Molecular Crystals and Liquid Crystals

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl20

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Version of record first published: 26 May 2010

Mol. Cryst. Liq. Cryst., Vol. 505, pp. 219/[457]–230/[468], 2009

ISSN: 1542-1406 print/1563-5287 online
DOI: 10.1080/15421400902950121

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This study describes the preparation of biocompatible patterned surfaces by a two-photon absorption technique. We have synthesized poly(2-methoxyethyl acrylate) copolymers, which exhibit biocompatibility and photocrosslinking moiety. Fabrication resolution can be controlled in the sub-micrometer range by changing the laser power, photoinitiator concentration, and scanning speed. The patterned surfaces showed excellent human platelet compatibility. Biocompatible patterned surfaces can be used in various medical devices, implants, biosensor chips, and tissue engineering scaffolds.

Keywords: biocompatible polymer; fabrication; platelet adhesion; poly(2-methoxyethyl acrylate); scaffold; two-photon

1. INTRODUCTION

Biocompatibility of materials is an important factor to be considered when developing medical devices and tissue engineering scaffold.

This work was supported by the Grants-in-Aid for Scientific Research (B) (No. 13558102) and Young Scientists (A) (No. 17680035) from the Ministry of Education, Culture, Sports, Science and Technology.

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The possibility of replacing damaged or diseased organs with artificial tissues, which are engineered from a combination of living cells and biocompatible patterned scaffolds, is becoming a reality through multidisciplinary efforts [1,2]. The virtue of a two-photon absorption technique [3–11] as a tool for fabricating microdevices as compared to that of photon or electron beam lithography and bottom-up technologies using self-organization [2] lies in its three-dimensional capability; this technique has found application in the fabrication of photonic devices and micromachines with feature sizes close to the diffraction limit. The two-photon absorption of photosensitive materials facilitates the fabrication of complex patterned surfaces. When femtosecond laser pulses are tightly focused into the volume of a liquid resin, which is transparent in the infrared (IR) spectrum, the two-photon absorption process is initiated. Ti:sapphire femtosecond lasers operating in the 700–1028 nm [3–12] wavelength range have been widely used to initiate two-photon absorption. Various patterned structures such as spirals [3], needles [4], bulls [5], oscillators [6], microchannels [7], and woodpiles [8] have been fabricated by focusing the femtosecond laser pulses on commercially available photosensitive polymeric materials. The flexibility of this technology and the ability to precisely define construct geometry allows issues associated with vascularization and patient-specific tissue fabrication to be directly addressed. The fabrication of reproducible scaffold structures by the two-photon absorption technique is important for conducting systematic studies on cellular processes and the better understanding of *in vitro* tissue formation. Biocompatible polymers are considered to be very promising materials for the fabrication of medical devices and tissue engineering scaffolds. However, thus far, the two-photon absorption technique employs polymers exhibiting toxicity.

Here, we report the preparation of biocompatible patterned surfaces used in medical devices and tissue engineering scaffolds by the two-photon absorption technique. In order to design novel biocompatible polymers and photoinitiators with strong two-photon absorption cross sections, we prepared poly(2-methoxyethyl acrylate) copolymers, which exhibit biocompatibility and photocrosslinking moiety. The possibilities of seeding human cells on the patterned surfaces were examined. Human platelet adhesion experiments were carried out to study the biocompatibility of patterned surfaces. These studies demonstrate that the two-photon adsorption technique has a great potential for the manufacture of biomedical scaffolds with controlled surface topology and properties.

2. EXPERIMENTAL DETAILS

2.1. Materials

The monomers 2-methoxyethyl acrylate (MEA), glycidyl methacrylate (GMA), and epoxycyclohexyl methylacrylate (ECHMA) were used in the preparation of patterned surfaces. These monomers were distilled under reduced pressure before use. The photoinitiators trans-4-[P-(N-methyl-N-hydroxylethylamino)styryl]-N-methylpyridium-tetraphenyl borate and N,N-Diphenyl-7-[2-(4-pyndinyl)eyhenyl]-9,9-diethyl-9H-Auoren-2awine, were procured from Fuji Film. Uvacure 1591 (Daicel) was used as a conventional UV initiator. 1,4-dioxane, hexane, tetrahydrofuran (THF), metylethylketone (MEK), which were used as solvents, were of analytical grade. Cover glass (Matsunami) was used as a substrate for coating the polymers.

2.2. Polymerization

PMEA was prepared by free-radical polymerization, as reported previously [12–14]. MEA copolymers (poly(MEA-co-GMA) and poly(MEA-co-ECHMA)) were prepared by free-radical polymerization initiated by 2,2′-azobis-isobutyronitrile (AIBN). The copolymerization of MEA with GMA or ECHMA was carried out for 8 h in 1,4-dioxane at 75°C. The resulting polymer was then recovered by precipitation in an excess of hexane and purified by the three-time precipitation of the THF solution. The feed composition of PMEA was 85 mol%, and the yields of the polymers were in the range of 60–80%.

2.3. Characterization

The average molecular weights (MWs) and molecular numbers (MNs) of the polymers were measured by gel permeation chromatography (GPC), with polystyrene (Aldrich) as a standard. GPC measurements were carried out using Tosoh SC-8020 equipped with a refractive index detector (RI-8020) and Waters Styragel HR4E columns. THF was used as mobile phase at a flow rate of 1.0 ml/min. The composition of the copolymer was determined by 400 MHz proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy using a Varian Unity Plus 400 spectrometer. $CDCl_3$ was used as an NMR solvent. Tetramethylsilane (TMS) was used as a reference.

2.4. Laser Setup

Our experimental setup is shown schematically in Figure 1. In our experiments, a Ti:sapphire oscillator (Spectra-Physics) with a

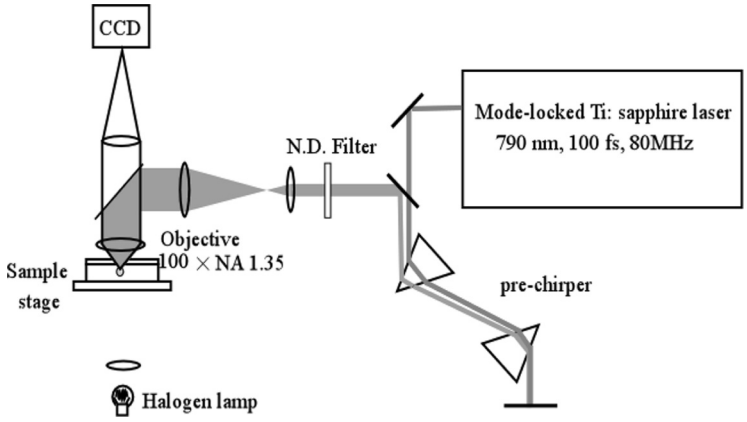

FIGURE 1 Experimental setup for the two-photon absorption system.

repetition rate of 80 MHz, pulse duration of 100 fs, and laser wavelength of 790 nm was used. The number of laser pulses and the irradiation time were controlled by a mechanical shutter having a minimum switching time of 5 ms. Laser power was 100–380 mW, and the scanning speed was 50–600 μm/s. Femtosecond laser pulses were focused by a × 100 immersion lens microscope objective with a numerical aperture (NA) of 1.35 (Olympus) and filled with a refractive index matching oil ($n_{oil} = 1.515$).

2.5. Preparation of the Patterned Surfaces

The polymer and the photoinitiator were dissolved in THF whose concentration was 125 mg/ml. The concentration of the photoinitiator varied between 0.001 and 1 wt%. 0.5 ml of the polymer solution was pipetted dropwise onto the cover glass. The cover glass containing the polymer solution was spun at 1,000 rpm for 10 s using a spin coater (1H-7D, Mikasa). A polymer film can be photocrosslinked by the two-photon adsorption technique. On the completion of pattern writing, an un-crosslinked polymer was washed away using MEK.

2.6. Platelet Adhesion Test

Patterned surfaces were washed three times with phosphate-buffered saline (PBS). A polyethylene terephthalate (PET) sheet was used as a negative control. The PMEA surface was used as a positive control. Blood was drawn from healthy volunteers and mixed with 1/9 volume

of acid citrate dextrose. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by the centrifugation of the blood at 1200 rpm and 3000 rpm for 5 min and 10 min, respectively. Platelet suspension plasma containing 1×10^5 cells/μl of platelet was prepared by mixing PRP with PPP. Then, 100 μl (platelet number: 1×10^7) of the platelet suspension plasma was placed on the sample surface and incubated for 60 min at 37°C. After the patterned surfaces were washed three times with PBS, they were immersed in 1% glutaraldehyde of PBS for 60 min at 37°C to fix the adhered platelets. The sample was freeze-dried, following which, it was sputter coated using palladium gold, prior to the observation by scanning electron microscopy (SEM, S-3500 N, Hitachi).

3. RESULTS AND DISCUSSION

3.1. Design and Synthesis of Novel Biocompatible Copolymers

In this section, we describe the experimental setup and discuss the results of the two-photon fabrication of biocompatible polymers. In this study, PMEA copolymers were designed for photocrosslinking experiments, because PMEA showed excellent compatibility with platelets, white blood cells, coagulation, and complement systems, and low protein adsorption with low denaturation [12,15,16]. Various medical devices can be coated with PMEA, which comes in direct contact with blood in order to reduce the formation of thrombus, and indeed, it has demonstrated significant clinical benefits in the field of artificial lung device [17]. In addition, we have already quantified the adsorption behaviors of the proteins on the surfaces of PMEA and common polymers in terms of their apparent association constant and adsorption and desorption rate constants [16]. These results suggest that the interaction between PMEA and proteins is weaker than that observed in other polymers. We hypothesized that the formation of a freezing-bound water structure was an important factor that contributed toward the excellent biocompatibility of PMEA [13,14,18–20]. Here, we designed and synthesized PMEA copolymers, which exhibit biocompatibility and photocrosslinking moiety. The copolymers used were poly(MEA-co-GMA), and poly(MEA-co-ECHMA), and their chemical structures are shown in Figure 2. The MWs (MW/MN) of poly(MEA-co-GMA) and poly(MEA-co- ECHMA) were 85,400 (3.1) and 64,000 (3.8), respectively. The composition of the monomer in the copolymer is determined by the NMR. The composition shown in Figure 2 agrees well with the feed composition (85 mol% PMEA). This result indicates that all the copolymers have a random monomer

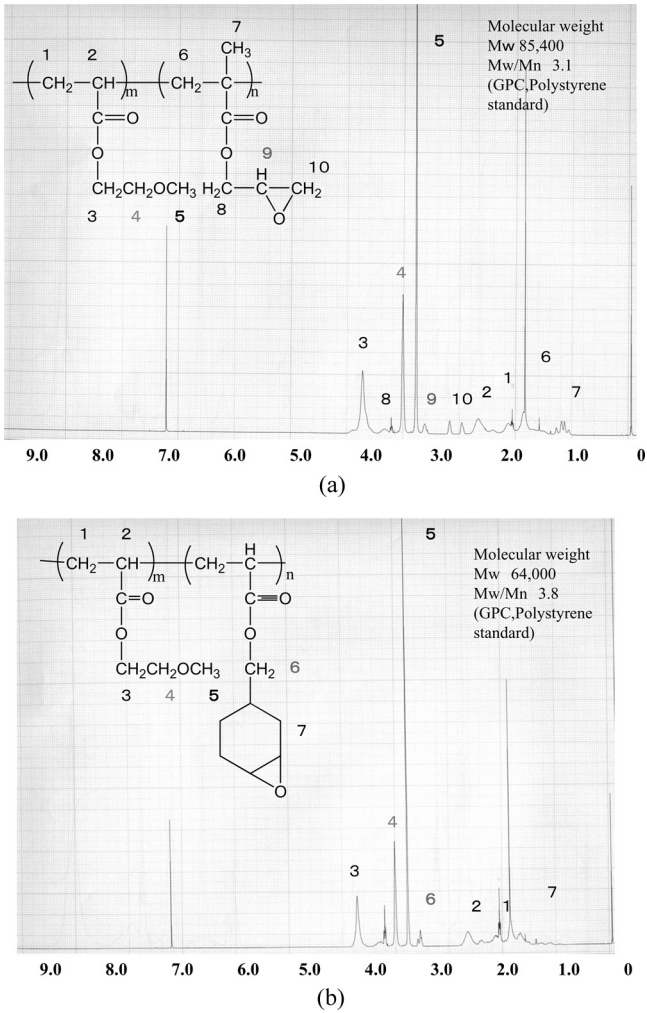

FIGURE 2 ^{1}H-NMR spectrum of (a) poly(MEA-co-GMA) and (b) poly(MEA-co-ECHMA). The composition of MEA was 85 mol%.

sequence. This implies that the copolymerization of MEA and GMA or ECHMA will obey the ideal mixing case.

3.2. Characterization of Photoinitiators

The two-photon absorption technique is based on the use of visible light and ultra-short pulse laser. When laser pulses are focused inside

a photocrosslinkable material, a reaction in which two photons are absorbed simultaneously is initiated; thus, resulting in the crosslinking of the material. Prior to structure fabrication, all photoinitiators were characterized to find suitable conditions for the two-photon absorption technique. A wavelength of $\lambda = 790$ nm was applied to induce two-photon crosslinking. Figure 3 shows the chemical structures and linear absorption spectra of three different photoinitiators in THF, which were determined using a spectrophotometer, i.e., trans-4-[P-(N-methyl-N-hydroxylethylamino)styryl]-N-methylpyridium-tetraphenyl borate (curve A), N,N-Diphenyl-7-[2-(4-pyndinyl)-eyhenyl]-9,9-diethyl-9H-Auoren −2awine (curve B), and Uvacure 1591 (curve C). From the adsorption spectra, it can be observed that there exists an absorption band near 500 nm resulting from the photoinitiator A (Figure 3a), and the solution is transparent in the 600–800 nm spectral range. In addition, two absorption bands exist in the 300–400 nm range due to the photoinitiator B (Figure 3b), and the solution is transparent in the 500–800 nm spectral range. Further, an absorption band exists near 300 nm resulting from the photoinitiator C (Figure 3c). However, the strong absorption band has a two-photon energy of less than 800 nm IR radiation; therefore, an effective two-photon absorption could be expected with a two-photon energy of less than 800 nm absorption. From these spectra, it can be inferred that B can be an appropriate photoinitiator, which satisfies two-photon absorption in the case of 790 nm absorption. The photoinitiators were transparent to an ultrashort-pulsed beam emitted from the Ti:sapphire mode-locked laser, and they allow the laser pulses to

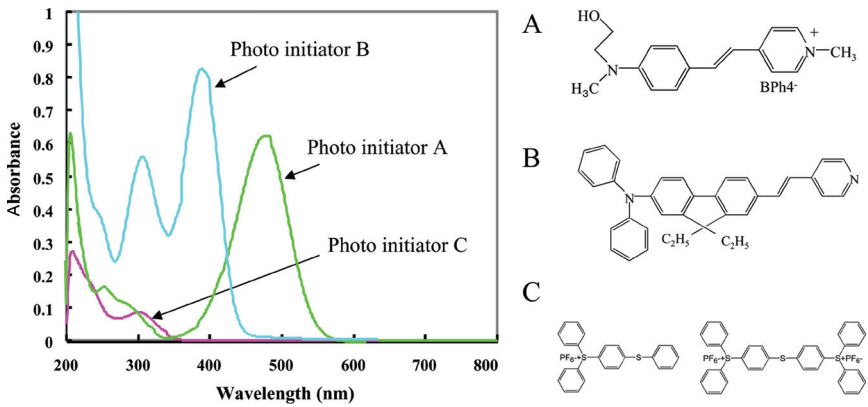

FIGURE 3 Chemical structures and liner absorption spectra of three different photoinitiators in THF.

penetrate deeply. It is known that the two-photon absorption cross sections such as cross sections for the generation of radicals (σ_2') of the photoinitiators.

A (trans-4-[P-(N-ethyl-N-hydroxylethylamino)styryl]-N-methyl-pyridium- tetraphenyl borate) and B are $\sigma_2' = 4.7 \times 10^{46}$ cm$^4 \cdot$ s [21], and $\sigma_2' = 0.97 \times 10–^{46}$ cm$^4 \cdot$ s [22], respectively. The photoinitiators were specially designed using π-conjugated compounds with higher two-photon sensitivity than conventional UV-adsorbing initiators such as the photoinitiator C.

3.3. Two-Photon Fabrication

When near-IR light, which has high peak power, is focused inside the polymer film, the spatial density of the photons increases at the focal spot. A photoinitiator, which absorbs a UV photon, absorbs two near-IR photons simultaneously and becomes a radical when the spatial density of the near-IR photons is sufficiently high. The resultant radical breaks the epoxy bonds of copolymers and photocrosslinked polymers. The crosslinked polymers do not dissolve in organic solvents; however, the un-crosslinked polymer is washed away with the solvent. The speed of the above reaction initiated by the two-photon absorption is proportional to the square of the photon density at each position in the polymer film, whereas the speed is proportional to the density itself for a reaction initiated by single-photon absorption. The point-spread function for two-photon absorption photo-crosslinking is defined as the square of the point-spread function for single-photon absorption polymerization [3]. This implies that if we use a pulsed laser of 790 nm wavelength, the lateral size of solidification at the focal spot would be almost the same as that of the diffraction-limited light spot at 395 nm, and the longitudinal size would be smaller than that of the diffraction-limited light spot [3]. The two-photon absorption process occurs only at the focused spot, as shown in Figure 4 (when scanning speed: 50 μm/s, concentration of photoinitiator: 0.1 wt%, and laser power: 380 mW). Line structures were fabricated on the focused spot of laser. The front spot of the line grows in the polymer film, because the focused laser spot locally photo-crosslinking, changing the refractive index locally. The original thickness of the polymer film was approximately 20 μm. After photocrosslinking, the un-crosslinked polymer was washed away with MEK. Typically, two-photon induced crosslinking was responsible for the formation of line patterns having a width of 0.5 μm, which were observed by SEM, as shown in Figure 5 (when scanning speed: 600 μm/s, concentration of photoinitiator: 0.1 wt%, and laser power: 190 mW). Various patterns such as dots,

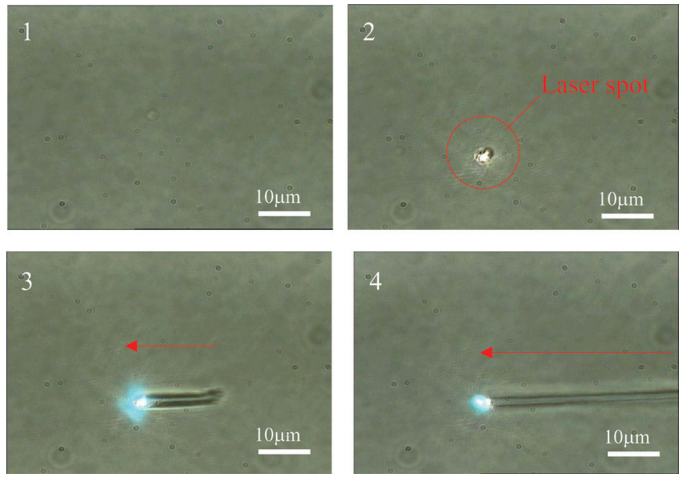

FIGURE 4 Two-photon crosslinking process in the polymer film. Photoinitiator B, scanning speed: 50 μm/s, concentration of photoinitiator: 0.1 wt%, and laser power: 380 mW.

holes, lines, and grids were successfully created using this technique. In the absence of photoinitiators, no writing occurred at powers below the damage threshold (laser power less than 700 mW in our system). The two-photon absorption technique allows a rapid, single-step fabrication of patterned surfaces with a resolution down to sub-micron size. Next, the fabrication parameters that affect spatial resolution are investigated. Fabrication resolution can be controlled by changing the laser pulse energy (laser power), exposure time, polymer type,

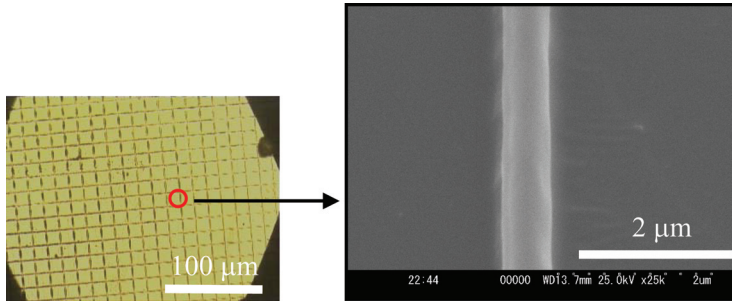

FIGURE 5 Typical optical and SEM images of patterned surfaces fabricated by two-photon absorption. Bar: 2 μm, scanning speed: 600 μm/s, concentration of photoinitiator: 0.1 wt%, and laser power: 190 mW.

initiator concentration, excitation wavelength, and scanning speed. To achieve sub-micron resolution, the scanning speed, absorption power, and initiator concentration were optimized. It was found that high scanning speed, low absorption power, and low initiator concentration induced the formation of a line structure having a small width. In particular, the scanning speed was the most effective parameter in the formation of line structures, and the fabrication resolution could be controlled in the sub-micrometer range by changing the above-mentioned parameters. It was also found that the fabrication resolution of both poly(MEA-co-GMA) and poly(MEA-co-ECHMA) was almost the same.

3.4. Biocompatibility Test

Micro-patterned scaffolds composed of polymers significantly influence cell behavior [1,2]. Platelet adhesion is one of the important tests performed to evaluate the biocompatibility of patterned surfaces. It is well known that platelet adhesion and thrombus formation take place when the material surface comes in direct contact with blood. The adhesion of platelets onto the surface of the polymer *in vitro* was investigated to evaluate biocompatibility with regard to in vivo evaluation. Figure 6 shows the number of platelets adhered to the surfaces of the copolymers. These polymers significantly affect platelet adhesion. Minimum number of platelets adhered onto the surface of the polymer; this number was almost the same for copolymers and PMEA, but it differed for PET. Since the morphology of a platelet is one of the factors that express the degree of platelet activation, the morphology of a platelet adhered on the patterned surfaces was

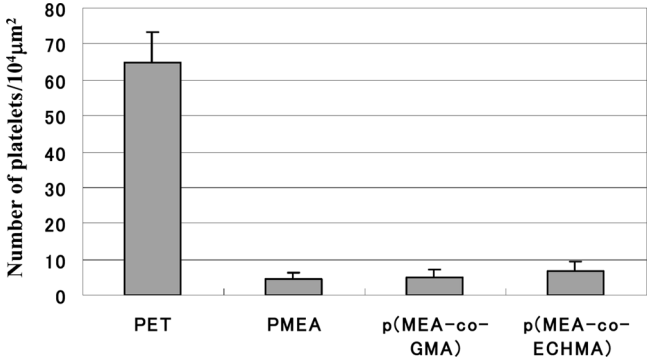

FIGURE 6 Number of platelets adhered onto the surface of polymers.

observed by SEM. The platelets adhered on the surface of PET spread with a pseudopod formation. Therefore, it is concluded that PET activates platelet function. On the other hand, the platelets that adhered on the patterned surfaces and the surfaces of PMEA retained their original spherical shape. This result implies that the patterned surfaces do not activate platelet function. In future, we plan to examine the adhesion behavior of other human cells. Patterned surfaces can be used in medical and biological applications.

4. CONCLUSIONS

We presented the results of the two-photon absorption technique, which can be applied for the precise fabrication of scaffolds. Submicron resolution was achieved using two different biocompatible polymers, namely, poly(MEA-co-GMA) and poly(MEA-co-ECHMA). Fabrication resolution can be controlled in the sub-micrometer range by changing the scanning speed, absorption power, and photoinitiator concentration. The two-photon absorption technique is used for the fabrication of complex structures that can be used as parts in tissue engineering scaffolds, cell culture platforms, biosensors, and medical devices.

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