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Tuning the surface properties of hydrogel at nanoscale with focused ion irradiation

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Abstract

With site-specific machining capability of Focused Ion Beam (FIB), we aim to tailor the surface morphology and physical attributes of biocompatible hydrogel at nano/micro scale particularly for tissue engineering and other biomedical studies. Thin film of Gtn-HPA/CMC-Tyr hydrogels were deposited on gold coated substrate and were subjected to irradiation with kiloelectronvolts (keV) gallium ion beam. The sputtering yield, surface morphology and mechanical property changes were investigated using Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM) and Monte Carlo simulation. The sputtering yield of hydrogel was found to be approximately 0.47 $\mu m^3/nC$ compared with Monte-Carlo simulation results of 0.09 $\mu m^3/nC$. Compared to the surface roughness of pristine hydrogel approximately 2 nm, the average surface roughness significantly increased with the increase of ion fluence with measurements extended to 20 nm at 100 $pC/\mu m^2$. Highly packed submicron porous patterns were also revealed with AFM, while significantly decreased pore size and increased porosity were found with ion irradiation at oblique incidence. The Young's modulus of irradiated hydrogel determined using AFM force spectroscopy was revealed to be dependent on ion fluence. Compared to the original Young's modulus value of 20 MPa, irradiation elevated the value to 250 MPa and 350 MPa at 1 $pC/\mu m^2$ and 100 $pC/\mu m^2$, respectively. Cell culture studies confirmed that the irradiated hydrogel samples were biocompatibity, and the generated nanoscale patterns remained stable under physiological conditions.

Keywords: hydrogel, Focused Ion Beam (FIB), ion irradiation, surface morphology, elastic modulus

1. Introduction

Hydrogels are crosslinked network composed of either natural or synthetic polymers, and the hydrophilic properties of hydrogels make them great materials for bioengineering [1, 2]. It is well known that the shapes and physiochemical properties of hydrogels have strong influence on cell growth and migration, and various approaches have been developed to tune these properties for improved cell growth and biocompatibilities. For example, porosity and pore size could be altered through additional porogens, freeze-drying, photopatterning, foam generation, etc. [3-7], while elastic modulus is a function of component concentrations [8, 9]. In addition to physical and chemical properties, topographic properties at micro and nanoscale have been recently suggested to play critical roles in cellular interaction with its surrounding environments [10-13]. For tissue engineering applications, it is now also advantageous to create nanoscale features on surface with properties similar to those in extracellular matrix in order to control cellular behaviour, enhance cell growth, adhesion and proliferation [10, 11, 13-15].

Previous studies on shape control of surface nanotopology were typically based on soft lithography[16] or the intrinsic elastic property[16, 17], dominantly on Polydimethylsiloxane (PDMS), there are only limited reports on altering surface topology of hydrogels at micro and nanoscale, possibly due to the technical difficulties involved in fabricating micro/nanoscale structure on soft materials, or limited knowledge of fundamental mechanisms involved. Recent reports showed that ion beam irradiation was capable of forming nanoscale wrinkle features on polymer surfaces [18-24]. Typically equipped with a gallium ion (Ga^+) or Helium (He^+) source, a modern Focused Ion Beam (FIB) instrument allows the accelerated ions to perform site specific milling with electrostatic lenses [25-27]. In conjunction with scanning electron microscope (SEM), micro- and nano-machining with resolution down to single digit nanometer can be done while imaging and analysing at the same time. FIB/SEM has been successfully applied for imaging applications such as three dimensional hydrogel [4], cell-material interfaces and even single cells [28-30]. However, the capability of site specific FIB milling is not fully utilized for biomaterial applications.

An example of periodic pattern of micron size dots presented in In Figure 1a was designed and preliminarily patterned on hydrogel surface within minutes, without the use of chemicals or sophisticated masks. This provided a prompt method for fabricating nano/micro scale features on hydrogel, and the final pattern was visible by optical microscope with clearly defined geometry (Figure 1b). Under SEM, however, some morphological artefacts on surface could be observed, and inconsistent geometries such as milling depth were also present due to lacking of information (Figure 1c). As such, the primary aim of the present research is to provide an in-depth investigation on the surface topology and physical properties of hydrogel at nano/micro scale by utilizing FIB, to achieve designed patterns of high precision. The engineering issues including yield and angular effects were investigated through both Monte Carlo simulation and experimental studies. Other questions such as surface roughness and modulus of hydrogel were measured by Atomic Force Microscopy (AFM) prior to and after ion irradiation. Based on these results, patterns on hydrogel with tailored surface topology and physical attributes could be precisely achieved by setting the beam parameters, e.g. acceleration voltage, ion fluence and incident angle. The compatibility and stability of the patterned hydrogels were also tested in cell culture to demonstrate the applicability of this patterning method for bioapplications.

2. Materials and methods

Sample preparation

Carboxylmehtylcelulose-tyramine (CMC-Tyr) and gelatin-hydroxyphenylpropionic (Gtn-HPA) were synthesized according to [4]. CMC-Tyr conjugate and Gtn-HPA conjugates were each dissolved in phosphate buffer saline (PBS) solution at a concentration of 5%. A hydrogel precursor solution was prepared by mixing Gtn-HPA and CMC-Tyr to 80:20 weight ratio, respectively. This precursor went through a vigorous vortex for a few minutes. Horseradish peroxide (HRP) and diluted H_2O_2 were then added to the precursor as cross-linking reagent, each reagent was pre-diluted with PBS solution. A final concentration of 15.5units/L of HRP and 49.8×10^{-6} M of H_2O_2 was used in this research. This precursor was then vigorously vortexed for a few minutes. The pH level of PBS solution, hydrogel before and after cross-linking is measured using a pH metre (Thermo Fisher Scientific, Scoresby, VIC Australia). For PBS solution, pH level was measured to be 7.51 and pH level of hydrogel before and after crosslinking was 7.35 and 7.41 respectively.

An aliquot of 100 µl of the precursor was deposited on a gold plated cover glass and any air bubbles were removed by gently stirring with the tip of micropipette. In order to achieve a thin, uniform layer of hydrogel, spin coating was performed in three stages. In the first stage, spin coating was span for 500 rpm for 10 seconds, followed by 3000 rpm for 40 seconds.

Finally, another 500 rpm for 10 seconds of spin coating provided a thin uniform layer of mixture with the thickness of approximately 1 μm. The precursor was then allowed to crosslink to form hydrogel.

Focused Ion Beam (FIB) irradiation

FIB milling was performed on a FIB/SEM system (FEI Helios NanoLab 600) equipped with a gallium liquid metal ion source (LMIS). Thin film of hydrogel samples were first transferred to the system chamber until high vacuum status is reached. Ion current used in the experiment ranges from 0.92 pA to 0.97 nA. Without additional notes, default overlapping and dwell time were 0% and 3 μs respectively. Ion fluence of the Ga^+ ranging from 0.05 pC/μm^2 to 600 pC/μm^2 was regulated to irradiate 10×10 μm square region. Default incident angle was kept at 0^o (normal incidence), and to study the effect of the incident angle, the stage was tilted from 0^o to 62^o. SEM images were typically acquired with a secondary electron detector with 5 keV acceleration voltage and 86 pA current.

Atomic force microscopy (AFM)

Surface properties were examined using an AFM instrument (Dimension Icon, Bruker Corporation. Santa Barbara, CA, US) under cleanroom environment. For measuring surface topology, cantilevers of 70 kHz of resonance frequency and 0.4 N/m of spring constant were used. By default, 20 × 20 μm regions were scanned to obtain reliable statistics. The sputtering yield of hydrogel was calculated by examining the volume removed by the ion beam irradiation and ion fluence applied. Surface roughness and the characteristics of regular patterns were calculated using software package (NanoScope Analysis 1.4, Bruker Corporation. Santa Barbara, CA, US). For the force measurement and modulus calculation, cantilever with 0.06 N/m spring constant was used to accommodate the low modulus of hydrogel sample with JPK NanoWizard2 AFM (JPK Instruments AG, Berlin, Germany) under ambient conditions. Calibration of the cantilever was conducted prior to the force mapping using mica sheet, measuring sensitivity and spring constant of the cantilever. Force mapping of the sample of 5 x 5 μm regions were done with 16 x 16 resolution. Analysis was carried on using JPKSPM Data Processing software (JPK Instruments AG, Berlin, Germany) which allows to do batch processing.

SRIM Monte-Carlo simulation

Monte-Carlo simulation of the sputtering process of hydrogel was performed with software package SRIM (The Stopping and Range of Ions in Matter) version 2013 [31]. Hydrogel was set up as a new compound consisting of carbon, hydrogen and oxygen with atomic stoichiometry of 8:8:1 respectively. Density of hydrogel was obtained by measuring the bulk weight and volume, and the average values 1.655 g/cm^3 obtained was input for all the simulations. At least 5000 ions were simulated in each run, and parameters including angle of incident and ion energy were varied based on the parameters used in the experiments.

Cell culture of COS-7 cells and cytotoxicity test

African green monkey kidney cells (COS-7 cells) were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 50 units/ml penicillin-streptomycin (P/S) at 37^{o}C in a 5% humidified carbon dioxide incubator. The patterned, unpatterned hydrogel samples and the tissue culture plate were sterilized under UV for 3 hrs before cell seeding. Cell seeding was conducted at a density of 3 x 10^4 cell per sample, and in each sample, the diameter of the hydrogel thin film was approximately 1 cm. The plate was incubated at 37 °C in a humidified 5% CO_2 incubator for 3 hrs for cell attachment to take place, and then 3 mL of fresh culture medium was added and the cells were allowed to further cultivate on the hydrogel. Visualization of the cells on patterned hydrogel was executed using an inverted optical microscope at different time point. The viability of cells after immobilization in the hydrogel was examined using a live/dead viability assay kit, and pristine hydrogels without patterning were used as control. The hydrogels were incubated in 5 µM calcein acetoxymethyl ester solution (Life Technologies, Australia) for half an hour, followed by 1.5 µM Propidium Iodide (Life Technologies, Australia) for 5 mins in DMEM at 37 °C. Images of live (green) cells were then acquired on day 5 by using an inverted laser confocal fluorescence microscope (Nikon A1 Rsi MP, Australia). For SEM imaging, the samples were then kept in the -20 ^{o}C freezer followed by lyophilization in a freeze-dryer (HETO PowerDry PL6000, Thermo Scientific, Australia) for 24 hrs.

3. Results and discussions

Milling yield of hydrogel by keV ion beam

The sputtering yield of hydrogel with ion beam irradiation was measured by volume loss method and compared with the results from Monte Carlo simulation based on SRIM. Ion fluence applied for determining the yield ranged from 0.05 pC/μm^2 to 600 pC/μm^2 to obtain an adequate amount of data points prior to significant redeposition, and sputtering rate was represented as volume (μm^3) removed per ion dose (nC) measured by AFM. The milling was performed on a typical hydrogel surface area of 10×10 μm, and total volume of material removed was calculated and plotted in Figure 2a. The sputtering rate of hydrogel by 30 keV Ga^+ estimated by the gradient of the plotted curve is 0.47 μm^3/nC, while the results of MC simulation with SRIM returned 0.09 μm^3/nC were also plotted in Figure 2a for comparison.

The difference between experimental and simulation results could be due to a few reasons. As MC simulation has certain limitations, including not all the factors such as binding energy of atoms, segregation of elements at the surface are clearly defined for hydrogel. Some estimations were supplemented during the setup phase based on previous reports and data [21, 22]. The surfaces of the irradiated hydrogel were roughened as discussed in later sections and contained porous features which may affect the measurments. Some additional effects such as charging during the irradiation of insulated hydrogel as well as redeposition may also have contributed to the variations in simulation [21, 22, 32, 33]. The experimentally determined yield of this study (0.47 μm^3/nC), however, is close to the reported yield of Polymethyl methacrylate (PMMA) with the same ion energy [34], and will provide a reference value for future tasks.

The effect of beam incident angle (angular effects) on yield was also investigated. As a rule of thumb, an inverse cosine rule was typically applied, in which the yield increases by $1/\cos^n(\varphi)$, where φ is the incident angle measured from the normal of the surface. The simulation result reasonably follows the trend with $n \approx 3$, consistent with the published results in literatures [22, 35, 36]. Incident angle larger than 62^o could not be directly measured due to the restrictions of the currently instrument, thus was not examined in this study. The yield of hydrogel with ions of different acceleration voltage in the keV range was also simulated with SRIM. The results suggested that sputtering yield gradually increased and reached the peak point close to 30 keV. With acceleration voltage approaching 60 keV, the yield of hydrogel started to decrease. This result also agrees with the trends found in previous reports for various materials [35, 37].

Surface morphology of irradiated hydrogel

Surface roughness of hydrogel surface prior to and after irradiation was investigated with AFM, with ion fluence ranging from 0.05 pC/μm^2 to 100 pC/μm^2. Two examples of ion fluence at 0.1 pC/μm^2 and 100 pC/μm^2 were presented in Figure 3a and b respectively. For each obtained images, measurements were performed at multiple locations in both pristine and irradiated regions. At ion fluence of 0.1 pC/μm^2, no significant morphological change was observed, and even the original swelling islands on the pristine hydrogel remained unchanged after the irradiation (Figure 3a). In Figure 3b, a dramatic porous pattern were introduced on bottom of the milled cavity after significantly extended irradiation (100 pC/μm^2) compared to the smoother pristine surface. Detailed measurements of the surface roughness are presented in Figure 3c including side-by-side comparisons with regard to ion irradiation. It is evident that surface roughness was at least doubled after irradiation; however, the average values remained below 10 nm at initial ion fluences. A surge occurred at ion fluence of 1 pC/μm^2, and average surface roughness was elevated to 20 nm at ion fluence of 100 pC/μm^2. This result suggest that nanoscale roughening occurs since initial radiation, while after achieving a certain threshold, erosion type of morphology becomes the dominant appearance on hydrogel surface.

Previous reports showed directional patterns on polymers [18, 35, 38-41], such as oriented ripples, wrinkles, etc., which are mainly induced by the interplay of sputtering erosion and surface relaxation mechanisms, e.g. surface expansion in the direction perpendicular to the direction of the ion beam [39]. In addition to surface roughness, these unique patterns and the distributions are also interesting phenomena and were the subjects to investigation with different ion irradiation parameters in this study. Figure 4a and b presented selected AFM images after FIB irradiation at 0 incident angles (normal) on hydrogel, while the incident angle was raised to 50º and 60º incident angles with results presented in Figure 4c-d respectively. A 2D profile of the AFM measurements is presented in Figure 4g to demonstrate the typical cross section. Overall, porous structures were observed across all the irradiated samples, with average diameters in the order of several hundred of nanometers. Contrary to previous reports, no significant orientation was observed in the patterns generated on the hydrogel surface in this study regardless of the ion fluence or incident angle. This may be due to the fact that synthetic hydrogel possesses more heterogeneous structures compared to single-component polymers, and the sputtering yield was varied across the surface to promote the formation of porous structures. Although it is feasible for hydrogel to have the controlled microscale porous morphology for scaffolding [4], the proposed ion irradiation approach provides unique controllable submicron pores which are challenging for other approaches.

The patterns measured by AFM were further analysed to obtain the details of the submicron pores. By converting the original AFM data to binary images as shown in Figure 4e-f, porosity, as defined by the percentage of void to overall area, could then be quantified. The measured pore size and porosity at varied ion fluence and incident angles were summarized in Figure 5 a-d. The results showed that the size of the porous patterns was not significantly changed with the increase of ion fluence, and the average pore size and porosity are approximately 600 nm and 0.45 respectively (Figure 5a and c). This also suggests that pore formation requires limited ion fluence even at 1 pC/μm^2, and then with the increase of ion fluence, hydrogel surface underwent uniform erosion with similar porous structures. In comparison, results based on higher incident angle (50 to 60 degree) showed similar porous structures (Figure 4c and d) but with significantly smaller pore size and higher porosity (Figure 5b and d). It is a common practice for ion sputtering at oblique incidence to introduce different morphology for inorganic materials [42]. For the current study, the average implantation depth of the incident gallium ion was reduced at higher incident angles, and the overall transferred kinetic energy is in closer proximity of the top surface layer. This allowed sputtering at top layer to be more effective compared to other dynamic surface mechanisms, and thus resulted in pores of higher density.

Modulus of irradiated hydrogel

Mechanical elasticity has been widely accepted as a controllable factor for cell growth and differentiation [9, 43]. Though it is feasible to tune the elasticity of polymers including hydrogel in bulk forms by varying the concentrations [8], controlled elasticity modification particularly at submicron scale has never been established. Previous studies [39, 44] showed significant higher modulus of PDMS after keV ion irradiation, and in the present study, we also aim to investigate the modulus through FIB irradiation *in situ*. The results measured by AFM force spectroscopy are presented in Figure 6 with ion fluence from 1 pC/μm^2 to 100 pC/μm^2. Compared to pristine hydrogel, the average modulus was significantly elevated with the increase of ion fluence, from less than 20 MPa for pristine hydrogel to 250 MPa at ion fluence 1 pC/μm^2 and 350 MPa at ion fluence 100 pC/μm^2. All the AFM modulus measurements were performed under ambient conditions, and although preferred, measuring modulus of hydrogel in liquid medium remains a challenge, as it is well known that hydrogel response in water is varied. Also in order to accommodate the estimated modulus range of hydrated hydrogel, soft cantilevers with spring constant of 0.03 N/m were initially used in a few attempts, and cantilevers were likely broken at the air-liquid interface, possibly due to surface tension at the air-liquid interface. The fittings based on a few successful measurements suggested that the modulus of hydrated is in the range of 100 kPa to 1 MPa, consistent with the reported hydration induced effects [45, 46]. The increased water content in the hydrogel may have resulted in larger probe-surface contact area, and the actual relationship of hydration-modulus after ion irradiation will be an interesting topic of future study.

It is interesting to note that site specific modulus tuning of post-crosslinking hydrogel accomplished in this study has not been feasible with other existing approached. Although focused electron beam was deployed for localized crosslinking of hydrogel [47, 48], the fabricated features were attached to substrate such as silicon and was not applicable for tissue engineering. Swelling effect of the electron patterned hydrogel was reported, but so far no details on modulus were provided. In the current study, nuclear stopping of the gallium ions is expected to be dominant in conjunction with the MC simulation results, and that causes substantial nuclear displacements and scission of bonds in the target hydrogel. A transition from one-dimensional chains into a three-dimensional matrix may provide the explanation for the elevated Young's modulus [49, 50], although a more conclusive study is needed in the future.

Results of cell culture on patterned hydrogel

To assess the stability of the generated patterns, ion irradiated samples were kept in physiological environment for 48 hrs, i.e. with the same culture medium and temperature but without seeding cells. After dehydration, the samples were finally investigated with both optical and SEM imaging (Figure 7a-b), and the original FIB milled patterns were clearly present without noticeable changes. This suggests that the FIB milled patterns are stable in cell culture environment, at least prior to the controlled dissolution of hydrogel. Although increased roughness after ion irradiation resulted in larger contact surface area, hardening of the top layer as shown by the increased modulus may provide additional barrier against physical and chemical modifications of the patterns.

To demonstrate the biocompatibility of FIB patterning on hydrogel, cell culture experiment was performed on the irradiated samples. Optical images recorded right after seeding and at 44 hrs after seeding are presented in Figure 7c-f. For both irradiated and non-irradiated samples, the COS-7 cells reached confluence, and displayed typical spread out morphology. The same morphology was observed in the 10 μm ion irradiated square regions (Figure 7d) compared to the cells grown on the irradiated hydrogel surface. In addition, confocal microscopy was performed to provide higher magnification investigations, with results presented in Figure 7g-h. It is clear that, for both irradiated and non-irradiated samples, COS-7 cells were firmly adhered to hydrogel, a scenario consistent with this type of hydrogel in the literature [4]. For the cells grown on the rectangle patterned regions as shown in Figure 7h, no measurable difference in morphology was found. The viability of these cells was confirmed by the consistent green fluorescent color, without the presence of any dead cells. These results confirmed that gallium ion beam irradiation has negligible effects on cell growth, although gallium is generally considered as toxic [51, 52]. From SRIM results, majority of the incident gallium will be eventually implanted in the target hydrogel, and possible source of the toxicity, the free gallium ions, are expected to be minimal on the hydrogel surface. It can also be inferred that patterns generated by other commercial available FIB sources based on noble gases such as Helium, Neon, etc. will likely be biocompatible, although additional studies will be preferred to confirm it. The results of both experiments presented above proved the stability and compatibility of these nano/micro scale patterns on hydrogel, and paved road towards various bioapplications.

Conclusion

In conclusion, Focused Ion Beam milling was performed on the thin films of hydrogels to tune various surface properties including surface morphology and modulus, characterized by electron microscopy and AFM. The sputtering yield of hydrogel by keV gallium ion was examined experimentally and compared to results obtained from Monte-Carlo simulation. It was revealed that the surface roughness was doubled after low dose irradiation, and significant increase of Young's modulus was also confirmed. During irradiation, unique nanoscale porous features in regular formation were also observed, and the pore parameters are subjects of ion incident angles. Cell culture experiments confirmed the biocompatibity and stability of the patterns generated based on FIB with gallium sources. Given the *in situ* high precision capability and sufficient yield compared to laser and electron beam based approaches, we expect the proposed approach will provide tunable submicron features on hydrogel which are unique for future research in tissue engineering and biosensing.

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