Biosci Bioeng **107**, 177 (**2009)**
[22] A. Lungu, I. Titorencu, M. G. Albu, N. M. Florea, E. Vasile, H. Iovu, V. Jinga, M. Simionescu, Dig J Nanomater Bios in press 2012
[23] M.G. Albu, Collagen gels and matrices for biomedical applications, Lambert Academic Publishing, Saarbrücken (2011)
[24] M. G. Albu, M. V. Ghica, M. Leca, L. Popa, C. Borlescu, E. Cremenescu, M. Giurginca, V. Trandafir, Mol Cryst Liq Cryst **523**, 97 (2010)
[25] L. He, C. Mu, J. Shi, Q. Zhang, B. Shi, W. Lin, Int J Biol Macromol **48**, 354 (2011)
[26] R. Servaty, J. Schiller, H. Binder, K. Arnold, Int J Biol Macromol **28**, 121 (2001)
[27] T. Taguchi, T. Ikoma, J. Tanaka**,** Inc. J Biomed Mater Res **61**, 330 (2002)