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Mechanical Properties of Architectured Gelatin-Based Hydrogels on Different Hierarchical Levels

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ABSTRACT

Preparation of three-dimensionally architectured porous biomaterials can be achieved in a one-step process by stabilizing gelatin with L-lysine diisocyanate ethyl ester (LDI) in water. The reaction of gelatin with LDI in presence of water leads to the formation of oligourea bridges between gelatin molecules and oligourea chains grafted on gelatin. The number and the length of the bridges, as well as of the grafted chains strongly depend on the concentration of the LDI used for the stabilization, and this has huge influence on the mechanical properties of the material on different hierarchical levels. Higher LDI concentrations yield materials with increased deformation resistance in tensile tests due to the higher number of covalent and physical netpoints in the material. However, mechanical properties determined on the micro-level by AFM indentation showed the opposite trend, i.e. a decrease of Young's modulus with increasing LDI content. This was interpreted by a decreasing number of shorter oligourea bridges between gelatin chains with decreasing LDI content.

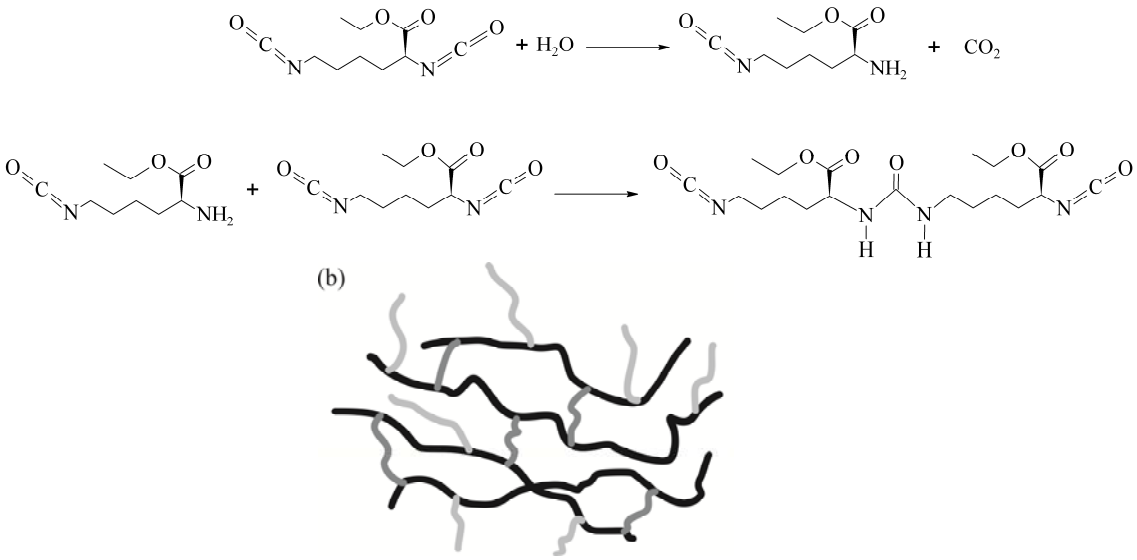
INTRODUCTION

3D architectured gelatin-based hydrogels (ArcGels), prepared in a one-step procedure through the reaction of gelatin with L-lysine diisocyanate ethyl ester (LDI) in water, in presence of poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) used as surfactant, have been shown to be very successful in inducing biomaterial-driven bone regeneration in critical size bone defects [1]. The stabilization of gelatin with LDI in the presence of water is very complex, due to occurrence of several competing reactions [2]. One of the reactions is between isocyanate groups from LDI and water resulting in formation of free amine groups, which can further react with other isocyanate groups and consequently LDI oligomers are formed (Scheme 1a). This leads to the formation of a complex 3D structure which comprises of protein chains (originating from gelatin), oligourea bridges, as well as grafted oligourea chains (both originating from LDI) (Scheme 1b). Differences in molecular structure in ArcGels prepared with different LDI amounts are related to changes in their mechanical properties. It should be noted that the pore size and porosity was constant for the ArcGels prepared with different LDI amounts [1]. Since mechanical properties of the material are crucial for the interaction with cells [3, 4], measuring the elasticity modulus of ArcGels on different length scales is of high importance. It was shown that ArcGels on the one hand showed similar behavior in compression tests [1], regardless of compositions, while in local indentation by an atomic force microscope (AFM) guided with an optical microscope [5, 6], the Young's moduli decreased with increasing LDI content [1]. This is principally in agreement with findings that

nanostructures and arrangements of material on the nano-level can cause different mechanical properties of foams on various scale lengths due to the different deformation mechanisms [7].

Here, the method of local indentation and data interpretation is discussed in more detail for ArcGels of two different compositions, highlighting potential sources of errors as well as discussing a potential molecular basis for the findings. In addition, the mechanical properties of the ArcGels in tensile tests are presented and discussed.

(a)



Scheme 1. (a) Oligomerization reaction of LDI in presence of water, (b) Structure of ArcGel: (—) gelatin, (—) mono or oligourea bridges and (—) grafted oligourea chains

EXPERIMENTAL PART

Materials

Gelatin from porcine skin (type A) with 200 bloom (Gelita, Port Neal, USA) and poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (PEPE) with an average $M_n \sim 14{,}600\ g \cdot mol^{-1}$ (Sigma-Aldrich, Steinheim, Germany) were used without purification. L-lysine diisocyanate ethyl ester (LDI) purchased from Shanghai Infine Chemical Co., Ltd. (Shanghai, China) was distilled under vacuum.

Synthesis of ArcGels

7.5 g of gelatin and 1 g of PEPE were dissolved in 67.5 g of water. The solution was stirred at 1500 rpm and 45 °C in a flat flange cylindrical jacketed vessel with bottom outlet valve (HWS Labortechnik, Mainz, Germany), and subsequently LDI was added. 3- and 8-molar excess of isocyanate groups to amino groups present in gelatin were applied to obtain two different

ArcGels: G10-LNCO3 and G10-LNCO8. After the reaction the formed foams were frozen, washed extensively with water and freeze-dried [1].

Characterization

Tensile tests were performed on Zwick Z2.5 (Zwick GmbH, Ulm, Germany) equipped with a temperature controlled tank with deionized water. Measurements were performed at 37 °C on dog bone-shaped samples 30 mm long and 2 mm wide. A pre-force of 10 mN and a deformation speed of 5 $mm \cdot min^{-1}$ were applied. Values are reported as median of 16 samples with standard deviation.

Micromechanical properties of the scaffold walls were determined by employing optical light microscope (Zeiss Observer Z1, Jena, Germany) together with an atomic force microscope (MFP-3D-Bio, Atomic Force F&E GmbH, Mannheim, Germany). Samples were pre-swollen in water at 37 °C and the measurements were performed at the same temperature. For indentation a pyramidal I-Drive/ BL-TR 400 PB AFM tip was used on 4-5 30 µm x 30 µm areas with 6 x 6 indentation points each on both sample compositions. In this way 144 to 180 data points were generated per ArcGel composition. The calculated Young's moduli are given as average values with standard deviation. Scanning electron microscopy was carried out on the SUPRA 40 VP (Carl Zeiss NTS GmbH, Germany) with a Schottky emitter at an acceleration voltage of 3 kV. Images were recorded on different positions and a representative image is given in the text.

RESULTS AND DISCUSSION

Tensile tests

Tensile test showed that the increase of the LDI content led to the overall increase of the ArcGels deformation resistance (Figure 1).

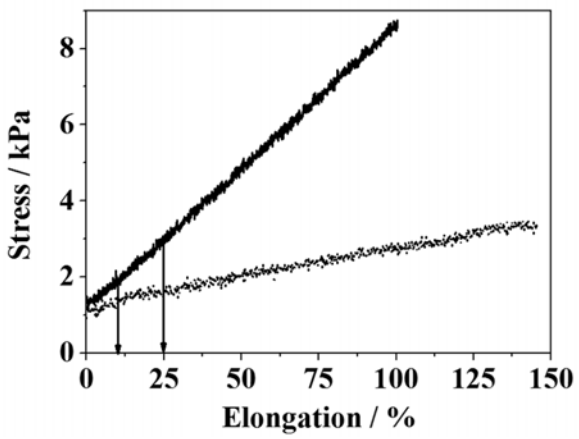


Figure 1. Representative stress-strain curves of (····) G10-LNCO3 and (—) G10-LNCO8

To quantitatively compare the two ArcGels with different compositions, Young's moduli (*E*) were not used since *E* cannot be determined for porous materials (due to not well defined area of the cross section). Instead, values of stress at elongations of 10% and 25% were

determined (Table 1). At 10% elongation, there was only a small difference in stress (1.4±0.1 vs. 1.8±0.1 kPa). To reach the elongation of 25% for G10-LNCO3, a stress of 1.8 kPa had to be applied, while for G10-LNCO8 a stress of 2.9 kPa was necessary. Additionally, the elongation at break decreased with the increase of the LDI content. The mechanical behavior of ArcGels in tensile tests depends on the deformation of the porous structure as well as the bulk properties of the materials. At low deformations, first of all a distortion of the 3D structure is likely, while at larger deformations also the polymer chains will be decoiled, oriented and stretched. The overall behavior can be rationalized by an increase of the number of oligoureas, resulting in covalent and physical netpoints, with increased LDI content. For samples with less LDI content, a lower number and shorter oligourea bridges between gelatin chains can be postulated. Since the macroscopic elasticity of gels is more influenced by the number of bridges than by their length, a higher LDI content caused the increase of the ArcGels deformation resistance. Similarly, theoretical considerations of collagen stabilization through chemical reactions showed that an increase of the crosslink density by factor of 2 increased the elasticity modulus approximately 40 times, while the rigidity of the bridges had a less pronounced influence on the macroscopic material properties [8].

Table 1. Mechanical properties of ArcGels measured by tensile tests

Material	Stress at 10% elongation / kPa	Stress at 25% elongation / kPa	Elongation at break / %
G10-LNCO3	1.4±0.1	1.8±0.1	137±17
G10-LNCO8	1.8±0.2	2.9±0.4	103±10

AFM indentation

Determination of local elasticities was enabled by a combination of an optical microscope with AFM-based indentation. For this purpose, the ArcGels had to be fixated in the AFM with a wire ring. In this way, changes of the material, as could potentially happen by gluing, could be prevented. The first step in the measurement was the identification of an area, which had difference in profile height of less than 3 μm (height of the AFM tip). This was important in order to avoid contact of the cantilever with the elevated parts of the ArcGel surface, which would lead to artifacts. Thereafter, the displacement of the AFM tip for the applied loading force was measured. The applied force is the function of the indentation depth and the mechanical properties of the material (specifically, the Young's modulus). For a tip with conical geometry (which was used here), according to the modified Hertz model [9], the relation between the loading force and the indentation depth is given through the following equation:

$$P = 2\, E_r \tan(\varphi)\, \delta^2\, \pi^{-1}$$

where P is the force, δ is the indentation depth, φ is the half-opening angle of the conical tip and E_r is the reduced elastic modulus. The reduced elastic modulus was determined as the slope of the curve obtained by plotting the loading force as the function of indentation depth. The local Young's modulus of the gelatin material was calculated using the following equation:

$$E_r = [(1-\nu_1^2)\, E_1^{-1} + (1-\nu_2^2)\, E_2^{-1}]^{-1}$$

where E_1 and ν_1 are Young's modulus and Poisson ratio of the ArcGel, and E_2 and ν_2 are the Young's modulus and Poisson ratio of the indentation tip. For every sample around 170 data points were obtained. The values were sorted by the relative frequency of repetition and corresponding histograms were plotted (Figure 2).

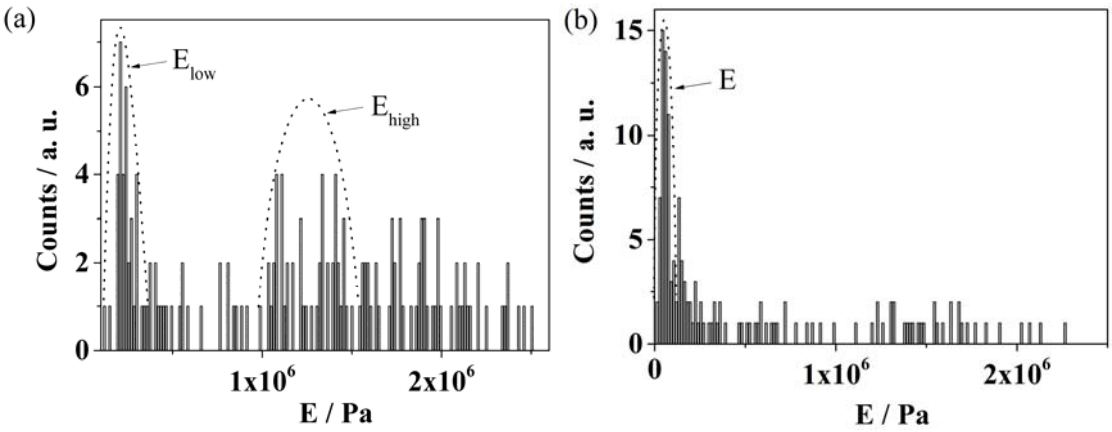


Figure 2. Histograms of Young's moduli measured by nano-indentation technique of (a) G10-LNCO3 and (b) G10-LNCO8

For G10-LNCO3, a broad distribution of Young's moduli was observed, which in the analysis have been arranged in two groups, as depicted in Fig. 2a. From these two groups, two Young's moduli were calculated for G10-LNCO3 ArcGels: E_{high} = 1250±140 kPa with a contribution of 26% to all data points, and E_{low} = 230±35 kPa with a contribution of 17%. Since the contribution of the higher modulus was greater, this value was taken to be the primary E for the G10-LNCO3. In the case of G10-LNCO8, the indentation results were more uniform so that only one Young's modulus of 55±30 kPa with a contribution of 40% to all data points was determined. Interestingly, the increase of the LDI content caused a decrease of the local elasticity modulus in the ArcGels. A potential explanation is that in the indentation experiments the covalent netpoints play a dominant role, and these and/or the shorter and therefore more rigid structure of the oligourea chains are of relevance in G10-LNCO3, while the larger number of physical netpoints postulated for G10-LNCO8 do not contribute strongly to the local Young's modulus.

Limitations of the AFM indentation method

Determination of local elastic properties on a wet, soft, and porous material is highly challenging. First of all, the determination of Young's modulus will be strongly dependent on the placement of the indentation tip. Positioning the tip on the junction of the pore walls or on a particularly thick part of the wall will result in detection of exceptionally high force (Figure 3). However, if the tip is placed on a broken wall, then resistance measured during the indentation is reduced because of the moving of the wall. Furthermore, because of the complexity of the

analyses, only a selected number of pore walls could be investigated, and potentially at other positions of the ArcGel, or in different batches of the same composition there might be a higher variability of elasticities than determined here. In addition to such experimental errors, the fitting of the model to the indentation curve, as well as the in- and exclusion of data points for the calculation adds systematic errors to the determined Young's moduli. Therefore, while the method and statistics are valid, the results should be evaluated in view of these limitations. It should be noted that the positioning of the AFM tip guided by optical microscopy was successful in terms of receiving consistent data. However, *E* values measured for the G10-LNCO3 varied over a broader range than for the G10-LNCO8 samples.

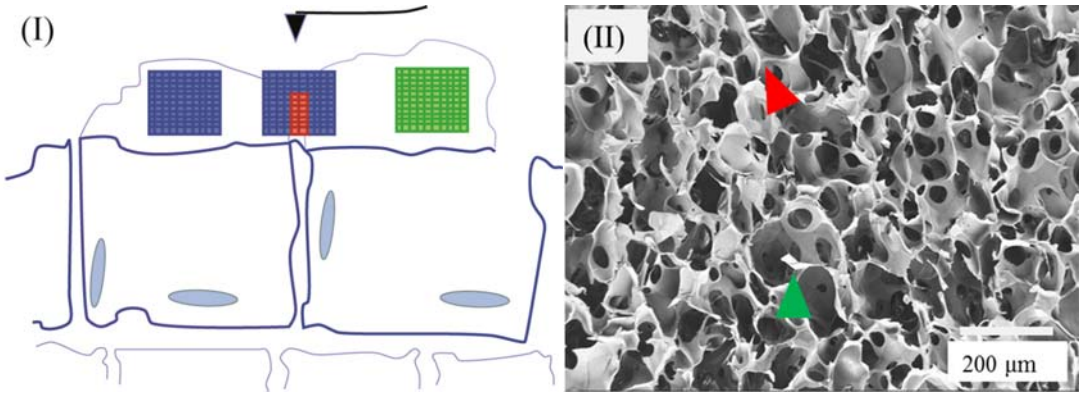


Figure 3. Indentation sites in ArcGels: (I) schematic - A) "correct" position to measure the indentation on an ArcGel wall; B) positioning on multi junction of walls, likely to lead to too high indentation resistance; C) positioning on a moveable piece of a wall; (II) Scanning electron microscopy image: (red) multi junction of walls and (green) movable piece of wall

CONCLUSION

The prepared ArcGels with two compositions have shown to have different mechanical properties and trends on different length scales. Properties on the micro-level, which are important in the interaction with cells, could be successfully determined by employing AFM-indentation guided by optical microscopy. Even though the technique is very sensitive to e.g. the positioning of the AFM tip, the method is highly relevant for determining local Young's moduli on soft, wet, and porous materials. While in these experiments the Young's modulus decreased with the LDI content of the sample, the necessary stress to yield 10 or 25% strain in tensile tests increased with LDI content. This different behavior on different length scales could potentially be rationalized by changes of the molecular structure of the networks. In earlier test [2], it could be shown that oligomerization occurs during the reaction of LDI with amines in aqueous solution, and that crosslinks as well as grafts are formed. It is likely, that at higher LDI content, oligomerization is promoted, so that covalent and physical netpoints contribute to the elastic behavior in tensile tests. In local indentation, however, the short covalent crosslinks play a more important role.

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