Reagent-free mechanical patterning of gelatin surfaces by two-step electron irradiation treatment

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ABSTRACT

Spatial control of mechanical properties of hydrogels such as gelatin is of high interest in order to develop highly functionalized substrates for applications in tissue engineering and biomedicine. Established methods to mechanically pattern hydrogels, including photolithography and ink-jet printing, employ additional reagents to induce changes in the network towards local differences in stiffness. Due to possible toxicity of these incorporated agents, reagent-free techniques are highly desirable for any biomedical field of application. Within the present work, we introduce a two-step process in which the focused electron beam of an electron beam lithography system is first utilized to obtain mechanical patterns in μm-range on air-dried gelatin substrates without any additional reagents. In a second step, these functionalized gelatin substrates are stabilized against gel-sol-transition for physiological conditions by global electron irradiation in the hydrated state by induction of polymer crosslinking. This work investigates precise mechanical patterning of gelatin obtained by this two-step functionalization process using first local and then global electron irradiation. Therefore, structural and mechanical characterization was performed by atomic force microscopy in the air-dried and rehydrated state. These qualitative and quantitative analyses show that excellent mechanical patterning of gelatin hydrogels can be achieved by the presented reagent-free two-step process.

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1. Introduction

Mechanically patterned hydrogels present highly promising functionalized substrates for a variety of applications such as engineered substrates [1,2], biomimetic tissues [3,4], drug delivery systems [5], bioactuators [6] and -sensors [7]. Established techniques to obtain local differences of mechanical properties in polymers such as hydrogels include photolithography [8,9], halftone gel lithography [10] or electrically assisted ionoprinting [11]. However, all these methods require additional reagents which can be inapplicable for biomedical applications due to possible toxicity. We believe that reagent-free mechanical patterning using a two-step process utilizing electron irradiation is an effective and promising method to precisely control spatial material mechanics of gelatin hydrogels with high resolution in the μm-range and even lower [12].

Modification of polymeric materials by focused electron irradiation is commonly used in electron-beam lithography where a highly focused electron beam is scanned in a programmable pattern over a polymer. Thereby, the solubility of the polymer is locally modified by irradiation-induced processes, either by crosslinking (reducing solubility) or by degradation (improving solubility) [14]. Afterwards, the pattern is obtained by applying a developer solution removing the soluble areas of the polymer. The patterned polymer is then used as an etching-mask. However, in the presented study the patterned gelatin is not intended as an etching mask. Here, focused electron irradiation as provided in an electron-beam lithography setup is utilized to directly functionalize air-dried gelatin in order to obtain a mechanically patterned surface.

While electron beam irradiation was already investigated to topographically pattern nm-thin biomolecule layers by degradation-induced ablation [15,16], here the potential of focused electron irradiation to mechanically pattern gelatin additionally is investigated. Therefore, the focused electron beam with a beam diameter of a few nm is scanned in a programmable pattern over an air-dried gelatin layer which was applied to a conductive n-doped silicon substrate to prevent charging effects by enabling electron flow to the ground (see Fig. 1). The gelatin substrates were irradiated in a well-defined pattern of irradiated and non-irradiated stripes each with a width of 1 μm. After irradiation, topographical and mechanical investigations were performed by atomic force microscopy (AFM).

After mechanical patterning, an additional step is necessary to stabilize gelatin against gel-sol-transition (further called "GST-stabilization") for use at physiological conditions. Since unstabilized gelatin has a GST-temperature below human body temperature [17], the stability of gelatin has to be enhanced. This can be achieved by polymer crosslinking [18] for which several methods exist such as polymerization of monomers, vulcanization [19,20], chemical crosslinking [21,22] or ionizing radiation [23–25]. An advantageous technique to

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Step I   Step II
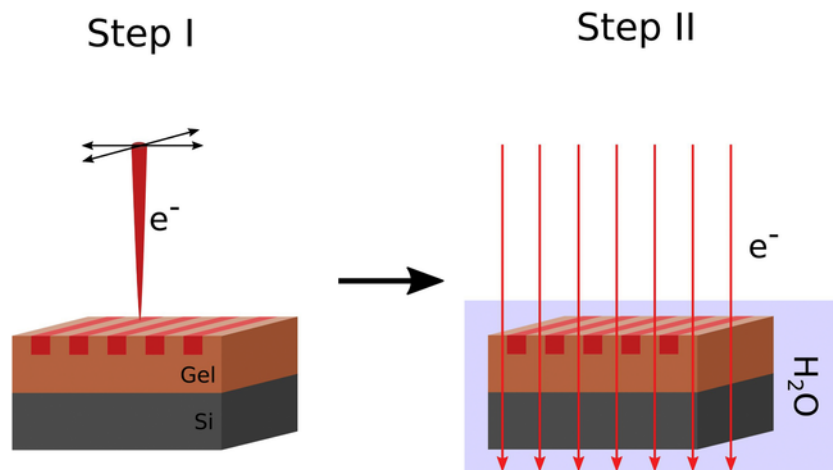
Fig. 1. Schematic image of the two-step process to obtain mechanically patterned gelatin hydrogels. Step I: The focused electron beam of an electron-beam lithography system is scanned in a stripe pattern over a dry gelatin layer which was applied onto a conductive n-doped silicon wafer. Step II: Global irradiation of the patterned samples completely covered with water was used to stabilize the gelatin surfaces against gel-sol-transition.

crosslink hydrogels utilizes homogeneous exposure to an electron beam. Thereby, no additional reagents are necessary such as chemical crosslinkers which could influence material toxicity [26]. Compared to other ionizing radiation techniques such as UV and gamma-radiation, electron irradiation achieves higher penetration depths [27] and crosslinking rates [28], respectively. During electron irradiation, the formation of radicals in the hydrated polymer network is induced, which then recombine into crosslinked covalent bonds [18]. Therefore, the presence of water is required. Primarily to obtain •OH-radicals which attack the polymer chain leading to formation of macroradicals which recombine into the desired covalent crosslinks [18]. In addition, the present water increases the mobility of these macroradicals which supports radical recombination [29]. In fact, this second stabilizing step is necessary because irradiation in the dried state as performed in the patterning step does not lead to a stabilizing crosslinking but to a breakdown of the polypeptide chains [23]. A breakdown of the polymer chains leads to an increase in solubility. The following work investigates the potential of this two-step process to obtain mechanically patterned and GST-stabilized gelatin surfaces with potential applications in biomedical fields.

2. Materials and methods

The gelatin solution was prepared by mixing gelatin powder (G2500; Sigma-Aldrich Chemie GmbH, Germany) with deionized water to obtain a concentration of 6 wt%. Gelatin powder was allowed to swell for 1 h. The mixture was heated to 50 °C until it was homogeneously liquefied and kept at 50 °C before processing. The liquefied gelatin solution was coated onto a conductive n-doped silicon wafer by spin coating (1000 rpm) to obtain layers with a thickness of approx. 1 μm. Gelatin layers were air-dried for one day at room temperature (RT). Thermogravimetric analysis evaluated a rest water content of approx. 12 wt% in the air-dried samples.

Sample patterning was performed by a focused electron beam provided in an electron-beam lithography system (eLine Worksation; Raith GmbH, Germany) with an acceleration voltage of 20 kV and a beam current of approx. 21 pA. Fields of 100×100 μm of the samples were irradiated a stripe pattern of irradiated and non-irradiated stripes each with a width of 1 μm. With a spot size of 4 nm and an exposure time of 9 μs per spot, surface charge densities of 1200 μC/cm^2

were obtained. In addition to the stripe pattern, a control field of 70×100 μm was homogeneously irradiated with the same irradiation conditions. The control field was used to identify the irradiated areas.

After patterning, the substrates were crosslinked by global electron irradiation to stabilize them against GST. Therefore, the samples have been rehydrated for 20 min in deionized water at RT. Thereby, they took up water twice as much as after preparation leading to a gelatin concentration of 4 wt% [25] (at swelling equilibrium). These rehydrated samples were irradiated by a 10 MeV linear electron accelerator (MB10-30MP; Mevex, Ontario, Canada) while completely covered with water. Irradiation was performed by a pulsed electron beam with a repetition rate of 180 Hz and a pulse duration of 8 ms. The electron beam was scanned over the sample with a scanning frequency of 3 Hz. The final dose of 40 kGy was achieved in steps of 5 kGy. The dose was measured with respect to a graphite dosimeter. Thereby, the dose is determined to an uncertainty of 10%. After irradiation, the samples were dried (rest water content of approx. 12%) until they were analyzed by AFM.

The structure and mechanics of the patterned samples were investigated by atomic force microscopy (Dimension Icon; Bruker Nano Surfaces, Germany) in peak force tapping mode. Therefore, tip-cantilever MPP-13120-10 (k=200 Nm^{-1}; Bruker Nano Surfaces, Germany), CONT-MP (k=0.08 Nm^{-1}; Nanosensors, Germany) and qp-BioAC (k=0.1 Nm^{-1}; Nanosensors) were used for the analysis of the dried, rehydrated and GST-stabilized samples, respectively. The two latter samples were measured in water after 20 min rehydration time where they reached swelling equilibrium (gelatin concentration of approx. 4 wt%). The measurements in water were performed with an accessory option (fluid cell) for the AFM (ICONEC-V2-NOPOT; Bruker AXS S.A.S., France). Stiffnesses were characterized by the elastic modulus (E-modulus) determined by the DMT-model [30]. The dry and wet AFM measurements after the first processing step (local irradiation in dry state) were performed at RT to avoid dissolution of the gelatin layers. The AFM measurements after the second processing step (global electron irradiation in wet state) were then performed at 37 °C to ensure thermal stability at physiological conditions. Altogether, three times 4 samples were prepared. Since we experienced excellent reproducibility of the patterns, exemplary data will be shown in the presented work.

3. Results and discussion

In order to identify the irradiated areas, analysis of the control field was performed. The control field was irradiated with the same conditions as the patterned samples but without any pattern. This analysis showed that the irradiated areas are characterized by decreased height and increased stiffness (not shown here) leading to the conclusion that the lower stripes are the irradiated ones. To verify that patterning had been successfully conducted, we investigated the gelatin surfaces by AFM after each processing step. First, we imaged the dry surfaces after the first step of electron beam patterning. Exemplary topographical and mechanical AFM images are presented in Fig. 2. The corresponding profiles are shown in Fig. 3. The substrate shows clear stripes with widths of 1 μm and well-preserved periodicity suggesting successful patterning using focused electron beams. The stripes have height differences of approx. 20 nm. The corresponding stiffness map representing the local E-modulus shows a clear stripe pattern with the same geometry as the topographical pattern. The mechanical profile shows a pattern with an excellent periodicity and moduli in the range of approx. 1.5–3 GPa, which corresponds well with previous studies on the mechanical properties of dried gelatin type B [31]. By analyzing the plateaus, the stiffness of the stripes can be obtained. This analysis leads to small differences in elastic modulus between the irradiated (approx. 2.25 GPa) and non-irradiated stripes (approx. 1.75 GPa), as shown in Fig. 3. The observed maximum and minimum peaks occur due to scanning direction and scanning speed. The stiffness was determined just in retrace-direction. Thereby, the cantilever tip runs into and falls down a ridge. Thereby, the cantilever senses higher and lower moduli, respectively. This could be reduced by reducing the scanning speed, but to avoid high measurement times, a faster scanning rate was used leading to the observed peaks. However, these peaks can be neglected and the moduli of the stripes can be obtained by analyzing the plateaus.

This first modification step (local irradiation) differs from the second (global irradiation) in three key aspects, viz. i) it is performed on dry gelatin, ii) it employs much lower electron energies (20 keV) and iii) realizes higher exposure doses of 1200 μC/cm^2, corresponding to approx. 15 MGy (as inferred from the electronic stopping powers [32]). Due to the absence of water (i) a hydroxyl radical mediated crosslinking mechanism becomes negligible, therefore chain scission will prevail in dried gelatin [33]. For this process, the significantly lower energies (ii) are still sufficient to initiate homolytic scission and in combination with very high doses (iii) electron beam treatment will certainly lead to very profound splitting of gelatin chains, even in

absence of water. Due to a very limited mobility within the dried state [34] crosslinking is only expected at "crossing points" of gelatin chains, e.g. around triple helices, whereas fragmentation is expected to dominate otherwise. The resulting network structure is thus expected to be a degenerated network, comprised of smaller units, crosslinked in regions related to triple helical structures. On the dry gelatin surface, irradiation-induced degradation might lead to additional surface removal in a range of a few nm as observed before [15,16] resulting in the obtained differences in stripe height. Furthermore, the stiffening of the irradiated areas can be explained by degradation-induced increase in density. Here, thermal effects such as heat induced ablation can be neglected since the electron power density ($\approx 3 \times 10^6$ W/cm^2) does not exceed the ablation threshold of condensed media ($\approx 10^8$ W/cm^2 [35]) and we were able to estimate an increase of local temperature of less than 5 K by solving the heat conduction equation for a bi-layered system (1 μm thick gelatin on top of a Si substrate) with finite element calculations and Monte Carlo simulations (not shown here). This change in local temperature is too low to induce thermal ablation. The maintenance of this mechanical pattern after re-hydration is discussed in the following.

Analysis of structure and mechanics of the rehydrated gelatin layers was conducted before thermal stabilization by the second processing step (global electron irradiation). The AFM investigations were performed in humid conditions at RT. Measurements at 37 °C already showed thermal instability leading to dissolution and thus loss of the patterned gelatin layer (not shown here). Exemplary AFM images and corresponding profiles of the measurements at RT are presented in Fig. 4 and Fig. 5, respectively. The images show well-defined stripes after rehydration and thereby well-maintained patterning. Compared to the samples in the dry state, the structures are not as distinct due to a reduced lateral resolution for AFM measurements of soft samples caused by an increased indentation depth and thus increased contact area [36]. However, the stripes are clearly visible and the pattern shows a well-preserved periodicity. Both, topographical and mechanical images show stripes with widths of approx. 1 μm. With a height of approx. 40 nm, the stripes are 1.5 times higher than in the dry state. This height difference demonstrates that the topography is affected by swelling and there are differences in swelling behavior between the irradiated and unirradiated stripes leading to the observed increase in height differences.

Mechanical investigations show a significant decrease in modulus from GPa in the dry state down to kPa-regime for the rehydrated samples. They show moduli of approx. 30 and 400 kPa for the unirradiated and irradiated areas, respectively. The irradiated stripes are
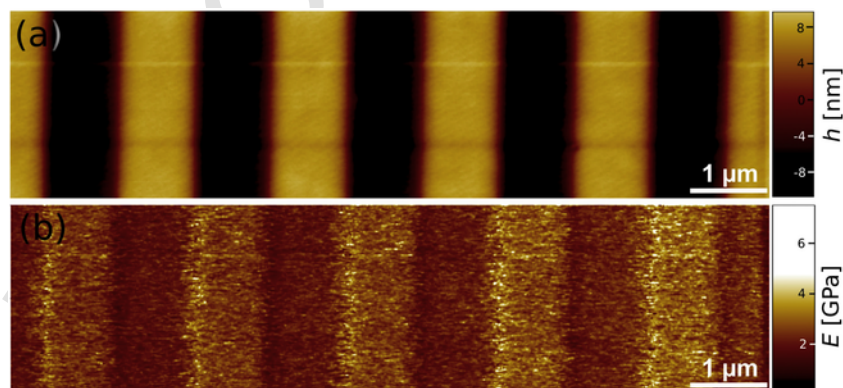
Fig. 2. AFM images of dry gelatin layers which were patterned by focused electron irradiation. (a) Topographical image showing height information of the layer. (b) Mechanical image showing local E-modulus.
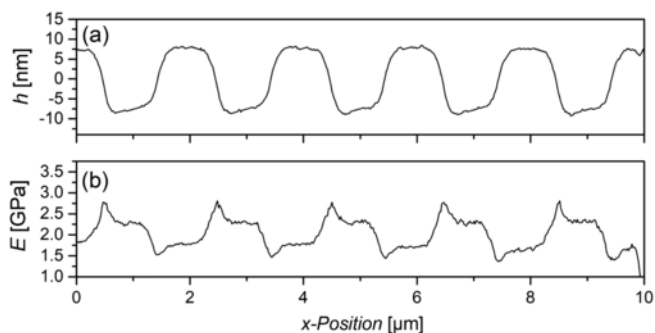
Fig. 3. Topographical and mechanical profile of the stripe pattern in the dry state of patterned gelatin using focused electron irradiation: (a) height and (b) E-modulus. Height and modulus profiles were obtained by horizontal averaging the whole image.

significantly stiffer than the unirradiated ones, indicating successful mechanical patterning of the sample.

In the second processing step, the gelatin layers were GST-stabilized against dissolution at physiological conditions. Therefore, global irradiation with a dose of 40 kGy was performed in the hydrated state. Afterwards, the patterning quality was again investigated by AFM. To demonstrate stability, these measurements were performed at physiological temperatures (37 °C). Exemplary AFM images of height and stiffness are shown in Fig. 6. The corresponding profiles are shown in Fig. 7. Apparently, the stripe pattern is excellently maintained after this procedure and stable at physiological temperatures. The pattern shows similar stripe heights as before (approx. 40 nm) but the mechanical investigations show a general stiffening of the sample. The lower stripes which were already irradiated in the first processing step show now an averaged modulus of approx. 1000 kPa while the previously unirradiated stripes show a modulus of 120 kPa. So both, the unirradiated as well as irradiated areas show an increase in elastic modulus by factor 3–4 indicating general matrix stiffening. That increase in stiffness corresponds well to our previous studies where it was shown that irradiation of gelatin hydrogels with 10 MeV electrons in wet conditions results in dramatic change of mechanical properties already at doses as low as some kGy [25]. These results have been interpreted in terms of covalent crosslinks inserted within the network due to energetic radiation [18]. In addition, we could confirm thermal stability at physiological conditions. This stabilization is also associated with the crosslinking induced by the second processing step.

Concerning stability of the mechanical pattern, long-term stability is expected since the irradiation-induced covalent crosslinking is highly stable over long periods of time [26,37]. Therefore, it is reasonable to assume that long-term stability in dried as well as hydrated state is given. From the performed experiments, we experienced excellent stability for at least hours and weeks for the hydrated and dried samples, respectively. This two-step process thus enables precise mechanical patterning with small topographical differences of max. 40 nm. In order to refer to pure mechano-sensing, the cellular response on this height difference has to be minimized. Since this response, so called contact guidance, is highly cell-type and geometry depending [38], this has to be adapted for the specific requirements and applications. In the case of fibroblasts on silicon wafers with similar grooved structures as described here, the contact guidance threshold was determined at 35 nm [39], but for the here presented samples, this has to be investigated in more detail in an additional project. However, the topographical differences can potentially be adapted by adjusting the process parameters such as irradiation dose. In addition, stiffness can further be modified by varying local and global irradiation dose as well as gelatin concentration for specific applications. Generalization to other bio-derived hydrogels, including collagen, seems feasible, while it is expected that the attainable spatial resolution of the patterns will clearly be affected by the polymer length.

4. Conclusion

The performed experiments show excellent patterning of gelatin surfaces by the described two-step-process involving local as well as global electron irradiation for patterning and thermal stabilization, respectively. Our investigations indicate that focused electron irradiation as used in electron-beam lithography is an effective and precise reagent-free technique to mechanically pattern hydrogels such as gelatin in order to obtain functionalized biomaterials and -surfaces. We were able to show that gelatin layers have a precise topographical and mechanical pattern with excellent periodicity after local irradiation in the dry state and following rehydration. Irradiation using highly focused electron beams further enables creation of various different patterns in the μm-range and even lower. Global electron irradiation in wet conditions further induces thermal stability of the functionalized layers for possible biomedical applications. By varying the hydrogel, irradiation conditions, as well as gel concentration, customization of the reagent-free mechanically patterned polymer substrate is possible.
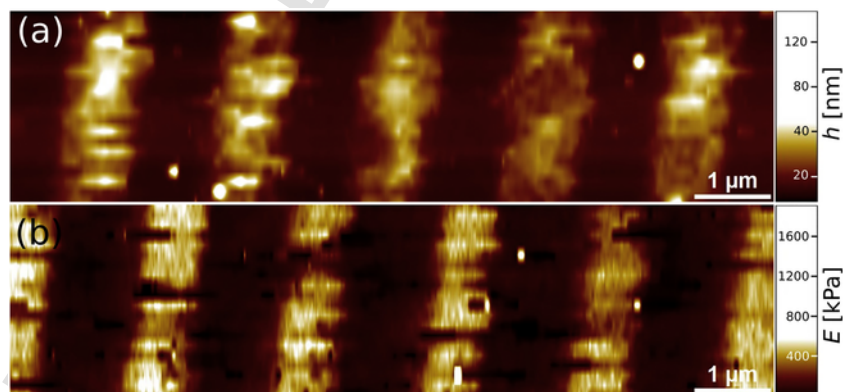
Fig. 4. AFM images of patterned gelatin layers in the wet state after 20 min rehydration. (a) Topographical image showing height information of the layer. (b) Mechanical image showing local E-modulus.
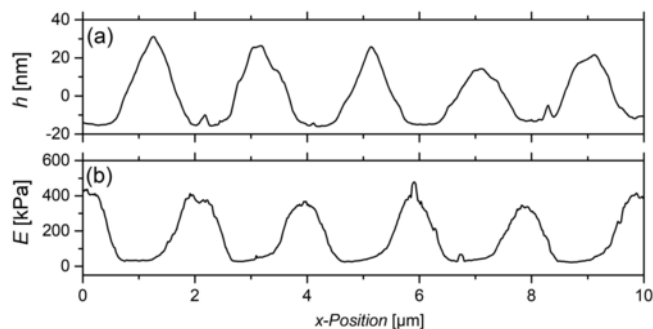
Fig. 5. Topographical and mechanical profile of the stripe pattern after rehydration: (a) height and (b) E-modulus. The profiles were obtained by horizontal averaging the whole image.
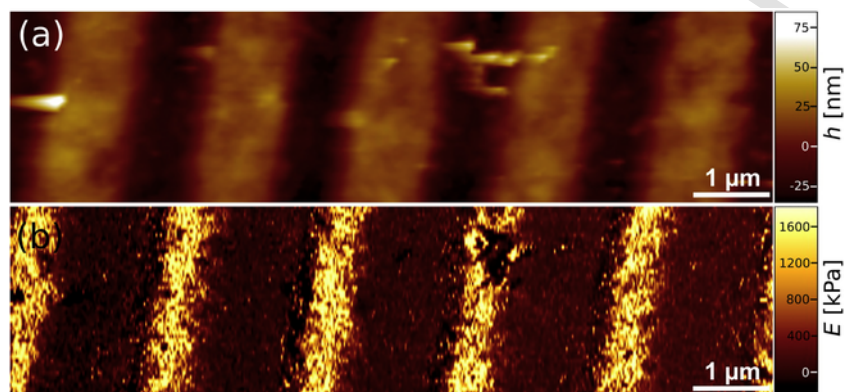
Competing interests
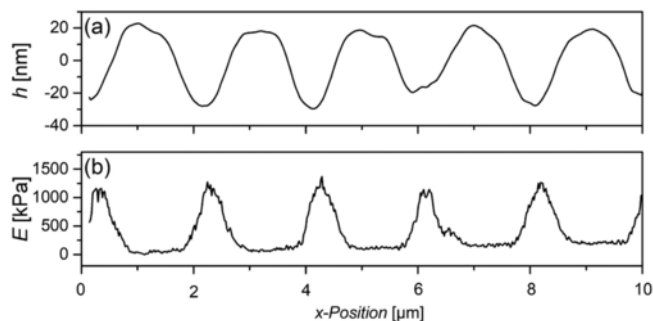
We have no conflicts of interest to declare.

Fig. 6. AFM images of patterned gelatin layers in the wet state after global irradiation. After irradiation, the samples were kept hydrated until measurement. (a) Topographical image showing height information of the layer. (b) Mechanical image showing local E-modulus.

Fig. 7. Topographical and mechanical profile of the stripe pattern after global irradiation in the wet state: (a) height and (b) E-modulus. The profiles were obtained by horizontal averaging the whole image.

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