X-ray ablation of hyaluronan hydrogels: Fabrication of three-dimensional microchannel networks

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We present a simple and highly versatile protocol for polymer ablation: hard x-ray irradiation makes it possible to rapidly depolymerize hyaluronan hydrogels and fabricate three-dimensional network of microchannels. Photodynamic and photochemical analyses show that x-ray irradiation directly cleaves the polymer backbone and the total dose controls the degradation kinetics. This nonthermal ablation protocol may offer opportunities for processing organic polymers and biological materials. © *2009 American Institute of Physics*. [doi:10.1063/1.3213358]

I. INTRODUCTION

Ablation of organic polymers and biological tissues has been extensively studied scientifically and technologically[1–6] since the first development using laser photons in 1982.[1,2] Laser ablation is a useful strategy to rapidly decompose polymers by photothermal and photochemical effects. However, high-intensity laser radiation may cause damage to matter through intensive heating, liquefaction, evaporation, and dielectric breakdown.[3] Organic polymers and biological materials can be very sensitively altered by thermal damage as far as their function and structure are concerned. Ultrafast laser pulses may reduce thermal damage but such sources are still under development.[7,8] It is thus desirable to develop a versatile and robust protocol to ablate polymers with a finely tunable "nonthermal" ablation technique.

Hyaluronan [salt form of hyaluronic acid (HA)] is an important extracellular and cell-surface associated polysaccharide.[9] It is commonly synthesized as a large, negatively charged, linear polysaccharide that is composed of repeating disaccharide units of glucuronic acid and *N*-acetylglucosamine: $[-\beta(1,4)\text{-GlcUA-}\beta(1,3)\text{-GlcNAc}-]_n$. HA has an important role in tissue homeostasis and biomechanical integrity via remarkable physicochemical characteristics such as viscoelastic and hygroscopic properties. These properties of HA lead to its widespread applications for bioengineered tissue scaffolds.[10–13] Related physiological functions stimulate interest on its role in cell biology, pathology, immunology, and cancer research.[9] The microfabrication of cell-laden HA architecture[12,13] to resemble three-dimensional (3D) cellular microenvironments[14–17] is also an important issue in HA. The HA molecular weight (MW) is, in general term, of critical importance because of its remarkable effects on cell activities.[10] Although low MW HA, required for biomedical applications, is produced by enzymatic[10] or nonenzymatic degradations,[11] there are few reports on reliable, effective methods to fabricate 3D architectures of HA hydrogels.

In this study, we introduce a protocol using a short x-ray irradiation to ablate bulky HA hydrogels based on well-controlled degradation kinetics: approximately 1 mg of HA rapidly degrades within 30 s of hard x-ray irradiation, with the same specific cleavage as in enzymatic degradation. With this fast x-ray ablation process, we are able to fabricate 3D HA hydrogel microchannels, as illustrated in Fig. 1. The ablation process occurs through a gel-to-sol transition as discussed later. Note that the depth and the width of the channels are tunable by adjusting the x-ray dose and the mask width. The x-ray irradiation of HA raw materials and HA-based hydrogels [cross-linked with divinyl sulfone (DVS)][18,19] is performed using synchrotron hard x rays (10–60 keV),[20–22] which are also used to image the induced degradation in real time. The fast degradation kinetics is due to a rapid chain scission associated with the formation of carbonyl or carboxyl groups in the HA backbone. In general, the x-ray ablation of the HA-based hydrogels could be quite effective in cleaving bulky HA architecture for 3D cellular microenvironments.

II. MATERIALS AND METHODS

We prepare HA-DVS hydrogel from sodium hyaluronate with 234 kDa (Lifecore), HA with a MW over 2000 kDa (Chugai Pharmaceutical), DVS (Tokyo Chemical Industry), and NaOH/methanol (Wako Pure Chemical Industries). HA (68 mg) is dissolved in 1.68 ml of 0.2*N* NaOH (pH=13). After complete dissolution, 20.02 μl of DVS is added to the

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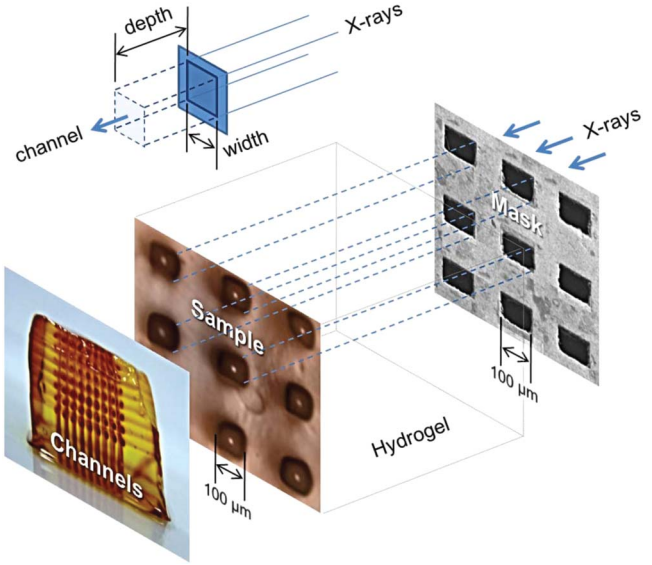

FIG. 1. (Color online) Schematic of x-ray ablation for HA hydrogels. The depth and the width in a single channel are tunable by adjusting the x-ray dose and the mask width. As an example, a HA architecture of 100-μm-width coherent microchannels fabricated using the x-ray ablation is demonstrated.

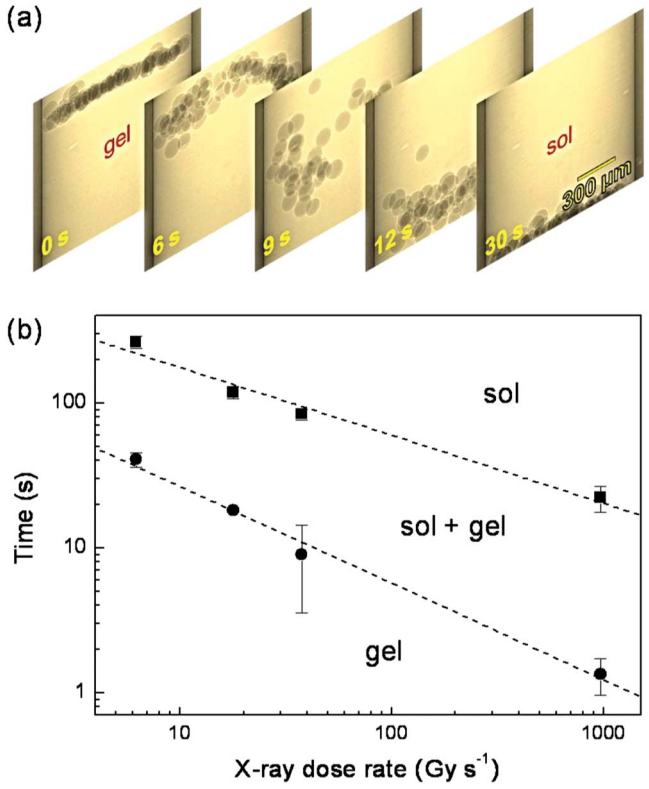

FIG. 2. (Color online) (a) Sequential *in situ* microradiographs showing a gel-to-sol transition of approximately 1 mg of HA hydrogel cross-linked with DVS during x-ray irradiation of approximately 1 kGy s^{-1}. Spherical silica balls initially remained in the top region of the "gel" HA medium, and then fell down to the bottom of the capillary tube during irradiation, clearly indicating a gel-to-sol transition. (b) Sol-gel phase diagram for the x-ray dose rate and the irradiation time. The ablation kinetics is determined by the total x-ray dose.

HA solution for the cross-linking reaction with the hydroxyl groups of HA. The molar ratio of DVS to hydroxyl group is 1:1. The final precursor solution is mixed completely, and 100 μl of the solution is inserted into each one of 15 syringes (1 ml). After incubation at 37 °C for 1 h to complete the cross-linking reaction for HA-DVS hydrogel preparation, the syringes are sealed with prewashed dialysis membrane tube (molecular weight cut off of 7 kDa) and dialyzed against PBS for 24 h. The ions (Na^+ and OH^-) are diffused out through the dialysis membrane neutralizing the *p*H inside HA-DVS hydrogels.

Irradiation and real-time phase-contrast imaging are concurrently performed using hard x rays (10–60 keV) at the 7B2 beamline of the Pohang Light Source (PLS) 2.5 GeV, 150 mA storage ring in Pohang, Korea.[21,22] Spatially-coherent synchrotron x-rays are used to track the detailed gel-to-sol transition during irradiation, using a $CdWO_4$ scintillator crystal and a charge-coupled device camera. All experiments are conducted at room temperature (25 °C) and normal humidity (~30%). The heating during irradiation is negligible.[21] The x-ray dose rate is controlled by adding silicon attenuators and measured with a previously calibrated ion chamber. The sequential microradiographs are taken with an interval time (acquisition time of 0.1 s and data transmission time of 0.4 s). Sequential snapshots in a movie are treated with the IMAGE-PRO PLUS software.

For photodynamic and photochemical analysis UV and Fourier transform infrared (FT-IR) spectroscopy and gel permeation chromatography (GPC) are performed. UV absorption spectra are obtained using SHIMADZU UV-2550 spectrophotometer at the range of 220–600 nm. FT-IR spectra are measured at a spectral resolution of 4 cm^{-1} with a Bomem DA8 FT-IR spectrometer equipped with a liquid nitrogen-cooled mercury–cadmium–tellurium detector. GPC analysis is performed using the following system: Waters 1525 binary high pressure liquid chromatography pump, Waters 2487 dual λ absorbance detector, Waters 717 plus autosampler, Ultrahydrogel TM 1000 and TM 250 columns (7.8 mm × 30 cm) (Milford). Eluant is 34 mM phosphate buffer (*p*H 6.6)/methanol=80:20(v/v) and the flow rate is 1 ml min^{-1}. Detection wavelength is 210 nm.

III. RESULTS AND DISCUSSION

Representative *in situ* microradiographs [Fig. 2(a)] demonstrate a real time gel-to-sol transition of HA hydrogel cross-linked with DVS[18,19] during x-ray irradiation. Spherical silica balls (~120 μm in diameter) that initially stayed in the top region of the HA hydrogel, fell down to the bottom with irradiation time, clearly indicating the x-ray-induced gel-to-sol transition. The irradiated mass of the HA-DVS hydrogel (MW=232 kDa, density≈1 g cm^{-3}) is approximately 1 mg in a capillary tube (approximately 1200 μm in diameter). The sol phase can be easily evaporated when exposed to the atmosphere, which is the reason for making a channel in the gel phase during x-ray irradiation as illustrated in Fig. 1. As already mentioned, the gel-to-sol transition is very fast: this mass degrades within 30 s of irradiation.

The ablation kinetics (photodynamics) mainly depends on the x-ray dose rate (or flux) and on the irradiation time, as illustrated in the sol-gel phase diagram of Fig. 2(b) [the x-ray

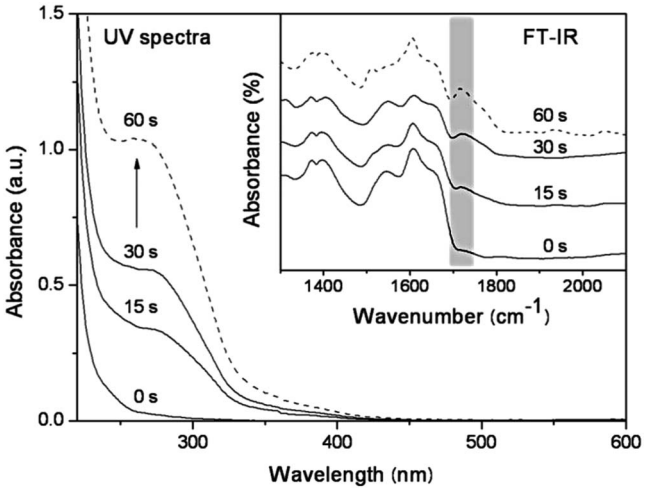

FIG. 3. UV spectra and FT-IR spectra (inset) of HA-DVS hydrogel sample after irradiation with x-rays of approximately 1 kGy s^{-1}. The arrow and the gray zone indicate the absorption band evolution at 260–270 nm in the UV spectra and at 1700–1750 cm^{-1} in the FT-IR spectra. The rapid band evolution within 1 min indicates a rapid x-ray-induced chain scission in HA.

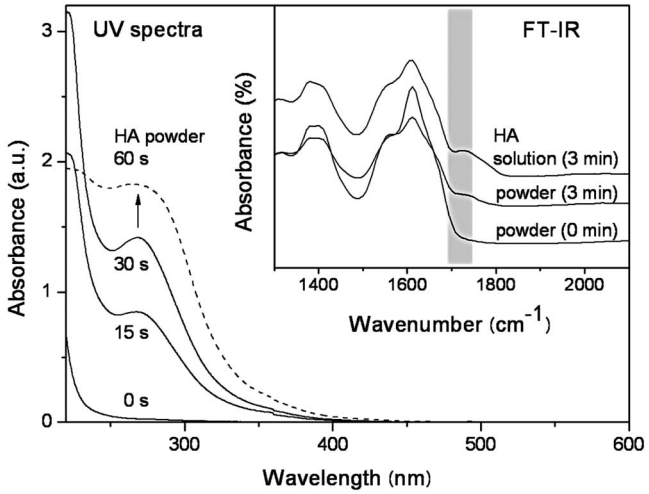

FIG. 4. UV spectra and FT-IR spectra (inset) of HA raw materials (powders or solutions, MW=232 kDa) after irradiation with x rays of approximately 1 kGy s^{-1}. The same band evolutions of HA raw materials and HA-DVS hydrogel suggest that the x-ray irradiation cleaves the HA backbone.

dose rate of Fig. 2(a) is approximately 1 kGy s^{-1}]. However, it is the total x-ray dose (equals the x-ray dose rate times the irradiation time) that actually determines the kinetics. The total dose required to "initiate" the transition is approximately fixed and ≈0.2–1 J g^{-1} (equals kGy) (from the lower dashed line), as shown in Fig. 2(b). The total dose to "complete" the transition is also approximately fixed, ≈2–4 J g^{-1} (from the upper dashed line). This suggests that the total energy (total x-ray dose) required for the transition is constant even if the flux changes. At a total dose of 1–2 J g^{-1} the gel and the sol states coexist. These findings are important since they show that the degradation kinetics can be controlled by modifying the total dose.

We now discuss the photochemical process. The HA-DVS hydrogel degradation at approximately 1 kGy s^{-1} is monitored with UV and FT-IR spectroscopes (Fig. 3). The absorption band at 260–270 nm (indicated by the arrow) in the UV spectra is due to carbonyl or carboxyl groups.[11,23] The intensity increase in the band with the irradiation time is due to the increase in the total dose [see Fig. 2(b)]. As shown in the FT-IR spectra in the inset of Fig. 3, a similar increase with the irradiation time is observed for the absorption band at 1700–1750 cm^{-1} (indicated by the gray zone) that also corresponds to carbonyl or carboxyl groups.[24,25] The same band evolutions were found in the UV and FT-IR spectra of the HA raw material (powders or solutions, MW=232 kDa) (Fig. 4). The UV and the FT-IR spectra of the two HA and HA-DVS hydrogel samples suggest that the x-ray irradiation cleaves the HA backbone. We note that the band evolution within 1 min is quite marked in the UV and the FT-IR spectra, indicating that the irradiation-induced chain scission is very rapid.

The GPC in Fig. 5 shows a significant reduction in the MW by x-ray irradiation in the HA and the HA DVS hydrogel samples. The splitting of the GPC spectra for hard x rays is similar to the depolymerization process of HA by soft x rays.[26] Our result corroborates the conclusion that the HA backbone is cleaved by x-ray irradiation. Also note that the x-ray-induced degradation of the HA-DVS hydrogels corresponds to minor changes in the FT-IR spectra except for the band at 1700–1750 cm^{-1}, similar to enzyme-induced HA degradation.[26] This indicates that the x-ray ablation process results from the controlled degradation of specific chains in the HA molecules. The formation of carbonyl or carboxyl groups is attributed to the scission of glycosidic linkages[23] between monosaccharide units in HA. This suggests that x-ray photons would be appropriate to use for the ablation of biopolymers based on polysaccharide with glycosidic linkages, such as agarose, alginate, chitosan, and hyaluronan. We thus conclude that the fast degradation kinetics is due to a rapid chain scission associated with the formation of carbonyl or carboxyl groups in the HA backbone. The fast kinetics with low doses ($\sim 10^4$ Gy) induces no radiation damage while high doses ($\sim 10^7$ Gy) may make structural damage of biological samples.[27] Further study is required to explore the role of photoelectrons and possible radical reactions under x-ray exposure.

IV. CONCLUSION

To summarize, we presented a protocol for microfabrication of HA-based hydrogels with a short hard-x-ray irradiation (x-ray ablation) in the 10–60 keV photon energy range. This protocol could be quite effective in cleaving bulky HA architecture for microelectronics and 3D cellular microenvironments. Compared to other approaches such as laser ablation, x-ray irradiation effectively allows a nonthermal photochemical process including many advantages such as high penetration, local irradiation, nonthermal character, and remote control—possibly opening potential opportunities in 3D HA hydrogel microfabrication or direct photoetching of polymers[28] for a variety of physical and biomedical applications.

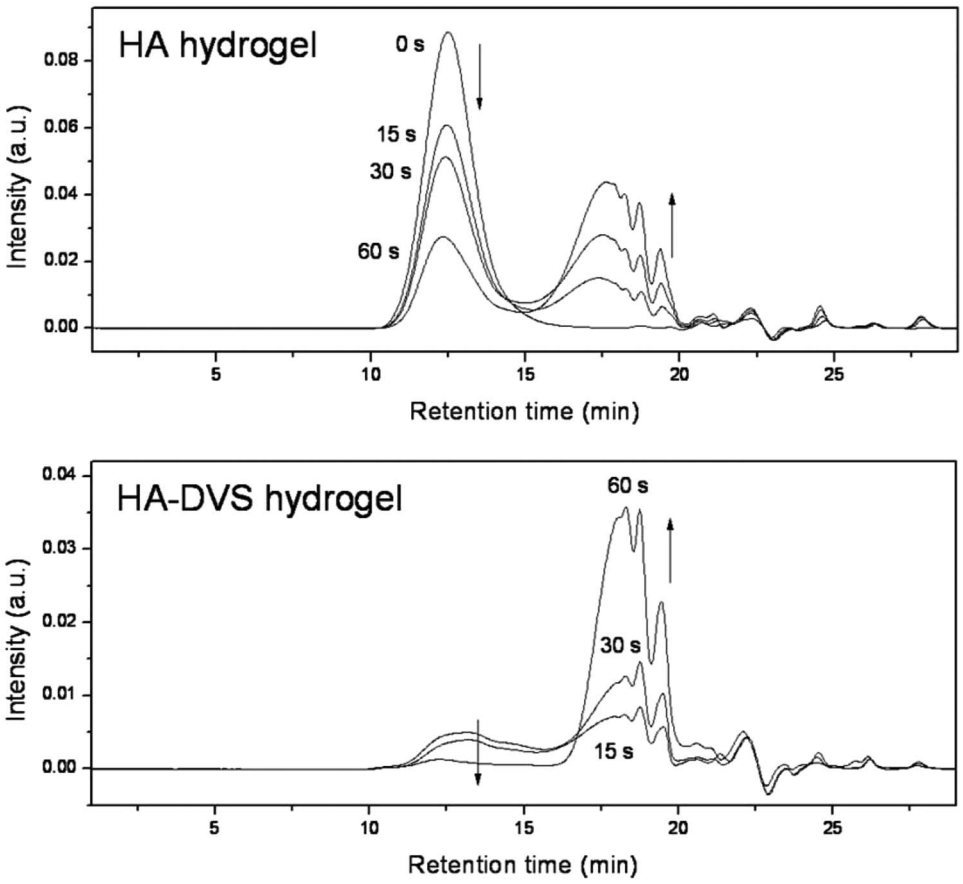

FIG. 5. GPC analysis of HA (MW =232 kDa) and HA-DVS hydrogel samples after x-ray irradiation up to 60 s. The GPC data show that the MWs of the HA hydrogels and the HA-DVS hydrogels significantly decreased with the x-ray irradiation (dose rate =1 kGy s^{-1}), indicating the chain scission by the x-ray irradiation.

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