Characterization of gelatin hydrogels derived from different animal sources

Authors Laura Michelini[†], Luca Probo[†], Silvia Farè*[,†,§], Nicola Contessi Negrini[†,§]

Affiliations

[†]Department of Chemistry, Materials and Chemical Engineering "G. Natta", Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milan, Italy

[§]INSTM, National Interuniversity Consortium of Materials Science and Technology, Local Unit Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milan, Italy

***Corresponding author**

silvia.fare@polimi.it

Abstract

Gelatin hydrogels are a valid alternative to produce scaffolds, wound dressings, and drug delivery systems. However, the animal source from which collagen is extracted to obtain gelatin and its treatment are often underestimated despite they can influence the properties of the obtained gelatin hydrogels. Here, three gelatin powders derived from different animal sources (i.e., porcine, bovine and cold water fish) are chemically crosslinked by two reactions and their physico-mechanical properties investigated. The non-cytotoxic hydrogels swelled differently in water (i.e., porcine > fish > bovine), which in turn influenced the mechanical properties of the obtained hydrogels, highlighting the importance of properly selecting the gelatin source when preparing gelatin hydrogels.

Keywords

Gelatin hydrogel; crosslinking; animal source; gelatin type; physico-mechanical characterization.

1. Introduction

Gelatin is a natural polymer derived from the processing of collagen, which can be obtained from different animal sources including porcine skin, bovine skin and fish [1]. Collagen can be either processed by acidic or lime-curing treatment to obtain type A and type B gelatin, respectively. When crosslinked, gelatin forms hydrogels that gained success as scaffolds for regenerative medicine [2], wound dressings [3] and drug delivery applications [4], thanks to its availability, intrinsic presence of cell-adhesive motifs (i.e., RGD sequence), *in vivo* degradability, and versatility [5]. Different fabrication technologies can be selected to produce gelatin hydrogels (e.g., freeze-drying, particulate leaching, emulsion, 3D printing, electrospinning) and their physico-mechanical properties can be tuned by varying gelatin hydrogel formulation and crosslinking parameters (e.g., concentration, reaction stoichiometry, temperature) [6].

Gelatin-based hydrogels have been successfully produced with desired properties by controlling the preparation and fabrication parameters. However, the choice of the collagen animal source and its treatment has been generally underestimated when producing gelatin-based materials. Indeed, the collagen source and the extraction process used to obtain gelatin might change its aminoacidic composition and physical properties (e.g., isoelectric point, molecular weight), thus heavily influencing the properties of the produced hydrogels [7]. Here, we prepared hydrogels by crosslinking gelatin obtained from three different sources and we compared their physico-mechanical properties.

2. Materials and Methods

2.1. Gelatin hydrogels preparation

All materials were purchased from Merck, unless differently specified. Three gelatin powders, obtained from different animal sources, were used to prepare the gelatin hydrogels: gelatin from porcine skin (gel_A, gel strength ~300 g Bloom, type A), gelatin from bovine skin (gel_B, gel strength ~225 g Bloom, type B) and gelatin from cold water fish skin (gel_F, type A).

Two *non-zero length* chemical crosslinking reactions were used to prepare the gelatin hydrogels, by using *N,N'*-methylenebis(acrylamide), MBA, or glutaraldehyde, GTA, as crosslinkers. A 15% w/v gelatin solution was prepared and crosslinked by adding 0.31 mmol of crosslinker per gram of gelatin. Crosslinking by MBA [2,8] was performed by dissolving gelatin (from porcine skin, MBA_A, from bovine skin, MBA_B, and

from fish skin, MBA_F) in water at 50 °C. After complete dissolution, the pH of the solution was increased at 10.5. Then, MBA was added to the solution and stirred for 10 min. The solutions were poured in Petri dishes, sealed, and stored for 24 h at 50 °C. Crosslinking by GTA [7] was performed by dissolving gelatin (from porcine skin, GTA_A, from bovine skin, GTA _B, and from fish skin, GTA _F) in water at 50 °C. Then, GTA was quickly added under fast stirring and, after 15 s, the solutions were poured in the Petri dishes, sealed, and stored for 24 h at 37 °C. Afterwards, samples were obtained by punching the crosslinked hydrogels. Samples were purified by subsequent immersion in ethanol for 24 h and deionized water for 24 h. Finally, samples were dehydrated by immersion in 50% v/v ethanol solution for 2 h, in 100% ethanol solution for further 2 h and dried under the fume hood.

2.2. Swelling and gel fraction

Swelling tests were performed by placing in multiwell-24 plates anhydrous samples (n = 4), subsequently immersed in 2 mL of deionized water 0.02% w/v sodium azide and stored at 37 °C. All dehydrated samples were characterized by comparable weights at the beginning of the test. At established time points, samples were removed from water, gently swabbed with paper tissue and weighted (W_t). The percentage weight variation ΔW% was evaluated by comparing the sample weight at the different time points to the weights of the anhydrous samples (W_0), by using equation (1):

$$\Delta W\% = \frac{W_t - W_0}{W_0} \times 100 \qquad (1)$$

The gel fraction G_f was calculated as percentage ratio of the weight of samples dehydrated after 7 days of immersion in water at 37 °C, normalized to their initial anhydrous weight [2].

2.3. Compressive mechanical properties

The compressive mechanical properties of the hydrogels were tested by Dynamic Mechanical Analyzer (DMA Q800, TA Instruments). Samples (n = 4) were tested after 1 week of immersion in water at 37 °C. Tests were performed at 37 °C in hydrated conditions. Each test (0.001 N preload) consisted in a load phase at 2.5% min^{-1} up to 30% strain, followed by the unload phase at 5% min^{-1} until 1%. The stress-strain curves were used to calculate the elastic modulus E (slope of the load curve in the 0-5% strain range), the maximum stress σ_{max}, the residual strain ε_{res}, and the hysteresis area H (area comprised between the load and unload curves).

2.4. *In vitro* cytotoxicity tests

Murine fibroblast cell line L929 (ECACC 85103115) was cultured in Dulbecco's Modified Eagle Medium (DMEM) with fetal bovine serum 10% v/v. Hydrogel samples were disinfected in ethanol 70% v/v and dried. *In vitro* indirect cytotoxicity test (ISO 10993-5) was performed by placing in multiwell-24 plates hydrogel samples (n = 3 per time point), immersed in 1 mL of culture medium and incubated (T = 37 °C, 5% CO_2) for 1, 3 and 7 days. Culture medium was also placed in empty wells and incubated as control. L929 cells were seeded in multiwell-96 ($1x10^4$ cells/well) and, when 70% confluent, the culture medium was replaced with the same volume of culture medium eluates or controls. Cells were cultured for further 24 h and alamarBlue™ assay was used to calculate the percentage cell viability (Tecan GENios Plus). Percentage viability was calculated as the fluorescent signal measured for cells cultured in culture medium eluates (f_{eluates}) normalized to the fluorescent signal measured for cells cultured in control medium (f_{control}), after subtracting the background fluorescence of the solution ($f_{\text{AlamarBlue}}$) [2], by using equation (2) :

$$\text{Cell viability}[\%] = \frac{f_{\text{eluates}} - f_{\text{AlamarBlue}}}{f_{\text{control}} - f_{\text{AlamarBlue}}} \times 100 \qquad (2)$$

2.5. Statistical analysis

Data (mean±standard deviation) were analyzed by one-way ANOVA test and Tukey's multiple comparison (GraphPad Prism), considering $p<0.05$ statistically different. Repeated measure ANOVA was used for weight variation tests.

3. Results and discussion

3.1. Weight variation and gel fraction

After immersion in water, all dehydrated hydrogels swelled, increasing their volume and weight over time, as representatively shown after 7 days of immersion (Figure 1A). Macroscopically, samples crosslinked by GTA showed the yellow color typical of GTA crosslinking, while samples crosslinked by MBA were more transparent. Hydrogels crosslinked with MBA reached stable weights after 72 h of immersion, while hydrogels crosslinked by GTA reached stable values at 7 days, with the exception of GTA_B, whose weight increased for all the duration of the test (Figure 1B, Table 1). Comparing the gelatin sources, gel_B hydrogels were characterized by the highest weight variation, followed by gel_F hydrogels and, finally, gel_A hydrogels that showed the lowest weight variation ($p < 0.05$), as previously proved for type A vs. type B gelatin [7]. This swelling response can be attributed to the different collagen source and treatment (acidic

vs. alkaline) used to obtain gelatin, resulting in different aminoacidic composition, protein structure, and molecular weight distribution [1], and isoelectric points and charges [7]. All hydrogels reported percentage gel fraction G_f values higher than 85%, confirming the successful formation of the gelatin hydrogel network (Figure 1C).

Table 1. Weight variation (ΔW%) and elastic modulus (E) of gelatin hydrogels derived from different animal sources and crosslinked by MBA or GTA, after 1 week in water at 37 °C ($^{a,b,c,d,e,f}p<0.05$).

Gelatin hydrogel	**MBA_A**	**MBA_B**	**MBA_F**	**GTA_A**	**GTA_B**	**GTA_F**
ΔW [%]	$872\pm90^{a,b}$	$2523\pm158^{a,c}$	$1283\pm103^{b,c}$	$481\pm24^{d,e}$	$1472\pm33^{d,f}$	$799\pm37^{e,f}$
E [kPa]	$29.7\pm3.4^{a,b}$	$14.2\pm2.1^{a,c}$	$22.3\pm2.5^{b,c}$	$42.4\pm2.7^{d,e}$	$14.3\pm1.3^{d,f}$	$26.6\pm5.8^{e,f}$

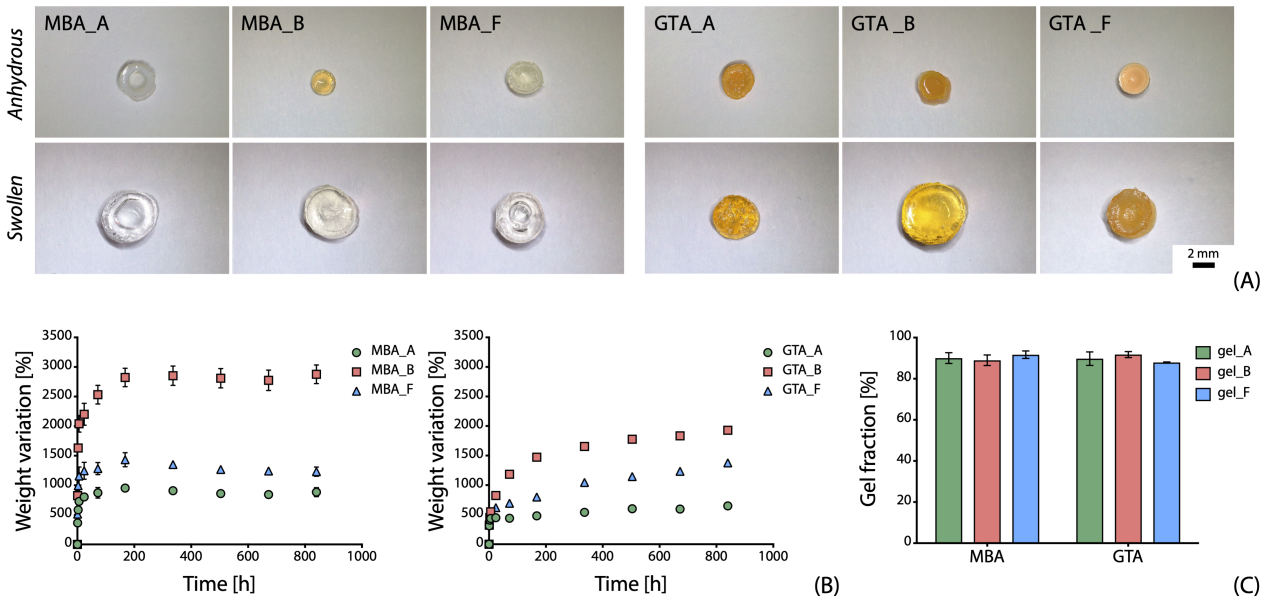

Figure 1. (A) Gelatin hydrogels from different animal sources, crosslinked by MBA or GTA in the dehydrated and swollen form (scale bar: 2 mm). (B) Weight variation of gelatin hydrogels; (C) percentage of gel fraction.

3.2. Compressive mechanical properties

All hydrogels were characterized by a typical viscoelastic response when subjected to a load/unload compressive cycle (Figure 2A).

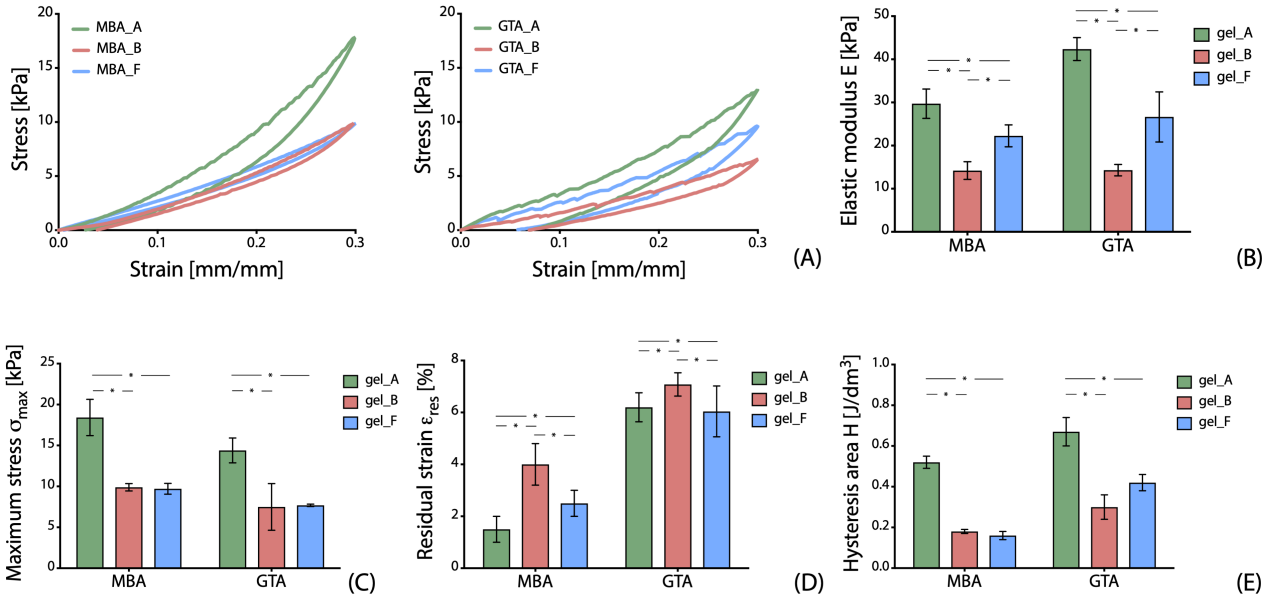

Figure 2. Compressive mechanical properties of gelatin hydrogels from different animal sources. (A) Representative compressive hysteresis cycle of hydrogels crosslinked by MBA or GTA. (B) Elastic modulus E, (C) maximum stress σ_{max}, (D) residual strain ε_{max} and (E) hysteresis area H (*$p < 0.05$).

The mechanical properties of the hydrogels (Figure 2) depend on their swelling. Hydrogels with higher swelling were characterized by lower mechanical properties. The elastic modulus (Figure 2B, Table 1) of gel_A hydrogels was the highest, the elastic modulus of gel_B was the lowest, while gel_F was characterized by intermediate values, using both MBA and GTA. The highest maximum stress (Figure 2C) was observed for gel_A, confirming its higher mechanical properties. The residual strain (Figure 2D) was different for all the hydrogels (gel_A<gel_F<gel_B), indicating higher elastic recovery for gel_A hydrogels. The highest hysteresis area (Figure 2E) was observed for gel_A, indicating higher energy dissipation.

3.3. *In vitro* biological tests

No indirect cytotoxic effects were observed for all the eluates obtained from hydrogels incubated up to 7 days (i.e., cell viability > 70%, Figure 3), despite increased pro-inflammatory response has been detected elsewhere for type A gelatin [9].

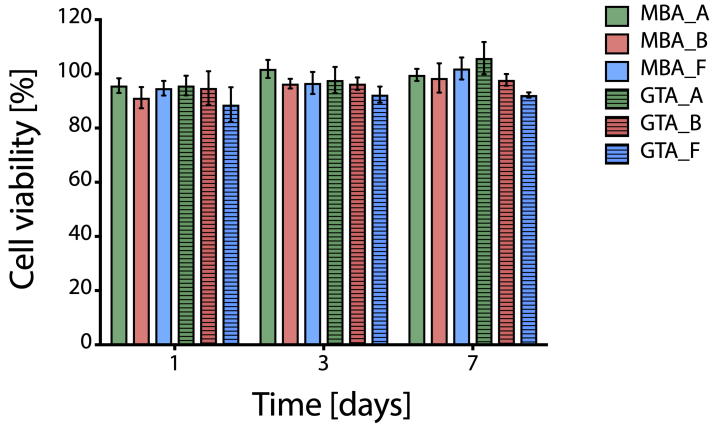

Figure 3. *In vitro* indirect cytotoxicity of gelatin hydrogels.

4. Conclusions

The animal source and treatment of collagen used to obtain gelatin hydrogels influence their physico-mechanical properties. Two different crosslinking mechanisms confirmed similar trends in the hydrogel properties: gel_A was characterized by higher swelling than gel_F and, finally, gel_B, with an inverse trend in the tested mechanical properties. The gelatin animal source and treatment must be considered to properly target desired physico-mechanical properties.

References

[1] S. Pahoff, C. Meinert, O. Bas, L. Nguyen, T.J. Klein, D.W. Hutmacher, Effect of gelatin source and photoinitiator type on chondrocyte redifferentiation in gelatin methacryloyl-based tissue-engineered cartilage constructs, J. Mater. Chem. B. 7 (2019) 1761–1772. doi:10.1039/c8tb02607f.

[2] N. Contessi Negrini, P. Tarsini, M.C. Tanzi, S. Farè, Chemically crosslinked gelatin hydrogels as scaffolding materials for adipose tissue engineering, J. Appl. Polym. Sci. 47104 (2019) 1–12. doi:10.1002/app.47104.

[3] J. Enrione, K. Pino, M. Pepczynska, D.I. Brown, R. Ortiz, E. Sánchez, C.A. Acevedo, A novel biomaterial based on salmon-gelatin and its in vivo evaluation as sterile wound-dressing, Mater. Lett. 212 (2018) 159–164. doi:10.1016/j.matlet.2017.10.049.

[4] M. Qin, J. Jin, Q. Saiding, Y. Xiang, Y. Wang, F. Sousa, In situ inflammatory-regulated drug-loaded hydrogels for promoting pelvic floor repair, J. Control. Release. 322 (2020) 375–389. doi:10.1016/j.jconrel.2020.03.030.

[5] M. Czerner, M. Prudente, J.F. Martucci, F. Rueda, L.A. Fasce, Mechanical behavior of cold-water fish gelatin gels crosslinked with 1,4-butanediol diglycidyl ether, J. Appl. Polym. Sci. (2020) 1–10. doi:10.1002/app.48985.

[6] C.E. Campiglio, N. Contessi Negrini, S. Farè, L. Draghi, Cross-Linking Strategies for Electrospun Gelatin Scaffolds, Materials (Basel). 12 (2019) 2476.

[7] C. Li, I.K.K. Liu, C.Y. Tsao, V. Chan, Neuronal differentiation of human placenta-derived multi-potent stem cells enhanced by cell body oscillation on gelatin hydrogel, J. Bioact. Compat. Polym. 29 (2014) 529–544. doi:10.1177/0883911514553903.

[8] M.C. Tanzi, S. Farè, I. Gerges, Crosslinked Gelatin Hydrogels WO2012164032 A1, 2014, WO2012164032 A1, 2014.

[9] M. Sirova, S. Van Vlierberghe, V. Matyasova, P. Rossmann, E. Schacht, P. Dubruel, B. Rihova, Immunocompatibility evaluation of hydrogel-coated polyimide implants for applications in regenerative medicine, J. Biomed. Mater. Res. - Part A. 102 (2014) 1982–1990. doi:10.1002/jbm.a.34873.