Riboflavin-UVA gelatin crosslinking: Design of a biocompatible and thermo-responsive biomaterial with enhanced mechanical properties for tissue engineering

Juan Manuel Galdopórpora[1], Claudio Javier Perez[2], María Victoria Tuttolomondo[1], Martín Federico Desimone[1]*

[1]Instituto de Química y Metabolismo del Fármaco (IQUIMEFA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET),Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Junín 956 Piso 3◦, Buenos Aires, 1113, Argentina
[2]Instituto en Investigaciones en Ciencia y Tecnología de Materiales, Universidad de Mar del Plata, (CONICET), Juan B. Justo 4302, Mar del Plata, 7606, Argentina

*Corresponding author: Tel: (+54) 1152874332; E-mail: desimone@ffyb.uba.ar

DOI: 10.5185/amlett.2019.2210
www.vbripress.com/aml

Abstract

The main objective of this study is to develop an economic, environmentally friendly and malleable biomaterial for tissue engineering applications. Water and glycerol have been used as solvents for the gelatin hydrogel synthesis. This solvent mixture led to a biomaterial with improved thermal properties. Indeed, a 16 °C increase in thermal transition temperature was achieved. Furthermore, to enhance mechanical properties, riboflavin was used as a crosslinking agent. Chemical crosslinking step was initiated with UV radiation to obtain riboflavin radical polymerization of gelatin chains, hence, rheological properties of gelatin hydrogel were improved. Thus, Gelatin-UV-Riboflavin hydrogel showed good swelling and increased mechanical properties, obtaining a novel material for drug delivery and medical purposes. Copyright © 2019 VBRI Press.

Keywords: Tissue engineering, biopolymer, crosslinking.

Introduction

The development of biomaterials and hydrogels has attracted great attention for wound healing, drug delivery and medical purposes. Gelatin is a hydrophilic protein obtained by partial hydrolysis of collagen, the main fibrous protein constituent in bones, cartilages and skins. Therefore, the source, age of the animal and type of collagen are all intrinsic factors influencing the properties of gelatins [**1**]. Worldwide, the most abundant sources of gelatin are pig skin, bovine hide, pork and cattle bone and fish bone [**2**]. Argentina is a cattle-breeder country and bovine based industry has a high impact in the region, hence we use bovine gelatin for its availability. Depending on the treatment procedure, there are two types of commercial gelatin known as type-A gelatin (isoelectric point at pH 8.00–9.00) and type-B gelatin (isoelectric point at pH 4.00–5.00) obtained under acidic or alkaline pre-treatment conditions respectively. Industrial applications call for one or the other gelatin type, depending on the degree of collagen cross-linking in the raw material. Because of the acid lability of cross-linking in immature collagens, such as in fish skins, reasonably mild acid treatment is enough to affect collagen solubilization [**3**].

Smart polymers experienced great attention nowadays. Smart systems are expected to have promising applications, because they have self-alterable dynamic properties and are sensitive to small change. This allows them to be used in applications such as tissue engineering, cell recovery, nanomedicine and drug delivery [**4, 5**]. In this work, we describe the synthesis of a thermo-responsive polymer, which its main characteristic property is a reversibly or irreversibly alterable phase transition that occurs in response to a change in temperature. Temperature has a remarkable effect on the hydrophobic interactions between hydrophobic polymer segments and the hydrophilic interactions between hydrophilic polymer segments and water molecules. Hence, a temperature change can interrupt the original equilibrium and trigger a sol–gel transition [**6**].

Riboflavin (Vitamin B2) acts as a photosensitizer producing oxygen free radicals, which induce the physical cross linking of collagen. This procedure is widely used for keratoconous treatment [**7-9**]. The

technique of corneal collagen cross-linking consists of photopolymerization of fibers by the combined action of a riboflavin and ultraviolet type A rays (UVA). Photopolymerization increases the rigidity of corneal collagen and its resistance to keratectasia [**10, 11**], as seen in a large amount of scientific reports. We investigate the rigidity enhancement in gelatin based on the effect of the treatment seen in collagen hydrogels. In addition, the use of gelatin in nanobiotechnology is ubiquitous as a cell-encapsulation, drug-delivery and nanoparticle platform material [**12-14**]. Hence, the main objective of this study is to develop a novel, biocompatible and malleable biomaterial using gelatin and this crosslinking procedure for potential application in tissue engineering and drug-delivery system.

Experimental

Material synthesis

Dried bovine Gelatin (125 bloom, type B) was used to produce the hydrogels, which were prepared using a 0.1% riboflavin solution:anhydrous glycerol (99.5%) ratio of 3:2 as the dispersant.

For 10 mL hydrogel production, 6 mL of a 0.1% riboflavin solution was heated at controlled temperature (100 °C) and 0.2 g of dry gelatin were added later. Once gelatin was dispersed, 4 mL of heated anhydrous glycerol was added. As the mixture temperature decreased, the hydrogel was formed. Crosslinking was achieved irradiating hydrogels under UVA light (λ = 365 nm) during 60 minutes [**15**].

Hydrogels can be storage in refrigerator (8 °C) or at room temperature (25 °C) under closed hermetically to prevent hydrogel dehydration. Hydrogels have a good storage stability of 1 month, as seen in previous works with crosslinked gelatin hydrogels [**16**].

Material characterization

Samples for scanning electron microscopy (SEM) were dehydrated in a graded series of ethanol (70% and two changes of alcohol 100%). Finally, the samples were subjected to CO_2 supercritical drying [**17**]. Recovered samples were platinum sputter-coated for observation with a Zeiss SUPRA 40 microscope.

ATR-FTIR (attenuated total reflectance) spectra of gelatin, gelatin-riboflavin and gelatin-riboflavin-UV hydrogels were recorded using a Nicolet iS50 Advanced Spectrometer (Thermo Scientific). ATR-FTIR spectra were recorded with 100 scans and a resolution of 8 cm^{-1}. Scanning range was 4000-500 cm^{-1}. All samples were previously freeze-dried.

Differential scanning calorimetry (DSC) was performed using a Perkin Elmer Pyris 1 DSC. The samples were tested from -45 to 100 ºC at a heating rate of 10 ºC min^{-1}, under nitrogen flow.

The rheological characterization of the hydrogels was carried out in a rotational rheometer (Anton Paar MCR 301), using parallel plates of 25 mm diameter. The elastic modulus, G´ and the plastic modulus G´´ were measured in small amplitude oscillatory shear flow as a function of frequency (from 0.1 to 500 s-1) at 20 ºC. Also at 1% deformation, G´ a temperature sweep was performed (from 0 to 55 °C). Strain sweep experiments were performed first in order to determine the linear viscoelastic range. No measurable degradation was observed in the tested sample.

Swelling studies

Freeze-dried gelatin-UV-Riboflavin hydrogels were placed in distilled water to allow them to swell for 0, 0.5, 1, 2, 4 and 6 hours. The hydrogels were removed and the excess liquid was wiped off. The equilibrium swelling ratios (%) hydrogels were calculated using the following equation:

$$\text{Swelling ratio (\%)} = (W_w - W_d) / W_d \times 100\%$$

where, W_w and W_d are wet and dry weights, respectively. Measurements were repeated until constant weights were obtained [**18**].

Cytocompatibility study

MDBK cells ($5x10^4$ corresponding to $2.5x10^4$ per cm^2) in passage 21 were added to a 24-well plate well, along with 1 mL of cell culture medium and incubated in a humidified 5% carbon dioxide chamber for 24 hours. The tested hydrogel was then added where relevant and the effect in cell viability was tested at 24 hours.

For cell metabolic assessment, medium was removed and replaced with 0.45 mL of cell culture medium and 0.05 mL of a 5 mg mL^{-1} MTT solution and incubated in a humidified 5% carbon dioxide chamber for 4 hours. Following incubation, MTT solution was removed, cell surfaces were washed three times with PBS and 1 mL of absolute ethanol was added before leaving to stand for 30 minutes. The absorbance was recorded at 570 nm and readings were converted to survival % compared to cells with no treatment. In all cases results are expressed as mean ± SD from triplicate experiments.

Results and discussion

Material characterization

SEM image for gelatin showed typical scoring in both treatments. The surface of gelatin hydrogels is featureless or mildly wrinkled, showing no significant change in its structure by riboflavin UV crosslinking (**Fig. 1**). For further analysis of the thermal properties, DSC curves were analyzed. Hydrogels showed an endothermic peak at 40 °C, showing the loss of water at that temperature (Tf). In addition, at approximately -25 °C we observe the glass transition temperature (Tg) for Gelatin and Gelatin-Rib hydrogels. Once irradiation is performed, Tg peak is no longer in the thermogram. We assume a stabilization of the

Copyright © 2019 VBRI Press

amorphous state of the hydrogel by the crosslinking agent. (**Fig. 2**)

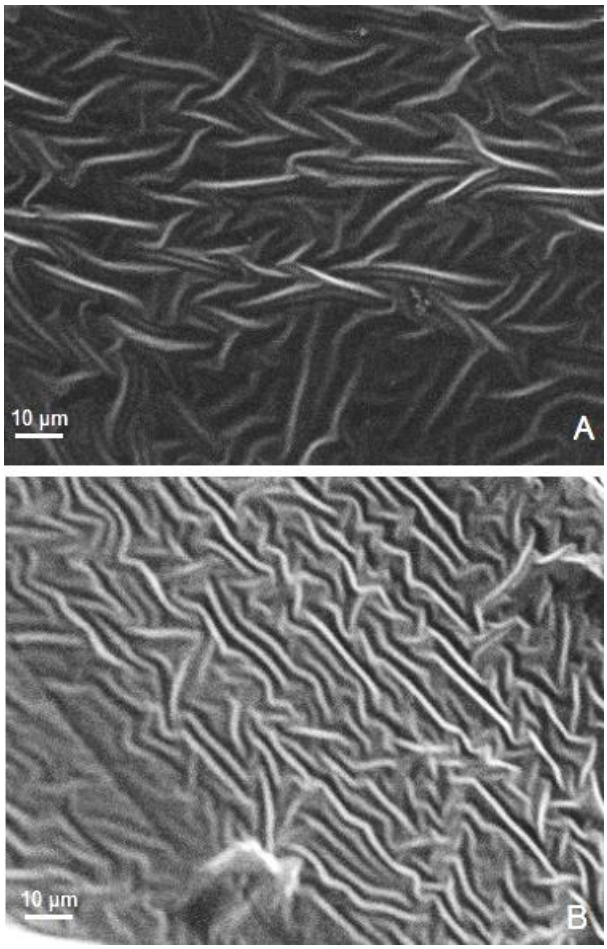

Fig. 1. SEM image for Gelatin hydrogel (A) and Gelatin-Rib-UV hydrogel (B).

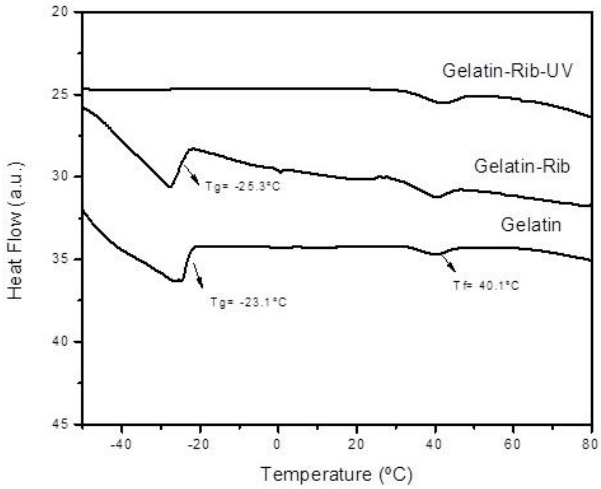

Fig. 2. DSC thermogram for Gelatin, Gelatin-Rib and Gelatin-Rib-UV hydrogels.

FTIR spectra of gelatin showed major peaks in the amide region, with vibration peaks at wavenumbers 1661 cm^{-1} corresponding to the amide I, 1554 cm^{-1} referring to the amide II, at 1239 cm-1 indicating amide III, at 2929.1 cm^{-1} corresponding to the amide B and at 3342.7 cm^{-1} indicating amide A [**19**]. The carbonyl bond stretching of C–N bond appears in the frequency range of 1620–1660 cm^{-1} indicating the Amide I band which is useful in the spectroscopic analysis of secondary structure of proteins [**20**]. C-H stretching modes were reported between 2900 and 3000 cm^{-1} [**21**]. None of the different treatments showed difference in FTIR spectra, due to low riboflavin concentration for UV crosslinking (**Fig. 3**).

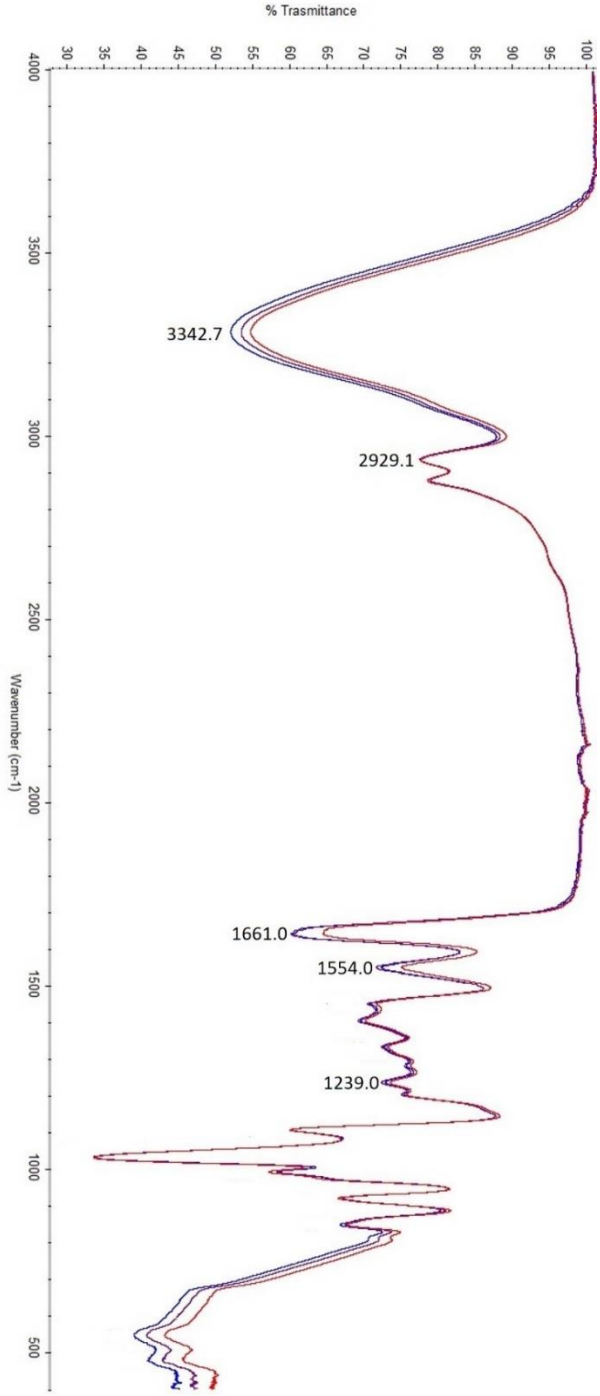

Fig. 3. FTIR spectra for Gelatin (blue), Gelatin-Rib (violet) and Gelatin-Rib-UV (red) hydrogels.

Rheological properties showed an increase of G' and G'' by 20% and 160% respectively when gelatin-

riboflavin UV crosslinking occur. On the other hand, when gelatin-riboflavin hydrogel is not exposed to UV radiation there is no significant change in its plastic or elastic modulus (**Fig. 4A**). In addition, to complement the results obtained from DSC curves, in Tf point, we observed a decrease of G' at 40 °C. The main objective of this measure was to establish thermoresponsive behavior (**Fig. 4B**).

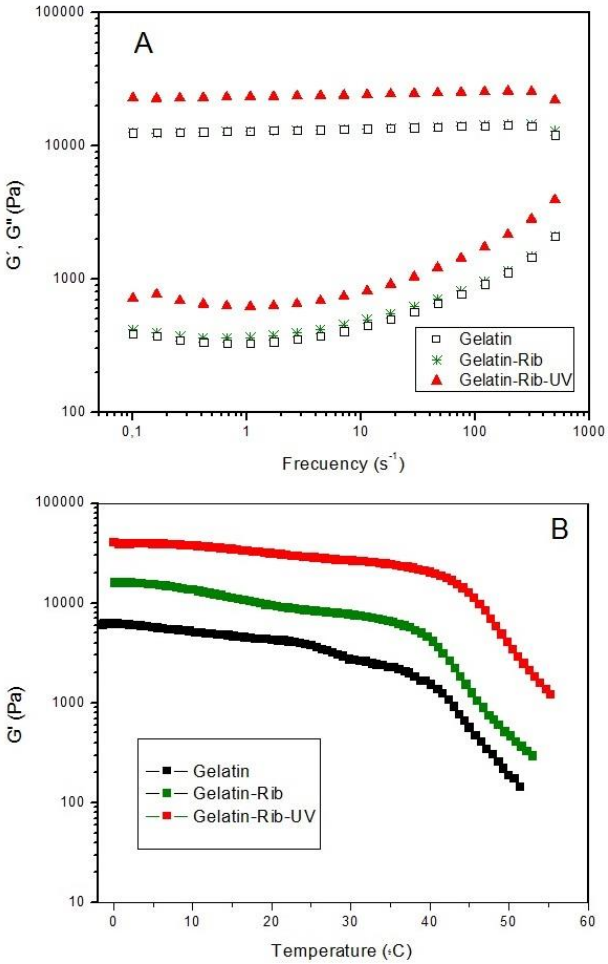

Fig. 4. (A) G' and G'' for Gelatin, Gelatin-Rib and Gelatin-Rib-UV hydrogels. (B) G' vs. temperature for Gelatin, Gelatin-Rib and Gelatin-Rib-UV hydrogels.

Swelling studies

Swelling studies were performed in Gelatin hydrogels. Gelatin-Rib and Gelatin-Rib-UV hydrogels and showed good swelling properties. Showing a 200% swelling ratio for Gelatin hydrogel and 280% for Gelatin-Rib and Gelatin-Rib-UV hydrogels. Gelatin hydrogel and Gelatin-Rib swelling profile reveals that the swelling ratio reached equilibrium in 4 hours. Due to crosslinking, Gelatin-Rib-UV hydrogels as can be seen in **Fig. 5**, reaches the equilibrium in longer time. This shows the equilibrium is delayed until the 24 hour mark is reached. This could be due because water molecules have a harder time filling the empty space in the confined pores left by the crosslinking with riboflavin [**22**]. Hydrogels which have high water content show good permeability and biocompatibility being clear advantages for biomedical applications [**23**] (**Fig. 5**).

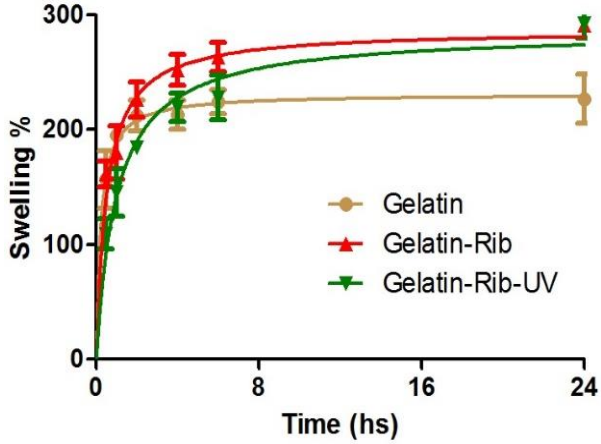

Fig. 5. Swelling ratio for Gelatin, Gelatin-Rib and Gelatin-Rib-UV hydrogels.

Cytocompatibility results

Cell viability in the selected scaffolds compared to cells without contact with the hydrogels was evaluated. Cells without hydrogels in media were studied as a viability control (100%). Gelatin hydrogel is a well-known biocompatible hydrogel [**24**]. When riboflavin was incorporated, cell viability significantly increased ($p < 0.01$) to 130%. Riboflavin is known for its application in cell culture medium as a free-serum supplement, despite its cytotoxicity due to radical oxygen species production while polymerization takes place [**25**]. In spite of the production of cytotoxic species, when riboflavin was treated with UV light, cell viability also increased. This apparently contradicting effect could be attributed to the scavenging of the released free radicals by the gelatin protein chains, leading to a decrease in free-radical-driven cell damage, resulting in an increase in cell viability. (**Fig. 6**)

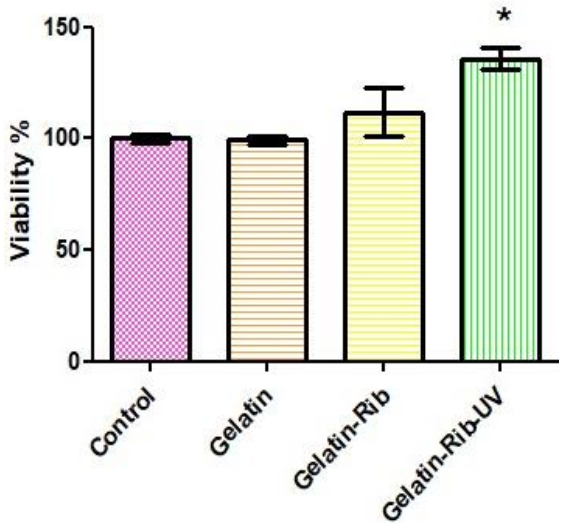

Fig. 6. Biocompatibility assay for Gelatin, Gelatin-Rib and Gelatin-Rib-UV hydrogels.

Copyright © 2019 VBRI Press

Conclusion

A novel hydrogel was synthetized using riboflavin and UV light as a crosslinking agent. In order to achieve thermo-responsive behavior at 40 °C, glycerol was used as plasticizer. In acute skin inflammation the local temperature can reach up to 40 °C, making this material useful for tissue engineering [**26**]. The use of plasticizers in gelatin-based hydrogels enhances the thermal stability of amorphous solid state [**27, 28**]. Thus, the use of crosslinking agents also stabilizes the amorphous solid state of gelatin hydrogel. Incorporation of riboflavin-UV treatment showed an enhancement in its rheological properties [**29**], not shown without UV treatment, confirming UV mediated crosslinking.

As a matter of fact, the scaling up this product can be easily done due to the economic and available methods of synthesis of both components. Also, the fabrication of the hydrogel is simple to achieve. Not of minor importance, the size and shape of the hydrogel can be designed, making the scaffold fit the injured area. Furthermore, the results after the analysis of its swelling properties and biocompatibility shows promise for this Gelatin-Rib-UV hydrogel to be widely used for medical purposes and tissue engineering.

Acknowledgements

J.M.G. is grateful for his doctoral fellowship granted by Universidad de Buenos Aires. The authors would like to acknowledge the support of grants from the Universidad de Buenos Aires UBACYT 20020150100056BA, from CONICET PIP11220120100657CO and from Agencia Nacional de Investigaciones Científicas y Técnicas PICT 2015-2922 (to M. F. D.).

References

1. Johnston-Banks, F. A., *Food gels*, **1990**, 233. **DOI:** 10.1007/978-94-009-0755-3_7
2. Gómez-Guillén, M. C.; Giménez, B.; López-Caballero, M. A.; Montero, M. P., *Food Hydrocolloids*, **2011**, *25*, 1813. **DOI:** 10.1016/j.foodhyd.2011.02.007.
3. Montero, P.; Borderias, J.; Turnay, J.; Leyzarbe, M. A., *J. Agric. Food Chem.*, **1990**, *38*, 604. **DOI:** 10.1021/jf00093a004
4. Hardy, J. G.; Palma, M.; Wind, S. J.; Biggs, M. J., *Adv. Mater.*, **2016**, *28*, 5717. **DOI:** 10.1002/adma.201505417
5. Schmaljohann, D.; *Adv. Drug Delivery Rev.*, **2006**, *58*, 1655. **DOI:** 10.1016/j.addr.2006.09.020
6. Chai, Q.; Jiao, Y.; Yu, X., Gels, **2017**, *3*, 6. **DOI:** 10.3390/gels3010006
7. Raiskup-Wolf, F.; Hoyer, A.; Spoerl, E.; Pillunat, L. E; *J. Cataract Refractive Surg.*, **2008**, *34*, 796. **DOI:** 10.1002/food.19910350433
8. Xiang, J.; Liu, P.; Zheng, X.; Dong, D.; Fan, S.; Dong, J.; Lv, Y.; *J. Biomed. Mater. Res. A*, **2017**, *105*, 2662. **DOI:** 10.1002/jbm.a.36126
9. Rich, H.; Odlyha, M.; Cheema, U.; Mudera, V.; Bozec, L., *J. Mater. Sci. Mater. Med.*, **2014**, *25*, 11. **DOI:** 10.1007/s10856-013-5038-7
10. Caporossi, A.; Baiocchi, S.; Mazzotta, C., Traversi, C.; Caporossi, T., *J. Cataract Refractive Surg.*, **2006**, *32*, 837. **DOI:** 10.1016/j.jcrs.2006.01.091
11. Wollensak G; Spoerl E; Seiler T., *J. Cataract Refractive Surg.*, **2003**, *29*, 1780. **DOI:** 10.1016/s0886-3350(03)00407-3
12. Billiet, T.; Gevaert, E.; De Schryver, T.; Cornelissen, M.; Dubruel, P., *Biomaterials*, **2014**, *35*, 49. **DOI:** 10.1016/j.biomaterials.2013.09.078
13. Elzoghby, A. O.; *J. Controlled Release*, **2013**, *172*, 1075. **DOI:** 10.1016/j.jconrel.2013.09.019
14. Levett, P. A.; Melchels, F. P.; Schrobback, K.; Hutmacher, D. W.; Malda, J.; Klein, T. J., *Acta biomater.*, **2014**, *10(1)*, 214. **DOI:** 10.1016/j.actbio.2013.10.005
15. Ahearne, M.; Coyle, A., *J. Mech. Behav. Biomed. Mater.*, **2016**, *54*, 259. **DOI:** 10.1016/j.jmbbm.2015.09.035
16. Ghassemi, Z.; Slaughter, G., Biopolymers, **2018**, 1. **DOI:** 10.1002/bip.23232
17. Tuttolomondo, M. V.; Galdopórpora, J. M.; Trichet, L.; Voisin, H.; Coradin, T.; Desimone, M. F., *RSC Adv.*, **2015**, *5*, 57395. **DOI:** 10.1039/c5ra08611f
18. Udoetok, I. A.; Wilson, L. D.; Headley, J. V., *ACS Appl. Mater. Interfaces*, **2016**, *8*, 33197. **DOI:** 10.1021/acsami.6b11504
19. Chakka, A. K.; Muhammed, A.; Sakhare, P. Z.; Bhaskar, N., *Waste Biomass Valorization*, **2017**, *8*, 2583. **DOI:** 10.1007/s12649-016-9756-1
20. Hashim, D. M.; Man, Y. C.; Norakasha, R.; Shuhaimi, M.; Salmah, Y.; Syahariza, Z. A., *Food Chem.*, **2010**, *118*, 856. **DOI:** 10.1016/j.foodchem.2009.05.049
21. Susi, H.; Ard, J. S.; Carroll, R. J., Biopolymers, **1971**, *10*, 1597. **DOI:** 10.1002/bip.360100913
22. Omidian, H.; Hasherni, S. A.; Askari, F.; Nafisi, S., *Iran. J. Polym. Sci. Technol.*, **1991**, *5*, 251. **DOI:** 10.1002/app.1994.070540211
23. Echazú, M. I. A.; Olivetti, C. E.; Anesini, C.; Perez, C. J.; Alvarez, G. S.; Desimone, M. F., *Mater. Sci. Eng. C*, **2017**, *81*, 588. **DOI:** 10.1016/j.msec.2017.08.059
24. Saghazadeh, S.; Rinoldi, C.; Schot, M.; Kashaf, S. S.; Sharifi, F.; Jalilian, E.; Yue, K., *Adv. Drug Delivery Rev.*, **2018**, *127*, 138. **DOI:** 10.1016/j.addr.2018.04.008
25. Wollensak, G.; Spoerl, E.; Reber, F.; Seiler, T.; *Eye*, **2004**, *18*, 718. **DOI:** 10.1038/sj.eye.6700751
26. Chanmugam, A.; Langemo, D.; Thomason, K.; Haan, J.; Altenburger, E. A.; Tippett, A.; Zortman, T. A., *Adv. skin wound care*, **2017**, *30*, 406. **DOI:** 10.1097/01.asw.0000522161.13573.62
27. Díaz-Calderón, P.; MacNaughtan, B.; Hill, S.; Mitchell, J.; Enrione, J., *Int. J. Biol. Macromol.*, **2018**, *109*, 634. **DOI:** 10.1016/j.ijbiomac.2017.12.030
28. Lin, J.; Wang, Y.; Pan, D.; Sun, Y.; Ou, C.; Cao, J., *Int. J. Biol. Macromol.*, **2017**, *108*, 947. **DOI:** 10.1016/j.ijbiomac.2017.11.015
29. Mi, S.; Khutoryanskiy, V. V.; Jones, R. R.; Zhu, X.; Hamley, I. W.; Connon, C. J., *J. Biomed. Mater. Res., Part A*, **2017**, *99*, 1. **DOI:** 10.1002/jbm.a.33152

Copyright © 2019 VBRI Press