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Direct Three-Dimensional Microfabrication of Hydrogels via Two-Photon Lithography in Aqueous Solution

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The ability to precisely microfabricate hydrogels in three dimensions has gained tremendous importance as it may be useful for a number of biomedical applications such as construction of biosensors, tailoring materials for drug delivery, and creating soft scaffolds that interact with living cells.[1−3] The two most important aspects to consider while making patterned structures are the ease of the technique and the freedom to make arbitrary structures. Two-photon lithography (TPL) stands out when both of these aspects are considered. Using a confocal laser scanning microscope equipped with a femtosecond mode-locked near IR laser, one can microfabricate structures, from commercially available monomers, of any shape and having resolution less than 100 nm.[4−7] TPL has been used to microfabricate a wide variety of arbitrarily shaped structures including cantilevers[8] and gratings[9] and for creating defects in photonic crystals.[10,11] One of the major current limitations of multiphoton lithography used in hydrogel fabrication has been the inability to use effective hydrophobic chromophores in aqueous media. This characteristic has limited the use of multiphoton lithography for biological applications, because the use of cytotoxic organic solvents such as toluene to solubilize the hydrophobic chromophores is very undesireable.[12] In addition, the direct net shape fabrication of hydrogels in water without the need to exchange solvents can offer advantages during the hydrogel production step. This is particularly useful since changes from organic solvents used in fabrciation to water in use can lead to significant structure distortions. Our objective for this work has been to fabricate hydrogels

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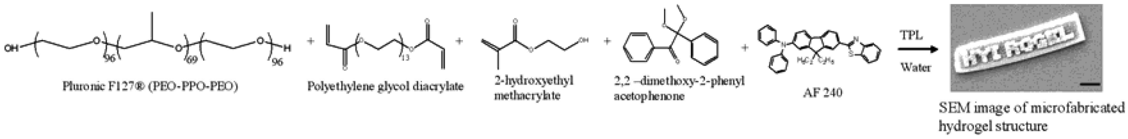
Scheme 1. Chemical Components Used for the Microfabrication of Hydrogels Directly in Water[a]



[a] The SEM image shown is representative of the possible hydrogel microstructures obtained after two-photon lithography. Scale bar = 10 μm.

capable of releasing growth factors from very precise 3D structures to be placed on neural electrodes.[13]

Chemical reactions in a two-photon process are tightly confined at the focal point, generally to the order of cube of laser wavelength. [14] Since the cross sections are very small, a very intense source of light is needed which often results in unwanted damage to the structures if insensitive chromophores are used. Hence, molecules having excellent two-photon sensitivity are needed which enable lower laser powers for two-photon excitation.[15] Researchers interested in biological imaging have previously studied a variety of methods to solubilize hydrophobic chromophores in water including synthesis of complex water-soluble chromophores using multistep reactions, using water-soluble quantum dots for multiphoton in vivo biological imaging, or the use of amphiphilic block copolymer complexes. [16−18] In common with this prior work we are interested in applying oil soluble chromophores for use in aqueous solution. We have instead elected to solubilize hydrophobic chromophores with a large two-photon absorption cross section using a nonionic surfactant (Pluronic F127 (PF127)) and applying the resulting complex with an oil soluble photoradical initiator to fabricate hydrogel microstructures which could be useful for biomedical applications. The hydrophobic chromophores were used for free-radical photopolymerization of 2-hydroxyethyl methacrylate (HEMA) and poly(ethylene glycol) diacrylate (PEG-DA) in an aqueous medium.

A simple one-step method is described to produce synthetic microfabricated hydrogel structures via TPL. To the best of our knowledge, this is the first report in which TPL has been performed using hydrophobic chromophores with a large two-photon absorption (TPA) cross section (δ) directly in aqueous solution. Previously, Basu and Campagnola[19] and Shear et al.[20] have cross-linked proteins in aqueous solution via multiphoton excitation. They utilized photosensitizers having low two-photon sensitivity which require extraordinarily high threshold laser powers for microfabrication., Their work is very promising for potential tissue scaffold fabrication; however, cross-linking of the proteins requires very long exposure times and high energies. The resulting protein aggregates may cause immunogenic response in vivo.[21] This communication reports the direct net shape fabrication of biocompatible acrylate and methacrylate hydrogels which require much lower exposure times for free-radical cross-linking, thereby reducing the fabrication time.

It was found that only when both the initiator 2,2-dimethoxy-2-phenyl acetophenone (Irgacure 651) and the 2-photon sensitive chromophore (AF240) were present in the polymerizable mixture could hydrogel microstructures be formed. While the exact mechanism for the observed photopolymerization is unknown and a mechanistic determination is beyond the scope of this work, there are at least two possibilities: (a) The chromophore behaves as a photosensitizer, which emits an up-converted fluorescent light in the UV−visible regime on simultaneous absorption of two near IR photons. Subsequently, the initiator (Irgacure 651) on absorbing the fluorescent light starts free-radical polymerization and (b) an intermolecular electron transfer from the two-photon-excited AF240 to Irgacure 651 during molecular collision. It has been reported by several groups that the apparent α-cleavage quantum efficiency (Φ_α) of Irgacure 651 (leading to generation of benzoyl and substituted radicals) in the wavelength region from 313 to 365 nm is close to 100%.[22] Therefore, it is not unreasonable to assume that Φ_α would be also significantly high in the longer wavelength region (i.e., 365−400 nm) where the emission tail on the "blue side" of AF240 could overlap. The overlap could occur because of two reasons: (a) generation of a small percentage of <400 nm photons as a result of the simultaneous absorption of two 780 nm photons and (b) in aliphatic hydrocarbon solvents the blue emission tail of AF240 extends below 410 nm.[23] Thus, it is postulated that the hydrophobic poly(propylene oxide) block in PF127 might have provided an environment similar to that by saturated hydrocarbon solvents, resulting in energy transfer in a micelle setting. Although the spectral overlap is expected to be small, the molecular confinement provided by PF127 is likely to enhance the energy transfer efficiency.

The structures of the chemicals used for TPL are shown in Scheme 1. PF127 was chosen as a surfactant for dispersion of the hydrophobic chromophores because it is also FDA approved and has been previously used with no cytotoxic

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effects, when used in appropriate concentrations.[24,25] The TPA chromophore, designated as AF240, has a linear D$-\pi-$A motif where the donor (D) is diphenylamine and the acceptor (A) is benzothiazole. It has a fluorene bridge which is thermally and photochemically stable, and by locking the biphenyl unit into the fluorenyl ring, greater electron delocalization through increased π molecular orbital overlap is provided.[26] This planar feature enhances molecular polarizability and TPA sensivity.[27] The monomers, 2-hydroxyethyl methacrylate and poly(ethylene glycol) diacrylate, and the initiator, Irgacure 651, are commonly used in synthesizing highly biocompatible hydrogels.[13,28] The presence of water in the prepolymer solution helps in controlling important properties of the resulting structure such as modulus and the pore size which can be fine-tuned for its intended application. It also enables precise net shape fabrication which is not possible when an organic solvent is used in the fabrication step only to be replaced with water.

Photophysical studies of the resins (prepolymer solution) with chromophores dissolved in different solvents are shown in Figure S1 (Supporting Information). The chromophores are prone to aggregate and subsequently precipitate, when dispersed in water alone, resulting in a reduced concentration of the solvated dye and a decrease in the action cross-section value, a product of fluorescence quantum yield (Φ_F) and two-photon absorption cross section (δ; GM $= 1 \times 10^{-50}$ cm^4 s photon^{-1} molecule^{-1}), as compared to when they are dissolved in toluene. When the solvent is water with added PF127, an increase and greater consistency in the $\delta\Phi_F$ values is seen. A similar effect was seen when Tian et al. solubilized different hydrophobic chromophores in water using the block copolymer poly(methacrylic acid)-*block*-polystyrene.[18] This effect is believed to be due to micelle formation which leads to a dilution effect and reduced aggregation of the chromophores. The large error bars (Figure S1, Supporting Information) when the solvent is water with no PF127 may be caused by reduced solubility of the chromophore. In this case, the monomers themselves help in solvating the chromophores in water. There was no significant difference when the chromophore:PF127 molar ratio was changed from 1:10 to 1:20.

Two parameters, namely, required TPL threshold power (P_{th}) and the polymerization window range available to make hydrogel microstructures, were chosen to compare the efficiency of different monomer mixtures. The TPL threshold power is defined as the locally absorbed energy density below which no polymerization occurs.[29] The polymerization window range was calculated using the formula P_{max}/P$_{th}$, where P_{max} was the maximum power with which the structures could be microfabricated, beyond which damage to the structures would occur. When two-photon initiated polymerization was performed with the chromophore dissolved in different solvents, the best performance, as expected, was when the solvent was toluene. Approximately, 12 times the concentration of AF240 was needed to achieve similar P_{th} values when the chromophore was dispersed in water in the presence of PF127 (1:10) (Table S1, Supporting Information). When the chromophore was solvated in water alone, in the absence of PF127, only single-layer structures could be fabricated, since any multilayer microstructures were damaged because of the very high threshold power required. The highly efficient AF240 dye was used in these studies to demonstrate a proof-of-principle that PF127 can be used to directly microfabricate hydrogels in water. We believe that the use of dyes having even higher TPA cross sections will eliminate the need of high concentrations of dye required for microfabrications, thereby reducing any potential toxicity issues.

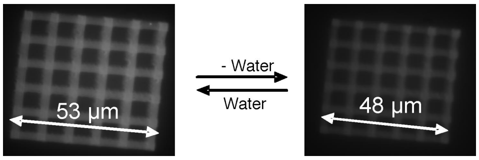

Figure 1. Fluorescence image of the microfabricated waffle in (a) swollen and (b) dry conditions. Swelling ratio of the microstructure is ~10%. The microstructure was obtained by polymerization of the monomers via TPL directly in water in the presence of PF127 (1:10). The laser power used was 21.5 mW, and the aspect ratio of the structure was 0.5.

Since the swelling of hydrogels is so important in a number of biomedical applications, the extent of swelling was studied using fluorescence microscopy of the microfabricated structures. The observed fluorescence is due to excitation of the AF240 chromophore which is retained in the hydrogels immediately after fabrication. It is possible to remove the chromophore from the hydrogel microstructures using extended extraction times. The swelling ratio was defined by the change in the width of the structure after immersion in water with respect to the width of dry gel. The width of the structures was calculated using Metamorph imaging software. Tanaka et al. have explained the lateral swelling of patterned hydrogels which depends on film thickness and the aspect ratio of the pattern.[30] As shown in Figure 1, when a net shape water microfabricated waffle was made with AF240 dissolved in water in the presence of PF127, the swelling ratio was ~10% (laser power = 21.5 mW). The water swollen gel was dried to assess the effect of water on the gel dimension. The patterned microstructure has an aspect ratio of 0.5 and a thickness of 100 nm. When the same structure was made with a higher laser power of 28 mW, the swelling ratio decreased to ~ 5%. When much thicker hydrogel slabs were made in the presence of water having a height of 3 μm, the swelling ratio was ~20%. By varying the thickness and the aspect ratio as well as the laser power used to microfabricate the structures, hydrogels having different

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swelling ratios could be produced. Hence, one can easily create microstructures with customized swelling properties using TPL. The structures remained adhered to the silanized glass coverslip after numerous deswelling−swelling studies. Delamination of structures from glass substrate was not observed at least up to fabrication of structures having an aspect ratio of 1.5.

In conclusion, this is, to the best of our knowledge, the first time synthetic hydrogel microstructures have been fabricated via TPL using hydrophobic chromophores having a large TPA cross section directly in aqueous solution. PF127, a nonionic surfactant, was used to enhance the dispersion and effectiveness of the chromophore in water. Although toluene is still a superior solvent for dissolving the hydrophobic chromophores, the use of water and the simple methods for using otherwise insoluble chromophores extends this technique for numerous biological applications.

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Supporting Information Available: Figure S1, Table S1, and experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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