



# Hyaluronic acid cryogels with non-cytotoxic crosslinker genipin

Johanna Roether\*, Claude Oelschlaeger, Norbert Willenbacher

Karlsruhe Institute of Technology (KIT), Institute for Mechanical Process Engineering and Mechanics, 76131 Karlsruhe, Germany

## ARTICLE INFO

### Article history:

Received 4 July 2019

Accepted 12 October 2019

Available online 14 October 2019

### Keywords:

Non-cytotoxic crosslinking

Genipin

Hyaluronic acid

3D scaffold

Cryogelation

## ABSTRACT

For the first time, macroporous, elastic, three-dimensional hyaluronic acid cryogels were prepared with genipin as non-cytotoxic crosslinking agent. These cryogels are characterized by a lamellar porous structure with a homogeneous pore size of  $\sim 100 \mu\text{m}$ , shear elasticity of  $\sim 2 \text{ kPa}$  and a swelling ratio of 2.5 in water. Additionally, multiple particle tracking based microrheology measurements reveal the formation of a heterogeneous network. This novel biomaterial owns great potential as non-cytotoxic alternative for application in drug delivery, as tissue engineering scaffold or wound healing substrate and can help reducing toxicity of artificial skin grafts or tissue equivalents.

© 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

For successful tissue engineering, a scaffold must be biofunctional, biodegradable, biocompatible, with a 3D porous architecture and high degree of pore interconnectivity. Furthermore, it should have appropriate mechanical properties to closely mimic mechanical, and ideally also chemical properties of the extracellular matrix. For fabrication of hyaluronic acid (HA) based-scaffolds, phase separation [1], freeze-drying [2] and electrospinning [3] have been established. Therefore, BDDE (1,4-butanediol diglycidyl ether) [4] and glutaraldehyde [1] were used as crosslinkers. The fabrication of one-component HA scaffolds via cryogelation [5] facilitates the formation of homogeneous pores at a relatively low polymer concentration. Crosslinkers that are reactive with HA at low temperatures are ethyleneglycol diglycidylether (EGDE) [6–8] and EDC/NHS (carbodiimide/N-hydroxysuccinimide) [9]. Mixed cryogels of HA with collagen, gelatine and chitosan were also crosslinked by EDC/NHS [10] and glutaraldehyde [11,12]. Most of the crosslinkers and especially their non-reacting residues are cytotoxic [13,14]. It is consequently desirable to use non-cytotoxic crosslinkers to form stable and biocompatible HA based hydrogels. Genipin is a natural product extracted from the gardenia fruit and it was shown that its cytotoxicity is significantly lower than that of common crosslinkers [15–20]. Genipin has been utilized to crosslink biopolymers, such as chitosan [19] and gelatine [20] or hybrid systems like chitosan/HA [21] but it has never been used to form pure HA gels before. We now proved the suitability of genipin as an alternative crosslinking agent for the fabrication of one component HA porous gels using the cryogelation

technique. Thus, we compared the swelling capacity, structural, micro- and macro-viscoelastic properties of HA scaffolds crosslinked with genipin to the corresponding features of such gels crosslinked with commonly used EGDE.

## 2. Materials and methods

### 2.1. Preparation of cryogels and determination of swelling capacity

Macroporous gels were prepared using the cryogelation technique as previously described [8]. Briefly, HA ( $M_w = 2.0$  to  $2.2 \text{ Mio Da}$ , Contipro) was dissolved in 1% NaOH and EGDE was added. For genipin crosslinking, HA was dissolved in PBS and mixed 2:1 with genipin in DMSO (20 mg/ml, considering the maximum solubility). After mixing for 20 min, solutions were frozen at  $-20 \text{ }^\circ\text{C}$  for 6 days. After thawing, the swelling ratio (SR) was determined by measuring the ratio of the mass of the gel equilibrated in water and un-swollen state.

### 2.2. Rotational rheometry

Gel bulk linear viscoelastic properties were characterized performing oscillatory shear experiments in the linear-viscoelastic regime, using a rotational rheometer Anton Paar MCR 501 (plate/plate, diameter 8 mm, gap 1 mm).

### 2.3. Multiple particle tracking

Local viscoelastic properties of the matrix, namely the pore walls, were investigated using the multiple particle tracking (MPT) technique [22,23]. In MPT experiments, the thermally driven

\* Corresponding author.

motion of inert microspheres that are evenly distributed within a sample is monitored. Here, we tracked the Brownian motion of green fluorescent polystyrene microspheres (diameter 0.19 μm). For performing measurements exclusively in the matrix, particles were added to the polymer solutions before freezing. The displacements of particle centers were monitored at a rate of 50 frames/s. Movies of the fluctuating microspheres were analyzed using a custom MPT routine, incorporated into the software Image Processing System (Visiometrics iPS) and a self-written Matlab program [24], based on the widely used Crocker and Grier algorithm [25].

### 3. Results and discussion

#### 3.1. Cryogels structural properties and swelling capacity

Independent of the crosslinker used, both types of cryogels obtained were spongy, elastic with large pores and they swelled instantaneously when immersed in water. Examples of 3D images of the structure of these cryogels in the wet state, obtained with laser scanning microscopy (LSM 510, Carl Zeiss), are shown in Fig. 1.

EGDE cryogels show interconnected round pores of size ~100 μm and thin matrix wall of ~5–20 μm [8]. For genipin cryogels, the images suggest the formation of a more lamellar porous structure with a pore size almost similar to that of EGDE gels. To our knowledge, this is the first time that pure HA hydrogels have been fabricated with non-cytotoxic crosslinker genipin. Different crosslinking mechanisms are the origin of the formation of these two morphologically different gels. In HA/EGDE gels, epoxy groups of the EGDE are covalently bond to the HA hydroxyl groups under alkaline conditions [6]. Genipin generally reacts with primary amino groups of biopolymers but HA does not have such groups. However, HA has multiple highly reactive hydroxy groups that are able to form e.g. glycosidic bonds, presumably with genipin [26]. The latter are known to be stable as seen in other polycarbohydrates, such as starch. Other gels, based on non-covalent bonds, are known to be stable in water, too. [27]. Although the crosslinking mechanism cannot be fully unravelled here, cryogelation of HA and genipin leads to stable intermolecular bonds, that are strong enough to allow for the swelling of HA/genipin gels in water without dissolving. Without genipin, no stable structures can be obtained.

Despite the higher molar ratio of crosslinker to polymer, HA/EGDE gels exhibit a higher degree of swelling with  $SR \geq 7.5$  compared to HA/genipin gels with  $SR \approx 2.5$ . This may be due to a heterogeneous gel structure with percolating domains of high

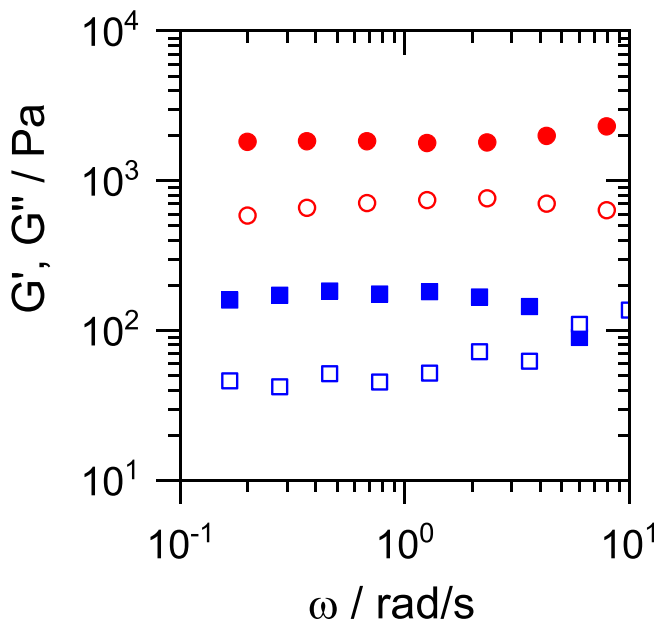


Fig. 2. Dynamic shear moduli  $G'$  (closed symbols) and  $G''$  (open symbols) of 3 wt% HA gels crosslinked with EGDE (blue) and genipin (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1  
Crosslinker concentrations in a 3 wt% HA hydrogel.

| Crosslinker | Concentration/wt% | Reactive groups/<br>μMol/ml | Solvent           |
|-------------|-------------------|-----------------------------|-------------------|
| EGDE        | 0.7               | 40                          | 1% NaOH           |
| Genipin     | 0.5               | 20                          | PBS with 30% DMSO |

crosslink density that limits the swellability of genipin gels. After immersion in water, both compositions show high long-term stability (several months) and shape fidelity, which can be considered mandatory in tissue engineering applications.

#### 3.2. Mechanical properties

In bulk oscillatory shear measurements, gels of both compositions show frequency independent elastic moduli and  $G'$  dominates over  $G''$  in the frequency range from 0.1 to 10 rad/s (Fig. 2).

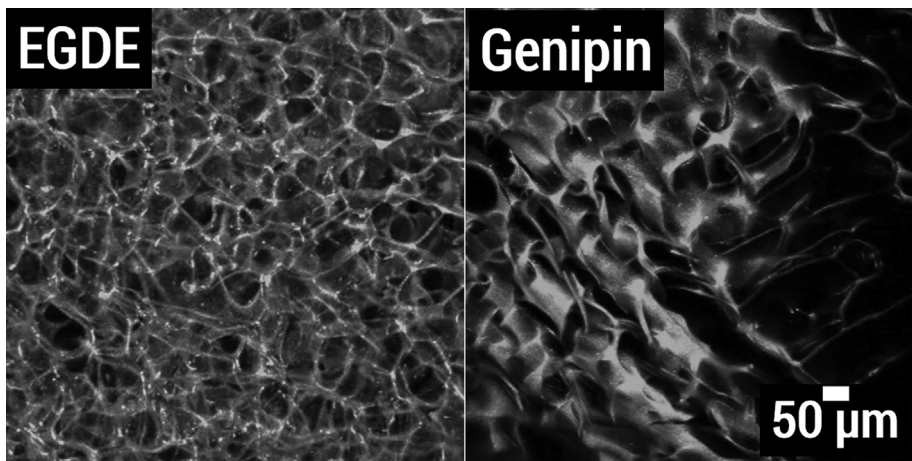
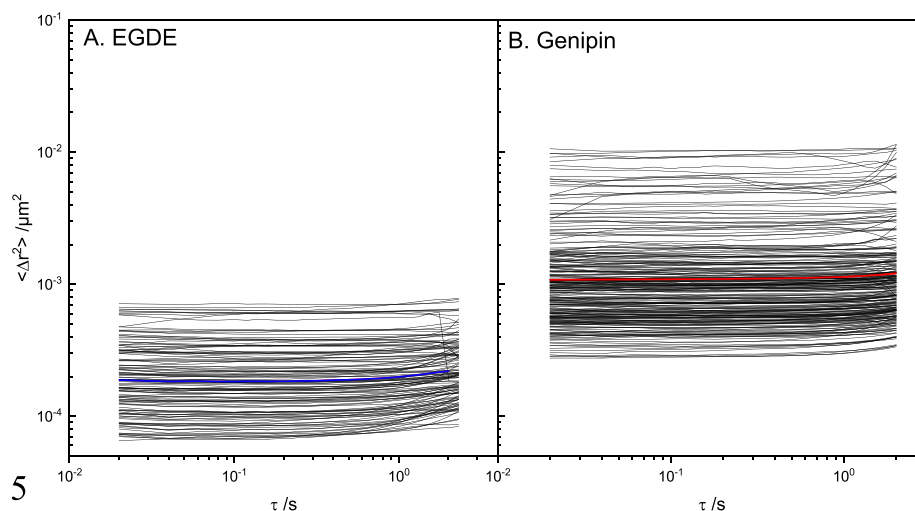


Fig. 1. LSM images of gels structures, crosslinked with EGDE and genipin, visualized by fluorescence of tracer particles embedded in the pore walls.



**Fig. 3.** MSDs of PS particles ( $d = 0.19 \mu\text{m}$ ) dispersed in the matrix of HA 3 wt% gels crosslinked with EGDE (A) and genipin (B). Blue and red curves are the ensemble-averaged MSD.

This is considered as a typical gel-like behavior. Corresponding shear elastic plateau modulus data  $G_0$  (average of  $G'$  values obtained in the probed frequency range) show that the less swollen gel made of genipin provides a higher elastic modulus value  $G_0 \sim 2000 \text{ Pa}$  compared to the highly swollen EGDE gel where  $G_0 \sim 200 \text{ Pa}$ . This is direct evidence of the different crosslink density of the swollen gels. The three-times lower water uptake of the genipin gel outweighs the two-times lower molar ratio of crosslinker to polymer compared to the EGDE gel (see Table 1 and see 3.1).

In order to characterize the local elasticity of the pore walls, microstructural and local viscoelastic properties of the gels were investigated by means of MPT microrheology. Fig. 3 shows the variation of mean square displacements (MSDs) as a function of lag time  $\tau$  for tracer particles with diameter  $0.19 \mu\text{m}$  dispersed in the gel network. In both cases, almost no time dependence of the individual MSDs is found and this result indicates that particles are highly constrained by the surrounding fluid which is consistent with an elastic trapping of particles in a gel-like network.

Additionally, for gels crosslinked with genipin (Fig. 3B), the range of displacements at a given lag time is much broader than for the gel crosslinked with EGDE (Fig. 3A). At  $\tau = 0.1 \text{ s}$ , MSDs vary about two orders of magnitude, from  $\sim 3 \cdot 10^{-4}$  to  $10^{-2} \mu\text{m}^2$  for genipin gels compared to only one order of magnitude from  $\sim 5 \cdot 10^{-5}$  to  $7 \cdot 10^{-4} \mu\text{m}^2$  for EGDE gels.

This indicates a more heterogeneous structure of the HA/genipin network with a non-Gaussian parameter [28]  $\alpha = 5.5$ , compared to the HA/EGDE gel where  $\alpha = 1.4$ . As already mentioned above, this might be the reason for the reduced swelling capacity of genipin gels (see 3.1). The higher absolute value of the average MSD for genipin gels indicates that particles explore a softer environment than in EGDE gels. The discrepancy between micro- and macrorheology which is more pronounced for genipin than for EGDE hydrogels, is presumably due to densely crosslinked regions in the more heterogeneous genipin hydrogels, that are inaccessible for tracer particles but seem to contribute to the overall mechanical strength of the constructs.

#### 4. Conclusion

Genipin can be used as crosslinking agent for producing non-cytotoxic macroporous hyaluronic acid cryogels. Bulk elasticity of genipin and conventional EDGE gels are in the same range,

whereas the local mechanical properties of genipin gels are more heterogeneous. Both gels show similar pore sizes in a well-suitable range for cell culture applications. In genipin gels, the pores appear more lamellar, but the major advantage of using genipin as crosslinking agent is its low cytotoxicity that allows the formation of stable cryogels with a broad range of potential applications, e.g. as cell culture scaffold, in drug delivery or wound healing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### References

- [1] M.N. Collins, C.J. Birkinshaw, *Carbohydr. Polym.* 2 (2011) 1040–1049, <https://doi.org/10.1016/j.carbpol.2012.10.028>.
- [2] H.J. Kim, K.K. Kim, I.K. Park, B.S. Choi, J.H. Kim, M.S. Kim, *Tissue Eng. Regener. Med.* 9 (2012) 57–62, <https://doi.org/10.1007/s13770-012-0007-7>.
- [3] L.J. Nesti, W.J. Li, R.M. Shanti, Y.J. Jiang, W. Jackson, B.A. Freedman, T.R. Kuklo, J. R. Giuliani, R.S. Tuan, *Tissue Eng., Part A* 14 (2008) 1527–1537, <https://doi.org/10.1089/ten.tea.2008.0215>.
- [4] A. La Gatta, C. Schiraldi, A. Papa, A. D'Agostino, M. Cammarota, R. Marcella, R. de Alfredo, M. de Rosa, *Carbohydr. Polym.* 96 (2013) 536–544, <https://doi.org/10.1016/j.carbpol.2013.04.022>.
- [5] V.I. Lozinsky, I.Y. Galaev, F.M. Plieva, I.N. Savina, H. Jungvid, B. Mattiasson, *Trends Biotechnol.* 21 (2003) 445–451, <https://doi.org/10.1016/j.tibtech.2003.08.002>.
- [6] A. Ström, A. Larsson, O.J. Okay, *Appl. Polym. Sci.* 132 (2015) 42194, <https://doi.org/10.1002/app.42194>.
- [7] C. Oelschlaeger, F. Bossler, N. Willenbacher, *Biomacromolecules* 17 (2016) 580–589, <https://doi.org/10.1021/acs.biomac.5b01529>.
- [8] J. Roether, S. Bertels, C. Oelschlaeger, M. Bastemeyer, N. Willenbacher, *PLoS One* 13 (2018), <https://doi.org/10.1371/journal.pone.0207397>.
- [9] T.M.A. Henderson, K. Ladewig, D.N. Haylock, K.M. McLean, A.J. O'Connor, *J. Biomat. Sci., Polym. Ed.* 26 (2015) 881–897, <https://doi.org/10.1080/09205063.2015.1065597>.
- [10] C.-H. Chen, C.-Y. Kuo, Y.-J. Wang, J.-P. Chen, *Int. J. Mol. Sci.* 17 (2016), <https://doi.org/10.3390/ijms17111957>.
- [11] T. Kutlusoy, B. Oktay, N.K. Apohan, M. Süleymanoğlu, S.E. Kuruca, *Int. J. Biol. Macromol.* 103 (2017) 366–378, <https://doi.org/10.1016/j.ijbiomac.2017.05.067>.
- [12] K. Tomihata, Y. Ikada, *J. Polym. Sci. Part A: Polym. Chem.* 35 (1997) 3553–3559.
- [13] J.M. Lohre, L. Baclig, E. Wickham, S. Guida, J. Farley, K. Thy-Agarajan, R. Tu, R.C. Quijano, *Am. Soc. Artif. Intern. Org. J.* 39 (1993) 106–113.
- [14] C. Nishi, N. Nakajima, Y. Ikada, *J. Biomed. Mater. Res.* 29 (1995) 829–834, <https://doi.org/10.1002/jbm.820290707>.
- [15] H.W. Sung, R.N. Huang, L.L.H. Huang, C.C. Tsai, C.T. Chiu, *J. Biomed. Mater. Res.* 42 (1998) 560–567, [https://doi.org/10.1002/\(SICI\)1097-4636\(19981215\)](https://doi.org/10.1002/(SICI)1097-4636(19981215)).

- [16] C.C. Tsai, R.N. Huang, H.W. Sung, H.C.J. Liang, J. Biomed. Mater. Res. 52 (2000) 58–65, [https://doi.org/10.1002/1097-4636\(200010\)52:1<58::AID-JBM8>3.0.CO;2-0](https://doi.org/10.1002/1097-4636(200010)52:1<58::AID-JBM8>3.0.CO;2-0).
- [17] Y. Chang, C.C. Tsai, H.C. Liang, H.W. Sung, Biomaterials 23 (2002) 2447–2457, [https://doi.org/10.1016/S0142-9612\(01\)00379-9](https://doi.org/10.1016/S0142-9612(01)00379-9).
- [18] B.S. Liu, C.H. Yao, Y.S. Chen, S.H. Hsu, J. Biomed. Mater. Res. 67A (2003) 1163–1169, <https://doi.org/10.1002/jbm.a.20017>.
- [19] F.L. Mi, Y.C. Tan, H.C. Liang, R.N. Huang, H.W. Sung, J. Biomater. Sci. Polym. Ed. 12 (2001) 835–850, <https://doi.org/10.1163/156856201753113051>.
- [20] M.F. Butler, N.G. Yiu-Fai, D.A. Pudney, J. Polym. Sci. A 41 (2003) 3941–3953, <https://doi.org/10.1002/pola.10960>.
- [21] S. Deb Nath, C. Abueva, B. Kim, B.T. Lee, Carbohydr. Polym. 115 (2015) 160–169, <https://doi.org/10.1016/j.carbpol.2014.08.077>.
- [22] M. Gardel, M. Valentine, D. Weitz, in: *Microscale Diagnostic Techniques*, Springer, Heidelberg, 2005, [https://doi.org/10.1007/3-540-26449-3\\_1](https://doi.org/10.1007/3-540-26449-3_1).
- [23] T.A. Waigh, Rep. Prog. Phys 68 (2005) 685–742, <https://doi.org/10.1088/0034-4885/68/3/R04>.
- [24] A. Kowalczyk, C. Oelschlaeger, N. Willenbacher, Polymer 58 (2015) 170–179, <https://doi.org/10.1016/j.polymer.2014.12.041>.
- [25] J.C. Crocker, D.G. Grier, J. Colloids Interface Sci. 179 (1996) 298–310, <https://doi.org/10.1038/ncomms8110>.
- [26] M.A. Selyanin, P.Y. Boykov, V.N. Khabarov, in: *Hyaluronic Acid: Preparation, Properties, Application in Biology and Medicine*, John Wiley, New York, 2015, <https://doi.org/10.1002/9781118695920.ch4>.
- [27] J. Zhu, R.E. Marchant, Expert Rev. Med. Dev. 8 (2011) 607–626, <https://doi.org/10.1586/erd.11.27>.
- [28] E.R. Weeks, J.C. Crocker, A.C. Levitt, A. Schofield, D.A. Weitz 287(2000), 627, <https://doi.org/10.1126/science.287.5453.627>