

Article

Photochemical Patterning of Ionically Cross-Linked Hydrogels

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Abstract: Iron(III) cross-linked alginate hydrogel incorporating sodium lactate undergoes photoinduced degradation, thus serving as a biocompatible positive photoresist suitable for photochemical patterning. Alternatively, surface etching of iron(III) cross-linked hydrogel contacting lactic acid solution can be used for controlling the thickness of the photochemical patterning. Due to biocompatibility, both of these approaches appear potentially useful for advanced manipulation with cell cultures including growing cells on the surface or entrapping them within the hydrogel.

Keywords: alginate; photoresist; iron; responsive hydrogel

1. Introduction

Ionically cross-linked hydrogels based on natural polysaccharides are commonly used in modern biomedical practice owing to their biocompatibility and slow dissolution under physiological conditions. The most common representative of these hydrogels is alginic acid (alginate) which is a linear heteropolymer composed from 1-4 linked guluronate (G) and mannuronate (M) residues. These residues are present in the form of homopolymeric GG and MM blocks as well as heteropolymeric GM blocks. In the presence of polyvalent metal cations, particularly Ca^{2+} , GG blocks of alginate chains form “egg-box” domains which ionically cross-link two neighboring GG blocks [1–3]. The resultant hydrogels are non-immunogenic, porous, and thermally stable. These properties are used for drug delivery [4–7], wound treatment [4,6,8], and cell encapsulation in calcium cross-linked alginate hydrogel [8,9].

Macroporous calcium alginate sponges with pore size of 70–300 μm are prepared by freezing of the hydrogel followed by lyophilization [10]. These sponges are widely employed as a degradable scaffold for tissue engineering [11]. While the surface of calcium cross-linked alginate hydrogel is highly hydrophilic and prevents direct cell attachment, the covalent derivatization of carboxylic group of alginate with RGD peptides provides excellent attachment to integrin receptors of cells [8,12]. Easy biodegradation of alginate hydrogels constitute their critical advantage for cell scaffolding allowing replacement of the scaffold with newly grown cells. The hydrogel undergoes slow dissolution under physiological conditions due to exchange of Ca^{2+} cations to Na^+ [13,14]. Renal clearance of the resulting alginate can be improved by its partial oxidative cleavage of diol groups in urinary residues without compromising its gel forming capabilities [15].

Fabrication of alginate proceeds in contact with calcium cations allowing easy encapsulation of a variety of cells in alginate beads and microbeads [16,17]. The instantaneous cross-linking substantially complicates fabrication of more sophisticated structures necessary for drug release and cell scaffolding. To overcome this problem, formation of the hydrogel can be slowed down by using suspensions of weakly soluble calcium salts such as calcium carbonate [16] and calcium sulfate [18] allowing production of bulk calcium alginate hydrogels. Currently, the creation of responsive ionically cross-linked hydrogels is done mainly through utilization of their pH and thermal responses. This approach was used for fabrication of calcium cross-linked alginate membranes actuated by chemoenzymatic systems [19,20], thermoresponsive alginate-PNIPAAm hydrogels [21,22], and electrical responsive alginate/poly(diallyldimethylammonium chloride) [23]. However, neither of these systems provides the spatial control that is necessary for fabrication of complex porous scaffolds and dynamic control of the gel formation and dissolution. Recently, several promising approaches have been reported. The most sophisticated approach to fabrication of alginate hydrogels involves inkjet printing [24,25] and photochemical UV photolysis of chelate calcium complex resulting in release of calcium cation and formation of ionic cross-links [26]. However, the problem of photodegradation of ionically cross-linked hydrogels has not been solved despite its importance for applications such as dynamically controlled cell detachment and migration as well as for 2D and 3D microfabrication.

Light is a particularly attractive tool for controlling biomaterials. Application of photochemical methods to biomaterials allows obtaining both high spatial selectivity (up to 1 μm) and easy fabrication of complex shapes. Conventional photolithographic methods that use short wave UV light and organic solvents are not suitable for sensitive macromolecules and cells. However, by using visible and to a lesser degree long wave UV light, it is possible to fabricate materials and dynamically change properties of biomaterials with already attached or embedded living cell cultures [27,28]. Photoisomerizable groups, including the azobenzene group [29], stilbenes [30], and spiropyrans [31,32] have been used to induce disassembly or dissolution of biomaterials. The most commonly used approach to control this material involves photolysis of covalent cross-links, such as 2-nitrobenzyl derivatives [27,28,33–35]. These materials were suggested for applications involving controlled drug release from micelles [36] and liposomes [37] as well as gene delivery using photoisomerizable cationic surfactants [38]. Similar methodology can be used for light induced release of cells encapsulated in a cross-linked hydrogel through photodegradable 2-nitrobenzyl bridge [39].

Micrometer sized patterning of surfaces with proteins is known to control a number of processes in attached cells including immunological and neural synapse activation [40,41], integrin clustering [42],

motility [43], and differentiation [44]. This approach can rely on functionalization of surfaces with biotin through a photocleavable linker [45] or cage [46,47] followed by attachment of biotinylated proteins to non-exposed surface using standard streptavidine chemistry. This approach has been extended for patterning of surface with two different biotinylated proteins using pH sensitive photoresist [48]. Photochemical patterning can also be applied to detachment of cells using a combination of a spiropyrone with thermosensitive N-isopropylacrylamide by a combination of UV exposure and low-temperature washing [49].

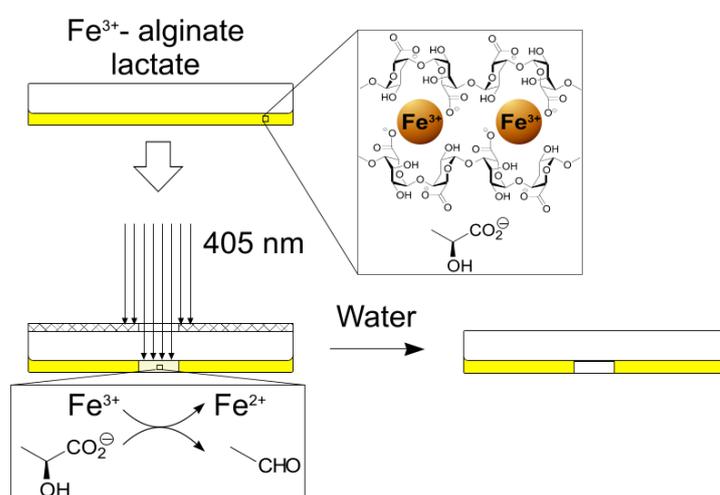
Patterning of hydrogels can be extended to 3D for preparation of scaffolds used in tissue engineering. The hydrogel can be 3D functionalized with cell binding RGD sequences in two steps. On the first step, agarose gel covalently derivatized with 2-nitrobenzyl or coumarine protected cysteine is irradiated by multiphoton excitation on a computer-controlled confocal microscope resulting in formation of free SH functionality inside the irradiated 3D pattern. On the next step the SH group is covalently derivatized with maleimide derivatives carrying cell-responsive glycine-arginine-glycine-aspartic acid-serine (GRGDS) ligand making the patterned path suitable for cell attachment [50]. An alternative approach involves 3D patterning by photoinduced cleavage of 2-nitrobenzyl group breaking cross-links in modified PEG diacrylate hydrogel followed by erosion of the hydrogel creating 3D channels using confocal-based laser scanning lithography [51]. The second approach permits external real-time spatial and temporal control over hydrogel properties allowing directed migration of cells attached to the surface. The main problems associated with hydrogels prepared from synthetic and semisynthetic photoresponsive polymers are biocompatibility and biodegradation. These hydrogels are prepared through free-radical or UV induced polymerization monomers which can affect the viability of cells that have to be placed inside. Biodegradation of the resultant hydrogels requires hydrolytic transformations that are substantially slower and less predictable than cation exchange in ionically cross-linked hydrogels while yielding potentially toxic degradation products.

Our attempts to combine advantages of ionically cross-linked and photoresponsive covalently cross-linked hydrogels are based on use of iron(III) cation as a responsive cross-link. Two common oxidation states of iron possess substantially different coordination properties. Iron(II) can be considered as a “soft” metal cation that tends to bind neutral ligands containing nitrogen and sulfur atoms and in smaller degree anionic ligands such as carboxylate group. In contrast, the iron(III) cation is a typical example of a “hard” metal cation that preferentially binds oxygen atoms in negatively charged ligands such as carboxylate, phenolate, or hydroxamate groups [52]. This difference in coordination chemistry of iron(III) and iron(II) cations has been previously utilized for speciation of iron(II) and iron(III) cations in solution, for preparation of molecular switches [53], and for mechanical actuation of hydrogels [54]. Iron(III) cations form stable alginate hydrogels [55] that have been successfully used as a support for growth of cell cultures [56,57]. While iron(II) cations are also capable of cross-linking of alginate [58] this process requires substantially higher concentration of ferrous cations while 20–30 mM solutions of iron(II) salts in 0.8%–1.2% *w/v* sodium alginate are viscous homogeneous liquids. Our recent experiments [20] demonstrate that oxidation of iron(II) cations to iron(III) results in formation of cross-linked alginate gel. Electrochemical oxidation of iron(II)-alginate solution at +0.8 V produces a film of iron(III) alginate on the anode that can grow up

to 1 mm thickness. Reversing the electrode polarity results in dissolution of the film or in the case of sufficiently thick film of the gel, in detachment of the hydrogel film from the electrode surface.

Exposure of the iron(II)-alginate solution to air results in slow oxidation and formation of a homogeneous iron(III) cross-linked alginate gel. The resultant iron(III) cations in the gel can be reduced photochemically in the presence of 10–20 mM of α -hydrocarboxylic acids salts such as sodium lactate that work as sacrificial reductants (Figure 1). Incorporation of lactic acid into iron(II)-alginate solution before the air oxidation stage produces photoresponsive gel that easily dissolves upon irradiation with UV or visible light after treatment with water or 0.9% saline [59].

Figure 1. Scheme of spatially selective photoinduced dissolution of iron(III) cross-linked alginate gel containing sodium lactate by 405 nm light. Binding of iron(III) cations by guluronate residues of alginate is shown schematically in analogy to the egg-box model established for calcium cross-linked alginate hydrogel.



Here we report on using the photoresponsive iron(III) cross-linked alginate hydrogel as a biocompatible photoresist and photochemical patterning of the hydrogel under biocompatible conditions.

2. Experimental Section

2.1. Materials and Instruments

Alginic acid sodium salt from brown algae was purchased from Sigma (BioReagent, MW 100–200 kD and low viscosity, 100–300 cP, catalog number 71238 and A2158). Iron(II) chloride, L(+)-Lactic acid and potassium ferrocyanide trihydrate were purchased from Fisher Scientific and used as supplied, without any pretreatment or additional purification. The preparation of the solution has been done using a Thermix stirrer model 120 M (Fisher Scientific, Pittsburg, PA, USA). Visible irradiation was done using a 300 mW focusable 405 nm laser. Light intensity was measured by a Hioki 3664 optical power meter. UV-Vis spectra of gels were measured using Agilent 8453 UV-visible spectrophotometers (Santa Clara, CA, USA).

2.2. General Procedure for Preparation of Iron(III) Cross-Linked Alginate Gel by Air Oxidation

To a solution of sodium alginate in distilled water (2.0% w/v, 3.0 mL) were added 2.7 mL of distilled water containing 0 to 44 mM of pH 7.0 sodium lactate and then a freshly prepared solution of iron(II) chloride in distilled water (0.3 mL of a 500 mM solution). To prevent formation of iron(II) alginate clumps the addition was done dropwise under vigorous stirring (≥ 1000 rpm) using an oval or octagonal stir bar. The addition decreased pH of the solution to 4.0–4.5 depending on the amount of sodium lactate. The resultant homogeneous solution (1.5 to 2.0 mL) was transferred to a Petri dish (30 mm in diameter) forming 1.5–2.0 mm layer that was oxidized by air for 24 to 48 h at 25 °C and 100% humidity.

2.3. Photochemical Patterning of Homogeneous Iron(III) Cross-Linked Alginate Hydrogel Containing Sodium Lactate

Homogeneous iron(III) cross-linked hydrogel containing 20 mmol of iron(II) chloride and 20 mmol of sodium lactate was prepared according the abovementioned general procedure followed by oxidation at 100% humidity for 48 h was irradiated for 60–390 s through a mask, in the center of the Petri dish by the 405 nm laser focused to give a 3 cm spot at distance of 50 cm from the sample with a light intensity of 44 mW/m². The exposed zone was dissolved by treatment with 0.9% saline (3 mL) using an orbital shaker (10 min, 50 rpm).

2.4. Determination of Rates of Surface Photodegradation of Iron(III) Cross-Linked Alginate Hydrogel Contacting Solution of Lactic Acid

Homogeneous iron(III) cross-linked hydrogel containing 20 mmol of iron(II) chloride and no sodium lactate was prepared in Petri dish according the abovementioned general procedure followed by oxidation at 100% humidity for 16 h. The Petri dish was topped with a solution of lactic acid (20 to 80 mM) and covered with a glass slide to prevent movement of the solution surface. A mask with a 5 mm round transparent window in the center was laid on the glass. The assembled system was irradiated for 60–360 s through the mask with the 405 nm laser distanced of 50 cm from the sample and focused to give a spot having a light intensity of 44 mW/m² in the center. The exposed zone was then treated with distilled water (3 mL) using orbital shaker (10 min, 50 rpm) to partially dissolve the alginate hydrogel from the exposed zone.

2.5. Photochemical Patterning of Surface Layer of Iron(III) Cross-Linked Hydrogel Contacting a Solution of Lactic Acid

Homogeneous iron(III) cross-linked hydrogel containing 20 mmol of iron(II) chloride and no sodium lactate was prepared in Petri dish according the abovementioned general procedure followed by oxidation at 100% humidity for 48 h. The resultant iron(III) cross-linked hydrogel was then incubated with a solution of potassium ferrocyanide (5 mM, 3 mL) for 5 min using an orbital shaker (50 rpm) to stain the surface of the hydrogel through formation of Prussian Blue particles. After removal of ferrocyanide solution the Petri dish was topped with a solution of lactic acid (20 mM) and covered with a glass slide to prevent movement of the solution surface. A mask with a 13 mm radial

resolution chart printed on polyester film in the center was laid on the glass, and the system was irradiated for 10 min through the mask with the 405 nm laser distanced of 50 cm from the sample, focused to give a spot having a light intensity of 44 mW/m^2 in the center. The exposed zone was then treated with distilled water (3 mL) using orbital shaker (10 min, 50 rpm) to reveal the radial pattern of the mask duplicated on the surface of the hydrogel as the blue stain.

3. Results and Discussion

3.1. Preparation of Iron(II) Alginate Solutions and Its Conversion to Iron(III) Cross-Linked Hydrogel

As reported previously, at concentrations up to 20 mM iron(II) cation can form homogeneous solutions with 1% w/v solutions of sodium alginate. Presence of sodium lactate in the gel increases this maximum concentration from 20 mM to 30 mM [59]. At higher concentrations of either iron(II) chloride or sodium alginate, the formation of white precipitate of iron(II) alginate took place, as has been observed in the literature [58]. Formation of the precipitate happened also during rapid addition of even small amounts iron(II) salts to sodium alginate solution as high viscosity of the solution causes locally high concentration of iron(II) cations. To prevent formation of the precipitate addition of 250 μL of a 500 mM solution of iron(II) to a solution containing 2.5 mL of a 2% w/v solution of sodium alginate and 2.25 mL of water. The solution of sodium lactate and iron(II) chloride has to be added on the side of an octagonal stirring bar to arrange instantaneous mixing. The initial pH of the alginates is 6.0–7.0 but after addition of the acidic FeCl_2 solution, the pH of the resultant solution decreased to 4.0–4.5 depending on the amount of added sodium lactate.

The obtained homogeneous colorless viscous aqueous solution of iron(II) chloride and sodium alginate undergoes slow oxidation of iron(II) cations into iron(III) which cross-link alginate to form a homogeneous hydrogel. Because cross-linking of alginate with the resultant iron(III) cations is instantaneous the rate of gelling is limited by rate of oxidation of iron(II) cations which proceeds rather slow and is accompanied by corresponding increase in absorbance below 400 nm.

Rates of the oxidation and hydrogel formation depend on concentration of iron(II) cations and lactate (Table 1). An iron(II) alginate solution that contains 20 mM of iron(II) chloride but no sodium lactate requires 12 h to form the hydrogel in a layer with 1.6 mm thickness. The analogous solution containing 20 mM of sodium lactate in addition will need at least 24 h to form the hydrogel. Smaller concentration of lactate vs. iron(II) chloride results in marginal decrease of gelling time.

Table 1. Minimal time required for production of hydrogel in solution containing 0.93% w/v of sodium alginate, 1 mm thickness.

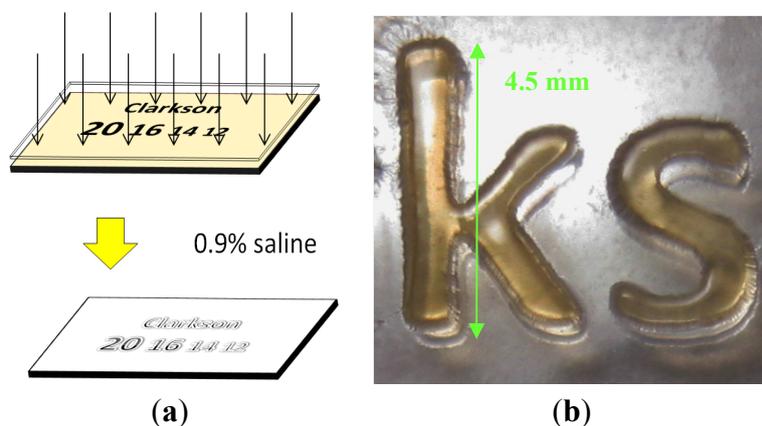
Entry	[FeCl ₂]	[Lactate]	Gelling process
1	15 mM	20 mM	>1 month
2	15 mM	15 mM	>7 days
3	20 mM	20 mM	24 h
4	25 mM	20 mM	20 h
5	25 mM	25 mM	20 h
6	30 mM	30 mM	<20 h

Even small excess of sodium lactate over the 1:1 ratio with iron(II) chloride causes a dramatic increase in gelling time. This change can be explained assuming arrangement of the resultant hydrogel that each iron(III) cation binds two carboxylate groups of alginate and one molecule of lactate. Presence of more than 1 equivalent of lactate would displace binding of alginate, thus preventing formation of hydrogel.

3.2. Photochemical Patterning of Iron(III) Cross-Linked Alginate Hydrogel Containing Lactate

Based on previously reported process of photodegradation of iron(III) cross-linked alginate hydrogel [59] we investigated properties of this material for photochemical patterning of surfaces. A layer of the hydrogel (2.0 mm thickness) was irradiated through a mask. The mask was prepared on a conventional laser printer on a transparent polyethylene terephthalate film. The irradiation was performed by diffused 300 mW 405 nm laser beam with intensity 44 mW/cm². After 6 min 30 s of irradiation, the hydrogel sample was treated with 0.9% saline solution for 10 min, which resulted in complete dissolution of exposed regions leaving shaded regions of the hydrogel intact (Figure 2).

Figure 2. (a) Schematic representation of photochemical patterning of iron(III) cross-linked alginate hydrogel containing sodium lactate; (b) enlarged image of the patterned surface.



The obtained results demonstrate the potential of iron(III) cross-linked hydrogels as positive photoresists. This application is of particular interest because of very mild conditions of preparation of the hydrogel, its photochemical degradation, and use of biocompatible materials. These features are particularly attractive for application of photochemical patterning to cell cultures attached to the surface of the hydrogel [4,60,61].

3.3. Photodegradation of Iron(III) Cross-Linked Alginate Hydrogel in the Presence of Lactate

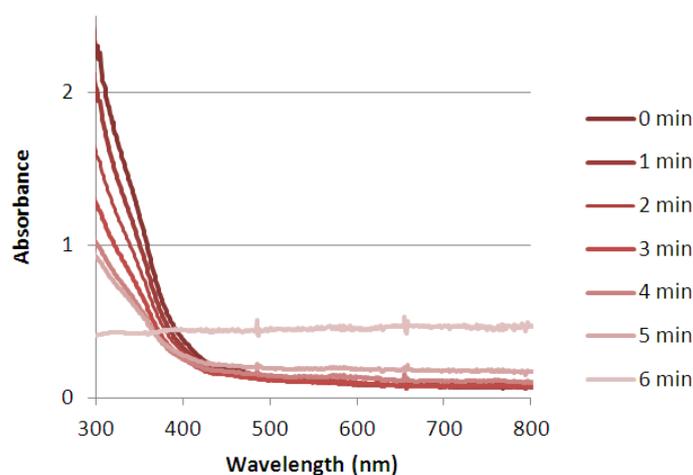
In contrast to known examples of photoresponsive hydrogels [62], photochemical degradation of iron(III) cross-linked alginate hydrogels requires presence of an α -hydroxycarboxylic acid such as lactic acid. The mechanism of photoreduction is most likely similar to well studied photoreduction of iron(III) citrate [63]. The latter process was reported to involve a photochemical ligand to metal charge transfer in a μ -oxo dimeric complex of iron(III)- α -hydroxycarboxylate resulting in formation of

α -hydroxycarboxylate radical and the first iron(II) cation. The resultant α -hydroxycarboxylate radical is immediately oxidized thermally by another iron(III) cation forming a ketone or aldehyde, carbon dioxide, and the second iron(II) cation.

This feature allows additional control over the process of photodegradation of iron(III) cross-linked hydrogel by restricting the photodegradation only to areas of the hydrogel where lactic acid is present. For example, the photochemical degradation can be performed in a layer of lactate-free iron(III) cross-linked hydrogel contacting an aqueous solution of lactic acid. In this system the photochemical degradation can proceed only in the narrow layer of the hydrogel containing sufficient concentration of lactate diffused from the aqueous solution. Because the rate of diffusion of lactate through the hydrogel is low, this photodegradation can only allow etching of a thin layer of hydrogel in irradiated sections of the hydrogel.

To determine the rate of the process of surface degradation of iron(III) cross-linked hydrogel the hydrogel (1.5 mL) composed of 1% *w/v* sodium alginate (low viscosity) and 25 mM FeCl₂ and no lactate was prepared 48 h before the experiment. After 48 h, the Petri dish containing the gel was filled to the top with a 20 mM solution of lactic acid and irradiated with 405 nm light. The process of photodegradation was monitored by measuring the absorbance of the gel between 300 and 800 nm (Figure 3). After 1 min of irradiation the Petri dish with the hydrogel was separated from the solution of lactic acid and the absorbance of the irradiated zone of the hydrogel was measured.

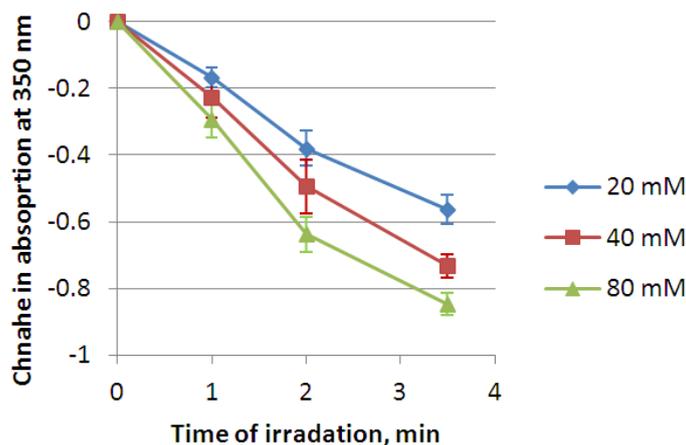
Figure 3. Change in UV-Vis absorption spectra of iron(III) cross-linked hydrogel contacting 20 mM aqueous solution of lactic acid upon irradiation with 405 nm light.



As expected, the absorbance of the gel below 400 nm decreased after every irradiation cycle for first five minutes due to decreased concentration of iron(III) cations which provide the most important contribution to UV absorbance [59]. Along with it there was a minor increase in absorption above 400 nm which can be attributed to increased roughness of the gel surface due to photodegradation which results in scattering of light passing through the hydrogel. The abrupt change in absorption at 6 min of irradiation is due to loss of gel structure and collapse of the hydrogel layer in the Petri dish left on its side during the measurement.

Predictably increasing concentration of lactic acid in the solution contacting iron(III) cross-linked alginate hydrogel resulted in increasing the rate of reduction of iron(III) cations (Figure 4). However, the increase was relatively minor (20%–25%) for doubling of concentration of lactate.

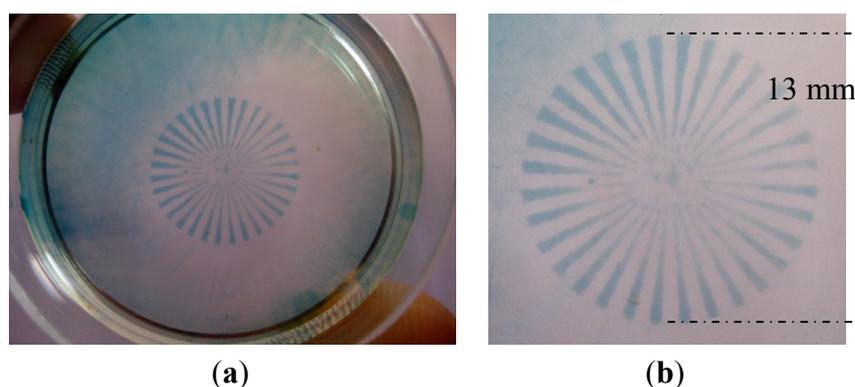
Figure 4. Changes in absorption iron(III) cations at 350 nm in iron(III) cross-linked hydrogel contacting 20–80 mM solutions of lactic acid and irradiated by 405 nm light.



3.4. Photochemical Patterning of the Surface of Iron(III) Cross-Linked Alginate Hydrogel in Lactate Solutions

Based on these result we attempted selective photochemical patterning of a thin layer of iron(III) cross-linked hydrogel contacting lactic acid solution with concomitant dissolution of the exposed regions of the hydrogel. To visualize the process a thin layer of the surface of the hydrogel was stained and the stain was afterward removed by photoinduced dissolution of the stained layer of the hydrogel. Iron(III) cross-linked alginate hydrogel was prepared by air oxidation of a 1% *w/v* solution of sodium alginate containing 25 mM of iron(II) chloride for 48 h. The surface of the hydrogel was then treated with 5 mM solution of potassium ferrocyanide for 10 min which formed a blue stain of insoluble Prussian blue particles formed in reaction of ferrocyanide anions with iron(III) cations in the thin layer of hydrogel contacting the ferrocyanide solution.

Figure 5. (a) Patterned stained iron(III) cross-linked alginate hydrogel after irradiation and washing; (b) Enlarged image of the patterned stained hydrogel.



The Prussian blue stained surface of the hydrogel was then covered with 40 mM pH 2.6 aqueous solution of lactic acid. The resultant hydrogel was irradiated by 405 nm laser beam with intensity 44 mW/cm² for 10 min through a mask of radial resolution chart (Figure 5). Subsequent treatment of the irradiated hydrogel with distilled water resulted in dissolution of the surface layer of the hydrogel and removal of the blue stain in the light exposed areas.

This qualitative experiment demonstrate the potential of photolithography of iron(III) cross-linked hydrogel for introduction of 3D control over the thickness of the photodegradation. The possibility for facile patterning or complete removal of a thin layer of the hydrogel under biocompatible conditions can be of interest for a number of applications, particularly for use of hydrogels for 2D and 3D scaffolding of cell cultures and photoinduced detachment of cells.

4. Conclusions

In conclusion, we demonstrate successful photochemical patterning of iron(III) cross-linked alginate hydrogel in the presence of lactate. Layers of homogeneous iron(III) cross-linked alginate hydrogel with 1–3 mm thickness incorporating 20 mM sodium lactate undergo photoinduced degradation thus serving as a biocompatible positive photoresist suitable for photochemical patterning. Alternatively, thickness control of the photochemical patterning can be done through surface etching of iron(III) cross-linked hydrogel contacting lactic acid solution. Due to biocompatibility, both of these approaches appear potentially useful for advanced manipulation with cell cultures, including the growth of cell cultures on the surface of the hydrogel or cells, entrapping them within the hydrogel.

Acknowledgments

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Conflicts of Interest

The authors declare no conflict of interest.

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