Protein adsorption and cell adhesion behavior of engineering plastics plasticized by supercritical carbon dioxide

Masaki WATANABE, Hanako MAEDA, Yoshihide HASHIMOTO, Tsuyoshi KIMURA and Akio KISHIDA

Department of Material-Based Medical Engineering, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo

Corresponding author, Akio KISHIDA; E-mail: kishida.mbme@tmd.ac.jp

We aimed to evaluate the biological properties of engineering plastics (PC, PSU, PAR) processed using supercritical carbon dioxide (scCO₂). Conventional mold process was used to prepare disk-shaped samples that were then plasticized by scCO₂ at temperatures lower than the glass transition temperature (Tg) of the polymers. Surface roughness, contact angle, and amount of adsorbed protein on the surface were increased after treatment. The surface roughness of PC was significantly changed by scCO₂ treatment. Cell adhesion and proliferation changed according to the differences in surface roughness. Initially, the cell adhesion decreased in all scCO₂-treated polymers. At 3 day, the cell proliferation on scCO₂-treated PC was lower than that on non-treated PC, while that on treated and non-treated PSU and PAR samples remained unaltered. These results suggest that when supercritical treatment is performed under conditions that affect the surface properties of the material, we should consider that cell adhesion and proliferation may change.

Keywords: Engineering plastics, Protein adsorption, Cell adhesion, Supercritical carbon dioxide

INTRODUCTION

Polymer materials are indispensable to fabricate medical and dental devices. In general, polymer materials are inferior in strength to metals or ceramics. Therefore, the application of polymer-based biomaterials in bone and tooth substitutes has been limited. In recent years, high-strength polymer materials, whose physical properties are comparable to those of metals and ceramics, have been developed. Such polymer materials are called “the engineering plastics”.

It is well known that engineering plastics have high mechanical properties, heat resistance, and chemical resistance, and are already used in the medical and dental fields. For example, polycarbonate (PC) has been applied in infusion tube joints, polysulfone (PSU) and polyarylate (PAR) are used in dialysis membranes and syringes, respectively, polyether ether ketone (PEEK) is commonly used in artificial bone, and polytetrafluoroethylene (PTFE) has been used in artificial ligaments. Due to their excellent properties, engineering polymers are also attracting attention as alternative materials to metal. On the other hand, as these engineering plastics have high mechanical and thermal properties, they are difficult to mold into the desired shape with predictable qualities. In order to solve this problem, new molding processes such as 3D printing, emulsion foaming, and electron beam technologies are being studied; however, these technologies also have disadvantages namely in terms of cost, scalability, and throughput. As the technology that can compensate those drawbacks, the supercritical carbon dioxide-based technology is expected to be a new engineering plastic processing method.

It is well known that supercritical fluids can create unique environments. Supercritical carbon dioxide is already applied in various fields, such as pharmaceuticals and semiconductors, because it allows to obtain materials that present different structures, such as nanoparticles, microcapsules, thin films, and foams. Kirby and McHugh reported a systematic research study on the phase behavior of polymers in supercritical fluid solvents. One of the most advantageous aspects of supercritical processing is that engineering plastics can be processed at low temperatures. Processing at low temperature is similar to general mold processing and may be useful from the viewpoint of green chemistry. However, little has been reported on the physicochemical properties of plasticized engineering plastics by supercritical fluids. It is important to know the effects of supercritical treatment as basic data for using engineering plastics in medicine and dentistry.

In this study, from the viewpoint of the application of supercritical carbon dioxide treated engineering plastics as biomaterials, we investigated the physicochemical and biological properties, protein adsorption and cell adhesion and proliferation, of engineering plastics processed using supercritical carbon dioxide.

MATERIALS AND METHODS

Apparatus

Figure 1 shows a schematic diagram of the apparatus used to prepare the samples. This system mainly consists of a high-pressure pump (NP-FX-25(J), Nihon Seimitsu Kagaku, Tokyo, Japan), a supercritical reaction chamber (Koatsu System, Saitama, Japan), and back pressure regulator (26-1700, TESCOM, Tokyo, Japan). CO₂ first passes through the pre-heater (Koatsu System), then...
it is further heated by a circulator (MA-4, JULABO JAPAN, Osaka, Japan), and finally it is pressurized to the supercritical state in the reaction chamber. The sample holder (Koatsu System) is set in the reaction chamber. The processing capacity is 35.2 m³/day. The reaction chamber specifications are such that the design pressure is 29.9 MPa (regularly used pressure: 25 MPa), the design temperature is 200°C (regularly used temperature: 150°C), and the vessel volume is 200 mL.

**Materials**

Engineering plastic such as polyarylate (PAR; U-100, Unitika, Osaka, Japan), polycarbonate (PC; K-1300Y, Teijin, Osaka, Japan), polysulfone (PSU; 182443, Sigma-Aldrich, St. Louis, MO, USA) were used. Table 1 shows the molecular structures, crystal structures and glass transition temperature (Tg) of the three polymers. These plastics were molded to disk shapes with a diameter of 9 mm and thickness of 0.2 mm to set the sample chamber. Fibronectin (FN) was purchased from Fujifilm, human mesenchymal stem cells (h-MSCs) and cell culture medium were purchased from Takara Bio (Shiga, Japan). All the other chemicals were purchased from Wako Chem (Osaka, Japan).

**Supercritical treatment**

Test samples with disk shape were first placed into the sample holder, which was then placed in the reaction chamber. As explained above, the CO₂ (99.9% purity) was first heated and then pressurized to the supercritical state in the reaction chamber. After reaching the desired conditions, the temperature was first lowered to near-room temperature and then the pressure was slowly decreased to get an unformed sample. The temperature and pressure conditions were shown in Table 2.

**Characterization**

1. **Surface properties**

The surface of samples before and after supercritical treatment were evaluated by laser microscopy (Keyence, Osaka, Japan), and the surface roughness (Ra) was calculated using the software. The hydrophilic/hydrophobic nature of samples was evaluated by contact

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**Table 1** Molecular structures, crystal structures, and Tg of the engineering plastics used in this work

| Polymer (abbreviation) | Molecular structure | Crystal structure | Tg (°C) |
|------------------------|---------------------|------------------|--------|
| Polycarbonate (PC)     | ![Polycarbonate](image) | Amorphous        | 150    |
| Polysulfone (PSU)      | ![Polysulfone](image) | Amorphous        | 190    |
| Polyarylate (PAR)      | ![Polyarylate](image) | Amorphous        | 193    |

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**Table 2** Experimental conditions

| Polymer | Temperature (°C) | Pressure (MPa) | Time (h) |
|---------|------------------|----------------|----------|
| PC      | 120              | 25             | 1        |
| PSU     | 150              | 25             | 1        |
| PAR     | 150              | 25             | 1        |
angle (FTA1000B, First Ten Angstroms, Portsmouth, NH, USA). A water droplet (1.1–1.2 µL) was added to the surface of the samples before or after supercritical treatment and then the contact angle was measured at 60 s.

2. Protein adsorption
Protein adsorption onto the engineering plastic samples before and after supercritical treatment was measured using FN/PBS[−] at a concentration of 100 µg/mL. Prior to the protein adsorption experiment, the engineering plastic samples were immersed in ultrapure water at room temperature for 12 h; the water was then discarded, and the samples were immersed in PBS[−] at room temperature for 2 h to reach a water absorption equilibrium state. The engineering plastic samples were then placed in 1 mL of protein solution at room temperature for 90 min. To elute the adsorbed protein from the surface of engineering plastic samples, the samples were washed several times with PBS[−] solution containing 2 wt% sodium dodecyl sulfate (SDS), and then incubated at 96°C for 10 min. After cooling to room temperature, the fluorescence intensity was measured using a protein analysis kit (Nano Orange Quantitative Kit, Invitrogen, Tokyo, Japan) to determine the concentration of the protein in the SDS solution14).

3. Cell adhesion
Cell adhesion onto the engineering plastic samples before and after supercritical treatment was evaluated using h-MSCs. The samples were placed in a 48-well plate and fixed with a tube (tygon®) with internal diameter of 7 mm. Cells (1×10^5 cells/mL; 200 µL of cell suspension) were seeded on each sample (2×10^4 cells/sample). After 1 day culture, the cells were stained with calcein-AM according to the manufacturer’s protocol and observed under a fluorescence microscope (BZ-X710, Keyence). After 3 days culture, the cell number was measured using the cell counting kit-8 (Dojindo Lab, Kumamoto, Japan) according the protocol of the manufacturer. Tissue culture polystyrene (TCPS) was used as a control.

**Statistical analysis**
All data are presented as mean±standard deviation based on five measurements for roughness and contact angle and on three measurements for protein adsorption and cell adhesion. The significance was evaluated using Student’s t-test and p<0.05 was considered statistically significant.

**RESULTS**

**Surface properties**
Supercritical carbon dioxide was used to treat the PC, PSU, and PAR disks. The treatment conditions are shown in Table 2. The temperature was set at 150°C, which is lower than the T_g of the samples. Figure 2 shows the appearance of PC, PSU, and PAR disks before and after supercritical treatment. The appearance is slightly changed by supercritical treatment, i.e., the samples were initially transparent and became opaque by supercritical treatment without size change. Figure 3 shows surface roughness (Ra) and static contact angle of samples before and after supercritical treatment. Both properties were increased in all samples after supercritical treatment.

**Protein adsorption**
Figure 4 shows the adsorbed amount of FN on the samples before and after supercritical treatment. The adsorbed amount of FN was increased after supercritical treatment.

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Fig. 2 Appearance of engineering plastics disks before and after supercritical fluid (SFC) treatment.

Fig. 3 Physical properties. (a) Surface roughness (Ra) and (b) contact angle.

*p<0.05
Fig. 4 Protein adsorption onto the engineering plastics before and after supercritical treatment. *p<0.05

Fig. 5 Correlativity between $Ra$ and adsorbed amount of protein before and after supercritical treatment.

Fig. 6 Fluorescence image of h-MSCs 1 day after cell seeding on the engineering plastics before and after supercritical treatment.
(a) TCPS, (b) PC before supercritical treatment, (c) PSU before supercritical treatment, (d) PAR before supercritical treatment, (e) PC after supercritical treatment, (f) PSU after supercritical treatment, (g) PAR after supercritical treatment

in PC and PAR, and there was no significant change in the amount of protein adsorbed on PSU before or after treatment. Figure 5 shows the correlativity between $Ra$ and adsorbed amount of FN. The correlation coefficient was 0.85, which indicates that the adsorbed amount of FN was increased after supercritical treatment.

Cell adhesion
Figure 6 shows the fluorescence microscope images of the h-MSCs seeded on the samples with and without supercritical treatment at 1 day after cell seeding. Many spindle-shaped cells were observed on all samples. Several spherical cells were also observed on the surface of the supercritical-treated samples compared with the non-treated samples. Figure 7 shows the ratio of h-MSCs adhered on the samples in comparison to that on TCPS after 3 days of cell culture. Although there was no significant difference between before and after treatment in PSU and PAR samples, the cell adhesion on PC was decreased by the supercritical treatment.

Fig. 7 h-MSC adhesion on the different samples before and after the supercritical treatment after an incubation period of 3 days. *p<0.05
DISCUSSION

Engineering plastics have several typical features, including high mechanical properties and heat and chemical resistance. Although engineering plastics are excellent materials to be applied as medical and dental biomaterials, the fabrication of engineering plastic-based materials is hindered because of these mechanical and thermal properties. Supercritical carbon dioxide can plasticize the polymer in a low temperature range below $T_g$, so it can lower the viscosity compared to existing molding processes. By combining supercritical carbon dioxide process with ongoing molding technology such as injection molding machines and 3D printers, it is expected that molding of complex shapes prosthesis, such as, over denture, partial denture, implant, abutment root, crown and crown bridge, will become easier and possible. In the present study, we investigated the physicochemical and biological properties of the engineering plastics PC, PSU, and PAR, which were treated with supercritical carbon dioxide as the first step of application of supercritical carbon dioxide treatment to engineering plastics.

The roughness of the supercritical-treated samples was increased compared to the non-treated samples (Fig. 3(a)). In addition to the change in appearance (from transparent to white), the supercritical treatment caused small voids and pores even when slow depressurization was applied, which means that the carbon dioxide was diffused in the polymer at high pressure and that the solvent (the supercritical carbon dioxide) expanded through pressure reduction. Nishikawa reported that polymers can absorb carbon dioxide in the supercritical state. In particular, if the polymers have a carbonyl group, e.g., poly(lactic acid), they can absorb high amounts of carbon dioxide because the molecular structure of the carbonyl group is similar to that of carbon dioxide. In this study, PC and PAR have a carbonyl group, and the $Ra$ after supercritical treatment was high ($Ra=8.89$ in PC and $Ra=5.91$ in PAR) compared with PSU ($Ra=3.90$). This means that PC and PAR can absorb more carbon dioxide than PSU, and that the $Ra$ easily increases in carbonyl-containing polymers following supercritical treatment. As shown in Figs. 4 and 5, there is a correlation between $Ra$ and adsorbed amount of FN protein, which is consistent with a previous study. It is considered that the contact angle is affected by $Ra$, thus we examined the correlation between these two factors. Figure 3(b) shows that the supercritical treatment caused an increase in the static contact angle. Thus, we assumed that the static contact angles of the samples after supercritical treatment increased because of the increase in roughness. It was reported that protein adsorption onto a material is the highest on surfaces with water contact angle of 60°–70°. The contact angle of all samples before supercritical treatment was approximately 65°–75°, and it decreased to nearly 10°–20° after supercritical treatment. It is generally accepted that the amount of adsorbed protein decreases when the contact angles are higher than 60°–70°; however, our results show that FN adsorption increased after supercritical treatment (Fig. 4). A possible reason for the large amount of adsorbed FN, even though the contact angles were higher than 75°, is related to the effect of the high surface roughness ($Ra$) after supercritical treatment. Although further investigation is needed, this shows that the supercritical treatment can alter simultaneously more than one of the parameters that control the adsorbed amount of protein.

The number of h-MSCs adhered on the supercritical-treated samples at 1 day was slightly lower than that of non-treated samples (Fig. 6). After 3 days of culture, there was no difference in cell adhesion between before and after supercritical treatment in PSU and PAR samples, while the cell adhesion on PC was further decreased on the supercritical-treated sample (Fig. 7). Missirlis et al. reported that cell adhesion on materials is reduced with increasing surface roughness. The roughness of PSU and PAR was slightly increased by the supercritical treatment, whereas that of PC was remarkably increased. Thus, we considered that the significant increase in surface roughness reduced cell adhesion in the case of PC. In general, when slow depressurization is applied, the voids and pores created by the carbon dioxide are often small, but in the case of polymers that have high carbonyl density, such as PC, the enhanced polymer-carbon dioxide interaction has some effect on the surface roughness of the polymer, which may affect cell adhesion. This indicates that supercritical carbon dioxide can be applied to treat surfaces or to mold polymer-based biomaterials, but if the biomaterials has carbonyl group, care must be taken regarding cell adhesion.

CONCLUSIONS

PC, PSU, and PAR were treated with supercritical carbon dioxide below the $T_g$ of the polymers, and the surface roughness and contact angle changed after supercritical treatment. It became clear that the amount of protein adsorbed on the surface of the materials correlates with their surface roughness, indicating that surface roughness has a significant influence on protein adsorption. Furthermore, it became clear that cell adhesion and proliferation were also changed according to the surface roughness. A previous report showed that the amount of carbon dioxide absorbed depends on the density of carbonyl groups in polymers. Thus, it is important to investigate the molecular structure of the polymers and the surface properties induced by supercritical treatment. In this study, the surface roughness of PC, which has a carbonyl group, was significantly changed by supercritical treatment. Initially, cell adhesion decreased in all supercritical-treated samples compared to the non-treated polymers. Cell proliferation was significantly reduced after 3 days of culture only in treated PC samples. Therefore, when supercritical treatment is performed under conditions that affect the surface properties, it is important to note that cell adhesion and proliferation can be affected as
Furthermore, this study showed that the treatment with supercritical carbon dioxide not only improves the moldability of engineering plastics but also has the potential as a surface treatment method. The next step is to study a new shape molding method and surface modification method using supercritical carbon dioxide for development of novel engineering plastics medical and dental devices.

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REFERENCES

1) Wagner AH, Yu JS, Kalyon DM. Injection molding of engineering plastics. Adv Polym Technol 1989; 9: 17-32.
2) Hu B, Duan X, Xing Z, Xu Z, Du C, Zhou H, et al. Improved design of fused deposition modeling equipment for 3D printing of high-performance PEEK parts. Mech Mater 2019; 137: 103139.
3) Hassanajili S, Pour A, Oryan A, Khozani TT. Preparation and characterization of PLA/PCL/HA composite scaffolds using indirect 3D printing for bone tissue engineering. Mater Sci Eng C 2019; 104: 109960.
4) Teo N, Gu Z, Jana SC. Polyimide-based aerogel foams, via emulsion-templating. Polymer 2018;157: 95-102.
5) Youn SW, Noguchi T, Takahashi M, Maeda R. Fabrication of micro mold for hot-embossing of polyimide microfluidic platform by using electron beam lithography combined with inductively coupled plasma. Microelectron Eng 2008; 85: 918-921.
6) Tom JW, Debenedetti PG. Formation of bioerodible polymeric microspheres and microparticles by rapid expansion of supercritical solutions Biotechnol Prog 1991; 7: 403-411.
7) Reverchon E, Porta GD, Taddeo R. Solubility and micronization of griseofulvin in supercritical CHF3. Ind Eng Chem Res 1998; 34: 4087-4091.
8) Kim JH, Paxton TE, Tomasko DL. Microencapsulation of naproxen using rapid expansion of supercritical solutions. Biotechnol Prog 1996; 12: 650-661.
9) Jung J, Perrut M. Particle design using supercritical fluids: Literature and patent survey. J Supercrit Fluids 2001; 20: 179-219.
10) Turk M, Hils P, Helgen B, Schaber K, Martin HJ, Wahl MA. Micronization of pharmaceutical substances by the Rapid Expansion of Supercritical Solution (RESS): a promising method to improve bioavailability of poorly soluble pharmaceutical agents. J Supercrit Fluids 2002; 22: 75-84.
11) Kayrak D, Akman U, Hortacsu O. Micronization of ibuprofen by RESS. J Supercrit Fluids 2003; 26: 17-31.
12) Alnaief M, Antonyuk S, Hentschel CM, Leopold CS, Heinrich S, Smirnova I. A novel process for coating silica aerosol microsphere for controlled drug release applications. Microporous Mesoporous Mater 2012; 160: 167-173.
13) Kirby CF, McHugh MA. Phase behavior of polymers in supercritical fluid solvents. Chem Rev 1999; 99: 565-602.
14) Nagahama K, Nishimura Y, Ohya Y, Ouchi T. Impacts of stereoregularity and stereocomplex formation on physicochemical, protein adsorption and cell adhesion behaviors of star-shaped 8-arms poly(ethylene glycol)-poly(lactide) block copolymer films. Polymer 2007; 48: 2649-2658.
15) Mishra S, Chowdhary R. PEEK materials as an alternative to titanium in dental implants: A systematic review. Clin Implant Dent Relat Res 2019; 21: 208-222.
16) Knaus J, Schaffarczyk D, Coefen H. On the future design of bio-inspired polyetheretherketone dental implants. Macromol Biosci 2020; 20: e1900239.
17) Campbell SD, Cooper L, Craddock H, Hyde TP, Nattress B, Pavitt SH, et al. Removable partial dentures: The clinical need for innovation. J Prosthetic Dent 2019; 118: 273-280.
18) Nishikawa S. The modification and processing of polymers with supercritical carbon dioxide. Nippon Gomu Kyokaishi 2004; 77: 19-23.
19) Deligianni DD, Katsala N, Ladas S, Sotiropoulou D, Amedee J, Missirlis YF. Effect of surface roughness of the titanium alloy Ti-6Al-4V on human bone marrow cell response and on protein adsorption. Biomaterials 2001; 22: 1241-1251.
20) Tamada Y, Ikada Y. Effect of preadsorbed proteins on cell adhesion to polymer surfaces. J Colloid Interface Sci 1993; 155: 334-339.
21) Deligianni DD, Katsala ND, Koutoukos PG, Missirlis YF. Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength. Biomaterials 2001; 22: 87-96.