Supporting information

Investigation of biological activity, mechanical properties and wound healing application of a novel scaffold based on lignin-agarose hydrogel and silk fibroin embedded zinc chromite nanoparticles

Reza Eivazzadeh-Keihan[a], Hooman Aghamirza Moghim Aliabadi[b,c], Fateme Radinekiyan[a], Mohammad Sobhani[a], Farzane khalili[a], Ali Maleki[a*], Hamid Madanchi[d,e**], Mohammad Mahdavi[f], Ahmed Esmail Shalan[g,h,+***]

[a.] *Catalysts and Organic Synthesis Research Laboratory, Department of Chemistry, Iran University of Science and Technology, Tehran 16846-13114, Iran. Email: maleki@iust.ac.ir; Fax: +98-21-73021584; Tel: +98-21-73228313*

[b.] *Faculty of Chemistry, K.N. Toosi University of Technology, Tehran, Iran.*

[c.] *Protein Chemistry Laboratory, Department of Medical Biotechnology, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran.*

[d.] *Department of Biotechnology, School of Medicine, Semnan University of Medical Sciences, Semnan, Iran. Email: hamidmadanchi@yahoo.com*

[e.] *Drug Design and Bioinformatics Unit, Department of Medical Biotechnology, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran. Email: hamidmadanchi@yahoo.com*

[f.] *Endocrinology and Metabolism Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran.*

[g.] *BCMaterials, Basque Center for Materials, Applications and Nanostructures, Martina Casiano, UPV/EHU Science Park, Barrio Sarriena s/n, Leioa 48940, Spain.*

[h.] *Central Metallurgical Research and Development Institute (CMRDI), P. O. Box 87, Helwan, Cairo 11421, Egypt. E-mail: a.shalan133@gmail.com; ahmed.shalan@bcmaterials.net*

[+] Currently on leave from CMRDI

Experimental section

Materials

Aside from silkworm cocoons, all of the applied chemical reagents and solvents together with lignin and agarose polymers, epichlorohydrin (≥99%), ethanol (≥99.8%), sodium hydroxide (≥98%,pellets (anhydrous)), tris(hydroxymethyl)aminomethane (≥99.8%), lithium bromide (anhydrous, ≥99.0%), zinc nitrate hexahydrate (98%), chromium (III) nitrate nonahydrate (≥99.99%), sodium bicarbonate (≥99.7%), ethylenediaminetetraacetic acid (anhydrous, ≥99.0%) as well as the dialysis tubing cellulose membrane (molecular weight cut-off = 14 kDa), were purchased in advance from Sigma-Aldrich.

Preparation of crossed-linked lignin-agarose hydrogel

The synthesis process of crossed-linked lignin-agarose hydrogel was performed as reported in the literature.[1] Briefly, 2.5 g of lignin powder was added to 10 mL of distilled water at 85 °C for 1 h. After the mentioned time, 0.75 mL of NaOH solution (30% w/w) was added to the mixture solution until the pH reached 12. Afterwards, the resulting alkaline suspension solution was cooled down at room temperature. Following that, a fresh agarose solution (5% w/v) was prepared using continuous stirring conditions at 70 °C for 30 min. Then, the prepared agarose solution was continuously added to the lignin solution at room temperature condition. In the next synthesis step, 10 mL of cross-linking agent, epichlorohydrin was rapidly mixed with the mixture polymeric solution and it was stirred for 30 min. Finally, the prepared cross-linked lignin/agarose hydrogel was cast into a petri dish and it was kept at -70 °C for 24 h for pre-preparing freeze-drying route. Later, after 24 h, the solvent sublimation process of freezed petri dish was conducted using a freeze-dryer machine. The dehydration process was continued for 24 h at (-60 °C) and 0.1 bar pressure.

Extraction of SF

The aqueous solution of the natural protein was conducted with some modification, based on the different degumming treatment methods for the extraction of silk fibroin.[2, 3] Briefly, the silkworm cocoons were cut into small parts. Next, the cocoon pieces were boiled in an aqueous $NaHCO_3$ solution (0.21% w/v) for 2 h, to remove the glue like sericin protein. Following the mentioned time (2 h), the elution process of degummed silk fibers was conducted with distilled water for several times. Afterwards, the eluted fibers were dried completely for 12 h at 23 ^{o}C. Then, the dried and degummed fibers were dissolved in aqueous LiBr solution (9.3 M, in H_2O) and the solution was stirred at 60 °C for 2 h. Thereafter, the clear solution was poured into a dialysis tubing cellulose membrane for conducting the dialysis procedure at room temperature. This process was continued for three days to remove the excess of LiBr and other impurities. After three days, the dialysis tube membrane containing silk fibroin solution was removed from distilled water and the purified silk fibroin solution was kept in the refrigerator (4 °C) for the next synthesis step.

Preparation of crossed-linked lignin-agarose/SF hydrogel

As mentioned in our previous research work,[2] in this synthesis step, the first 1.5 mL of crossed-linked lignin-agarose hydrogel was mixed with 1.5 mL of fresh SF solution. The mixture solution was kept under the stirring condition at room temperature for 2 h. Then, the gained crossed-linked lignin-agarose/SF hydrogel compound was cast into petri dish and as well, to pre-prepare it for the freeze-drying process, it was kept at -70 °C for 24 h. After the mentioned time, the solvent sublimation process of freezed petri dish was conducted using a freeze-dryer machine. The dehydration process was continued for 24 h at (-60 °C) and 0.1 bar pressure.

Synthesis of $ZnCr_2O_4$ NPs

As mentioned in the reported research literature,[4] the synthesis process of $ZnCr_2O_4$ NPs was conducted using coprecipitation-calcination method. In this typical method, first, 12.747 g of $Zn(NO_3)_2.6H_2O$ and 34.293 g of $Cr(NO_3)_3.9H_2O$ as metal nitrate precursors were dissolved in 250 mL of distilled water. Next, under the stirring condition, 400 mL of fresh NaOH solution (1 M) was dropped wisely added to the first mixture solution. Following the addition of NaOH solution, the obtained suspension solution was kept under the stirring condition for 20 h. After the mentioned time, in order to remove impurities, the obtained precipitate was filtered, washed with distilled water, and dried (100 °C) overnight. In the next step, the dried solid product calcined at 800 °C for 8 h.

Preparation of crossed-linked lignin-agarose/SF/$ZnCr_2O_4$ nanobiocomposite scaffold

In order to synthesize the crossed-linked lignin-agarose/SF/$ZnCr_2O_4$ nanobiocomposite scaffold, in a small scale, 1.5 mL of fresh crossed-linked lignin-agarose/hydrogel was mixed with 1.5 mL of fresh SF solution. Then, a determined amount of synthesized $ZnCr_2O_4$ NPs (0.5% w/w) was added to the mixture solution. In the next step, the suspension solution was stirred at room temperature for 2 h. Following that, the prepared homogeneous compound was poured into a petri dish and like the previous preparation stages; it was pre-prepared for the freeze-drying method.

Characterization

In order to monitor the functional groups and formation of new chemical bonds, all of the FT-IR spectra were engaged by means of Fourier-transform infrared spectrometer (RT-IR) (Shimadzu FT-8400s model, Japan). In this analysis, a determined amount of each sample (0.1 to 1.0%) was well mixed with 200 to 250 mg of fine KBr powder for sample pellets preparation. Considering the spectral resolution (4 cm^{-1}) in 400 to 4000 cm^{-1} frequency range and as well, the room temperature condition, all of the FT-IR spectra were recorded with the average number of scans between 6 to 18. Furthermore, the structural elements of the

designed cross-linked lignin-agarose/SF/$ZnCr_2O_4$ nanobiocomposite were identified using energy-dispersive X-ray (EDX) spectrometer (Numerix DXP-X10P, Czech Republic) and ultrathin window detector. Moreover, the distributed pattern of structural elements was well checked via the elemental mapping images. Besides, Field-emission scanning microscope (FE-SEM) (ZEISS-Sigma VP model, Germany) was operated at 15 kV, to evaluate and observe the structure, morphology, and size of synthesized scaffolds. By using double sided carbon tape on stainless steel stub, each synthesized sample was mounted and sputter-coated with gold (Agar Sputter Coater model, Agar Scientific, England). In addition, the thermal stability as well as thermogravimetric behaviour of cross-linked lignin-agarose/SF/$ZnCr_2O_4$ nanobiocomposite was evaluated by thermogravimetric (TG) analysis (Bahr-STA 504 instrument, Germany). In this analysis, 5 mg of synthesized nanobiocomposite was exposed to the argon atmosphere. The flow rate of argon was 1L/h. Correspondingly, at a constant heating rate (10 °C/min), the thermal cycle checked between 50 °C to 700 °C.

According to the standard method of ASTM D 882-88, the sample scaffolds were cut into rectangular strips (2.54 cm × 15 cm) using a double blade cutter (Model LB.02/A, Metrotech, S.A., San Sebastian, Spain) and their tensile properties, including tensile strength, elongation at break, and elastic modulus were measured using a universal testing machine (SANTAM, Model STM-20). The machine was operated in a tensile mode with an initial grip separation and a cross-head speed set at 50 mm and 50 mm/min respectively.[5]

In-vitro cytotoxicity assay

MTT assay was used to assess the toxicity and biocompatibility of the synthesized scaffolds. First, human skin fibroblast cells (Hu02) were prepared from the cell bank of Pasteur Institute of Iran and cultured at 1×10^5 cell/well in 96 well plate on the scaffolds under optimal conditions (37 °C, 5% CO_2 in humidified incubator) for 1, 2 and 3 days. Then, their proliferation rate was compared with cells cultured in a culture plate including cisplatin (as a positive control). The negative control was also prepared with supplemented Roswell park memorial institute medium

(RPMI) without scaffolds and cells in each well. In the following, the cells were treated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (10 µL solution of freshly prepared 5 mg/mL MTT in phosphate-buffered saline (PBS)) for 4 h at 37 °C. Next, media with MTT solution was replaced with 2-propanol at 100 µL/mL and plates were shaken gently to facilitate formazan crystal solubilization.[6] Finally, the absorbance of each well was measured at 590 nm using a microplate reader (STAT FAX 2100, BioTek, Winooski, USA). The percentage of toxicity and cell viability were intended via using eqs (1-2):

$$Toxicity\ (\%) = \left(1 - \frac{mean\ OD\ of\ sample}{mean\ OD\ of\ control}\right) \times 100 \quad (1)$$

Cell viability (%) = 100 (%) – Toxicity (%) (2)

In-vitro hemocompatibility assay

RBCs hemolytic assay was applied to detect the blood compatibility of the synthesized scaffolds on human erythrocytes. Primarily, after completing the informed consent form, fresh blood samples were taken from a volunteer with blood type O and a suspension of human RBCs (15% v/v) was prepared in 0.9% NaCl solution. Subsequently, fragments of 4×4 mm were cut from the crossed-linked lignin-agarose/SF hydrogel, crossed-linked lignin-agarose/SF/$ZnCr_2O_4$ nanobiocomposite and 100 µL of this suspension was added to their surface in a 96-well plate. Also, 1% Triton X-100 solution that lysates 100% of RBCs, was considered as a positive control and sterile 0.9% NaCl solution was considered as a negative control. Furthermore, all of these steps were repeated in three wells for each treatment.[7] Next, the plate was incubated for 1 hour at 37 °C and centrifuged at 3500 rpm for 10 min. Then, 150 µL of the supernatant was transferred to a new 96-well plate to measure the absorbance at 414 nm using a

microplate reader (STAT FAX 2100, BioTek, Winooski, USA). The hemolysis percentage of the scaffolds was calculated as follows in eq (3):

$$Hemolysis\ (\%) = \left(\frac{mean\ OD\ of\ sample - mean\ OD\ of\ negative\ sample}{mean\ OD\ of\ positive\ control - mean\ OD\ of\ negative\ control}\right) x\ 1$$

Anti-biofilm assay

In order to evaluate the antimicrobial properties of the synthesized scaffolds, the tissue culture plate (TCP) anti-biofilm assay was used with some modifications.[8] According to this method, 1 cm^2 pieces of crossed-linked lignin-agarose/SF hydrogel, crossed-linked lignin-agarose/SF/$ZnCr_2O_4$ nanobiocomposite, and a polystyrene piece (as a positive control) were sterilized in 70% ethanol aqueous solution and then dried in a sterilized incubator at 37 °C. Next, the samples were placed in three sterilized tube containing selected bacteria (Pseudomonas aeruginosa ATCC 27853) at concentration of 107 CFU/mL in NB culture medium and incubated in a shaker incubator for 24 h with shake speed 150 rpm at 37 °C. Then, the cross-linked lignin-agarose/SF hydrogel, designed nanobiocomposite, and polystyrene pieces were removed from the tubes and washed twice by PBS for anti-biofilm assay. Following that, specimens were stained by 0.1% crystal violet solution for 5 min and washed by 33% acetic acid solution in order to dissociate the bacteria from their surface. Finally, the absorbance of the resulting solutions was measured using a microplate reader (STAT FAX 2100, BioTek, Winooski, USA) at 570 nm.

Statistical analysis

Statistical analysis for the comparison of all biocompatibility, blood compatibility and anti-biofilm results was shown via a t-test by SPSS Statistics 22.0 software (SPSS Inc. Chicago, IL, USA). The values of $P \geq 0.05$ (*), $P \leq 0.05$ (**) and $P \leq 0.001$ (***) were measured as statistically insignificant, significant and very significant, respectively.

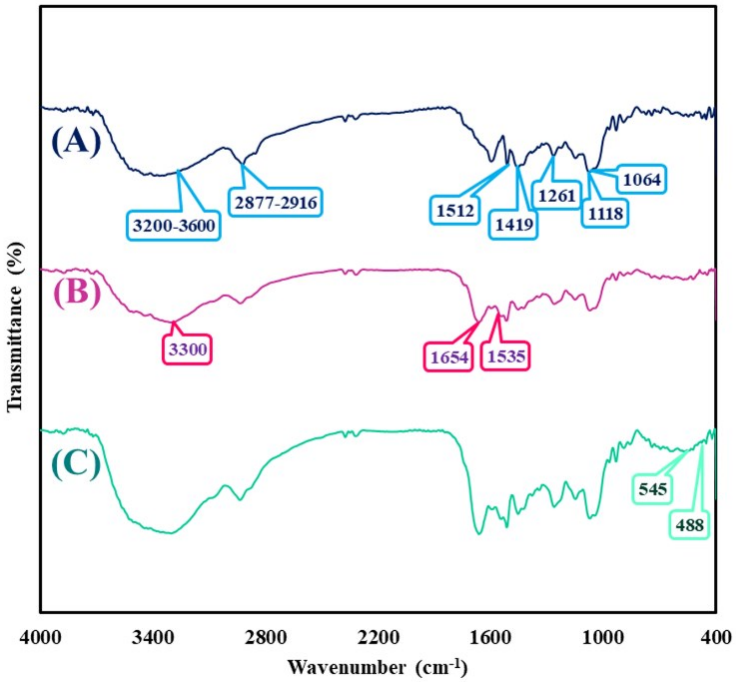

Fig. S1 FT-IR spectra of (A) cross-linked lignin-agarose hydrogel, (B) cross-linked lignin-agarose/SF hydrogel, and (C) cross-linked lignin-agarose/SF/$ZnCr_2O_4$ nanobiocomposite.

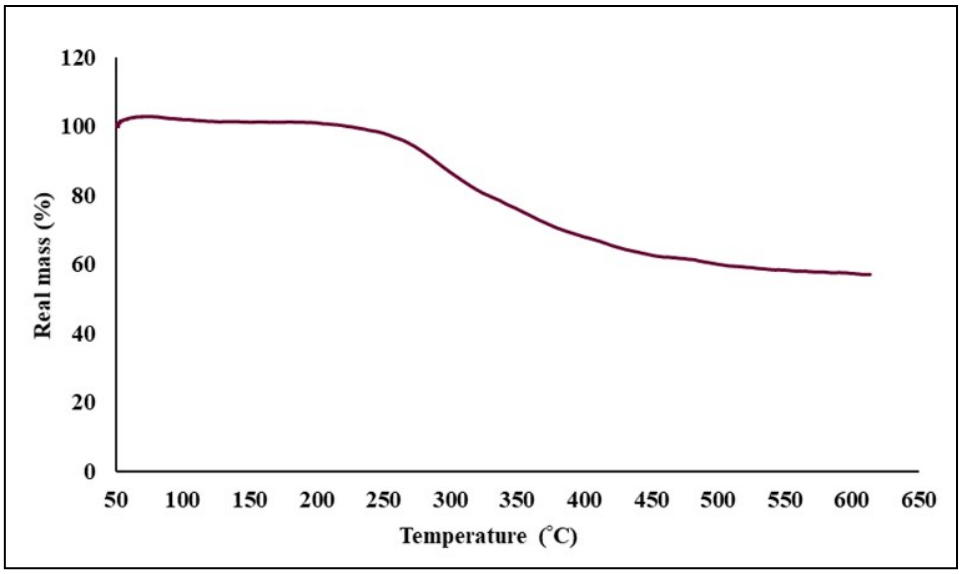

Fig. S2 TG curve of cross-linked lignin-agarose/SF/$ZnCr_2O_4$ nanobiocomposite.

References

1. S. Sathawong, W. Sridach, K. a, Journal of Polymers and the Environment, 2018, **26**, 3307-3315.

2. R. Eivazzadeh-Keihan, F. Khalili, H. A. M. Aliabadi, A. Maleki, H. Madanchi, E. Z. Ziabari and M. S. Bani, *International Journal of Biological Macromolecules*, 2020, **162**, 1959-1971.

3. R. Eivazzadeh-Keihan, F. Radinekiyan, H. Madanchi, H. A. M. Aliabadi and A. Maleki, *Carbohydrate Polymers*, 2020, **248**, 116802.

4. Y. Gao, L. Zhang, A. J. Van Hoof, H. Friedrich and E. J. M. Hensen, *ACS Catalysis*, 2019, **9**, 11104-11115.

5. L. Liu, C. He, M. Xiao, Z. An and S. Lv, *Applied Catalysis A: General*, 2020, 117541.

6. M. Gholipourmalekabadi, M. Mozafari, M. Salehi, A. Seifalian, M. Bandehpour, H. Ghanbarian, A. M. Urbanska, M. Sameni, A. Samadikuchaksaraei and A. M. J. Seifalian, *Advanced Healthcare Materials*, 2015, **4**, 918-926.

7. D. Archana, B. K. Singh, J. Dutta and P.K. Dutta, *Carbohydrate Polymers*, 2013, **95**, 530-539.

8. E. F. Haney, M. J. Trimble, J. T. Cheng, Q. Vallé and R. E. W. Hancock, *Biomolecules*, 2018, **8**, 29.