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ORIGINAL ARTICLE

A novel substrate for testosterone: biodegradable and biocompatible oil gel

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The androgen testosterone is less expensive than bone morphogenetic proteins and has been shown to effectively repair bone fractures. However, testosterone is lipophilic and insoluble in water, making it difficult to load into hydrogels, which are common drug carriers. In this study, we prepared a novel oil gel composed of poly(L-lactide) and a poly(trimethylene carbonate) derivative and studied the release of testosterone from the gel. Dimethyl sulfoxide and dimethyl carbonate, which are organic solvents with relatively low toxicities, were used as dispersion media. The oil gel in dimethyl sulfoxide released testosterone faster than that in dimethyl carbonate. In addition, the dimethyl carbonate oil gel was vacuum-dried to reduce the gel porosity and thus slow testosterone release. Therefore, oil gel is a promising substrate for lipophilic drugs, including testosterone.
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INTRODUCTION

Bone fractures due to strong impact on the body are an important medical problem. Partial bone loss is a more serious problem and can be caused by trauma, tumor removal or surgery to correct congenital deformations. Segmental defects are difficult to correct because they usually require many surgeries to regenerate the bone and regain its function. Current surgical techniques, such as autografts, allografts and distraction osteogenesis, have limited success[1–3] and can have serious consequences, such as leg shortening or amputation, if they fail. Therefore, delivering therapeutic drugs to the segmental defect using substances such as hydrogels for tissue engineering has been studied.[4–7] Bone morphogenetic proteins (BMPs) are generally used as therapeutic drugs. For example, Lutolf and coworkers[7] reported the synthesis of polyethylene glycol-based hydrogels containing pendant oligopeptide ligands for cell adhesion and substrates for matrix metalloproteinase, which served as cross-linkers. The gels were used to deliver recombinant human bone morphogenetic protein-2 (rhBMP-2) to large defect sites in rat crania.[7] However, clinical applications of BMP-2 have been hindered by its high cost.[6] Therefore, alternative methods to aid in fracture repair must be developed.

Testosterone is an androgen that has been reported to induce callus formation within 14 days of treatment in mice. Extrapolating from the results of the study, 8.3 μg of testosterone would be required to treat a segmental defect of similar size in humans.[6] Testosterone can be obtained more easily than BMP-2 and is therefore a viable alternative. However, testosterone is lipophilic and insoluble in water and cannot be loaded into hydrogels efficiently.

Thus, we focus on oil gel substrates. Oil gels are semi-solid systems in which an organic liquid phase is immobilized within a three-dimensional network. They are typically employed as chemomechanical materials[8] and oil absorbents,[9] among other uses. Because oil gels contain organic solvents, it should be possible to load testosterone into them effectively. To our knowledge, the loading of oil gels with testosterone has not been reported,[10] probably because of a lack of information on the biocompatibility and toxicity of the solvents and gelators.[11] In addition, physical gels might not retain testosterone effectively because of their structural instability, resulting in their collapse.[12] Therefore, chemical gels composed of low-toxicity components are needed.

Polylactide (PLA) is a well-known biodegradable and biocompatible polymer that has been studied for use in biomedical material applications (for example, drug carriers,[13–15] resorbable sutures[16] and nanosheets[17]). To exploit these PLA properties in drug delivery systems, we studied nanoparticles composed of poly(γ-glutamic acid)-graft-poly(lactide) copolymers, which utilize PLA stereocomplexes.[13] We also reported the rapid fabrication of PLA stereocomplexes using an inkjet printer to alternately deposit precise amounts of poly (L-lactide) and poly(D-lactide) to form LbL composites without needing to rinse them.[18] This system can be employed to fabricate substrates for water-soluble drugs.[19] Moreover, we described oil gels composed of a chemically cross-linked copolymer of PLLA and poly (trimethylene carbonate) derivatives.[20] Poly(trimethylene carbonate) derivatives are also biodegradable and biocompatible[21–23] and have been used to create cross-linking points. In addition, a compound containing oligo(ethylene glycol) groups, which resemble the biocompatible polymer polyethylene glycol, was used.[24] Oil gels are assumed to be good candidates for substrates for lipophilic drugs, including testosterone, because they are composed of biocompatible polymer networks and are considered to be safe for human use. However, drug release profiles for oil gels have not been measured.

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In this study, we investigated the release of testosterone from oil gels (Figures 1 and 2). Low-toxicity organic solvents (that is, dimethyl sulfoxide (DMSO) and dimethyl carbonate (DMC)) were used as the dispersion media. The oral rat LD_{50} of these solvents is comparable with that of ethanol, which is considered to have low toxicity (DMSO: 14 500 mg kg^{-1} (Material Safety Data Sheet of Tokyo Chemical Industry Co., Ltd., Japan), DMC: >5000 mg kg^{-1} (Material Safety Data Sheet of Ube Industries, Ltd., Japan), ethanol: 6.2–17.8 g kg^{-1} (Material Safety Data Sheet of Kanto Chemical Co., Inc., Japan). To the best of our knowledge, this is the first report on the medical application of a PLA chemical gel with a low-toxicity organic solvent.

MATERIALS AND METHODS

Materials

L-lactide (Musashino Chemical Laboratory, Ltd., Tokyo, Japan) was recrystallized from ethyl acetate. Benzyl alcohol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was distilled using 4 Å molecular sieves. Stannous 2-ethylhexanoate ($Sn(Oct)_2$), toluene, chloroform, palladium-activated carbon (10% Pd, Pd/C), dichloromethane, testosterone, DMSO and DMC were obtained from Wako Pure Chemical Industries, Ltd. Palladium hydroxide on carbon (20% $Pd(OH)_2$/C) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and used as received. 1,2-Bis(2-aminoethoxy)ethane, ethyl chloroformate and triethylamine were purchased from Tokyo Chemical Industry Co., Ltd., and distilled before use. Phosphate-buffered saline (PBS) was prepared by dissolving the following reagents in ultrapure water: NaCl (8 mg ml^{-1}), KCl (0.2 mg ml^{-1}), KH_2PO_4 (0.2 mg ml^{-1}), and Na_2HPO_4 (1.15 mg ml^{-1}). Ultrapure water was prepared using a Millipore Academic A10 system (Merk Millipore, Darmstadt, Germany).

Measurements

The molecular weight of the polymer was determined by gel permeation chromatography. A JASCO Chem NAV system (JASCO Corporation, Tokyo, Japan) equipped with a PU-2089 pump, AS-2055 autosampler, CO-2065 column oven and RI-2031 detector was used at 40 °C. The measurements were calibrated using poly(methyl methacrylate) standards. Two commercial columns (TSKgel SuperH3000 and TSKgel SuperH7000, TOSOH CORPORATION, Tokyo, Japan) were connected in series, and DMSO was used as the eluent. ^1H nuclear magnetic resonance spectra were measured with an nuclear magnetic resonance spectrometer (JEOL FX400, JEOL Ltd., Tokyo, Japan) at 400 MHz.

Oil gel preparation

Poly(2-methyl-2-carboxy trimethylene carbonate-*co*-L-lactide) was synthesized as a prepolymer (see Supplementary Information). The prepolymer (m: n = 25:75, number-average molecular weight $M_n = 26\,000$, 0.803 mmol carboxy groups, weight-average molecular weight $M_w = 37\,000$, 300 mg) was dissolved in dichloromethane (0.68 ml). Then, ethyl chloroformate (0.11 ml, 1.16 mmol) and triethylamine (0.12 ml, 0.883 mmol) were added to the solution, which was chilled in an ice bath. After 1 h, a given amount of 1,2-bis(2-aminoethoxy) ethane was added, and the reaction mixture was heated at 40 °C for 3 h. After the reaction, excess chloroform was added to remove the unreacted compounds. More than 12 h later, an oil gel in chloroform was obtained (Scheme 1).

Testosterone release from the oil gels

Oil gels (30 mg) were immersed in DMSO or DMC containing dissolved testosterone (DMSO: 7 mg ml^{-1}, DMC: 10 mg ml^{-1}) for 12 h to load 180 μg of testosterone into them. The amount of loaded testosterone was determined by the absorbance of the organic solvent at 329 nm. After rinsing the gel with the organic solvent, it was immersed in 30 ml of PBS or 0.01 M aqueous NaOH at 37 °C, and the supernatants were analyzed by high-performance liquid chromatography (Shimadzu liquid chromatograph, CBM-20A system controller, LC-20AD pump, SIL-20A autosampler, SPD-20A detector, CTO-20A column oven, DGU-20A3R degassing unit, eluent: acetonitrile/water = 6/4, column: Mightysil RP-18 GP Aqua 250-4.6 (Kanto Chemical Co., Inc., Tokyo, Japan), UV detector: 244 nm). The release percentages were determined and compared with the total amount of loaded testosterone. The vacuum-dried gels used in the release experiments were prepared by vacuum-drying the oil gels in DMC loaded with 180 μg of testosterone for 12 h at room temperature.

Degradation of the vacuum-dried gels

The oil gels in DMC (30 mg) were vacuum-dried for 12 h at room temperature to produce vacuum-dried gels. Then, the gels were immersed in 0.01 M aqueous NaOH or PBS (300 μl) for a certain amount of time. The gels were subsequently collected and washed with ultrapure water. After the oil gels were dried under reduced pressure for 12 h at room temperature, they were weighed, and the percent remaining was calculated based on the weight loss.

RESULTS AND DISCUSSION

It is notable that 180 μg of testosterone could be loaded into the oil gels in DMSO and in DMC. This amount of testosterone is adequate for medical applications (8.3 μg)[6]. Figure 3a and b show the testosterone release profiles for the oil gels in DMSO and in DMC, respectively. The drug was released more quickly from the oil gels in DMSO than from those in DMC. For example, after 15 min, approximately 60% of the testosterone was released from the oil gels in DMSO (Figure 3a), while approximately 20% was released from those in DMC (Figure 3b). The difference in the release profiles is attributed to the different solubilities of the organic solvents in water; DMSO is soluble in water, while DMC is not (the solubility of DMC

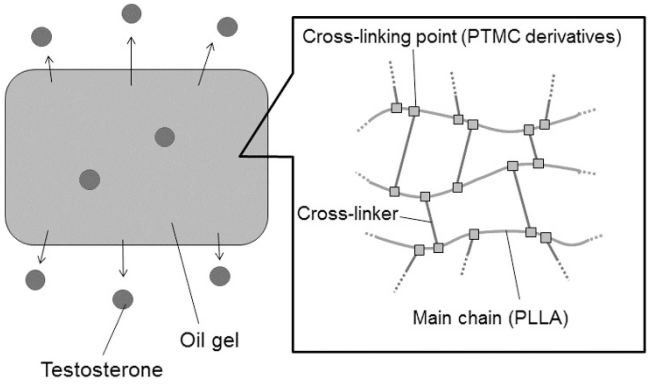


Figure 1 Schematic illustration of testosterone release from an oil gel composed of poly(L-lactide) and a poly(trimethylene carbonate) derivative. A full color version of this figure is available at *Polymer Journal* online.

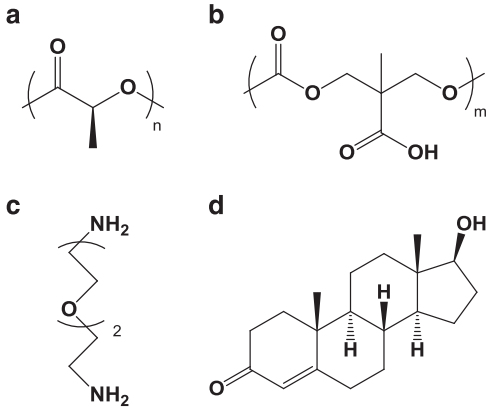
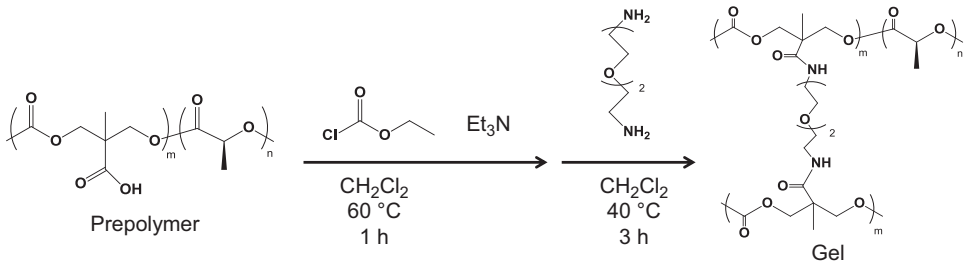


Figure 2 Chemical structures of poly(L-lactide) (**a**), poly(2-methyl-2-carboxy trimethylene carbonate) (**b**), 1,2-bis(2-aminoethoxy)ethane (**c**) and testosterone (**d**).



Scheme 1 Oil gel preparation.

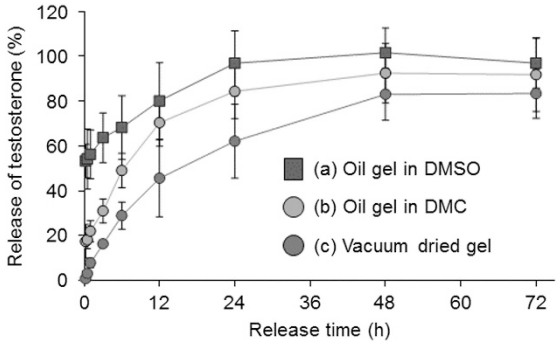


Figure 3 Testosterone release from oil gels in DMSO (a) and in DMC (b) and from a vacuum-dried gel (c). The amount of testosterone loaded into the gels was 180 μg ($n=3$). A full color version of this figure is available at *Polymer Journal* online.

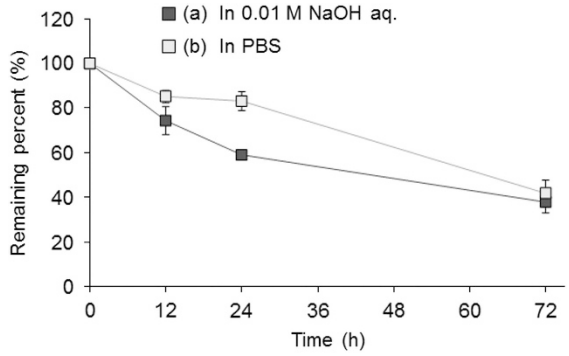


Figure 5 Degradation of a vacuum-dried gel in 0.01 M aqueous NaOH (a) and in PBS (b) ($n=3$). A full color version of this figure is available at *Polymer Journal* online.

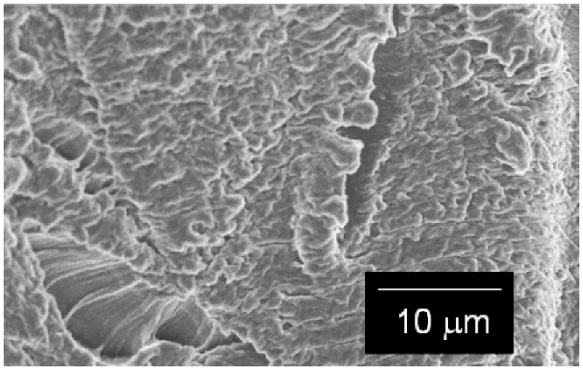


Figure 4 Scanning electron microscopy images of a vacuum-dried gel.

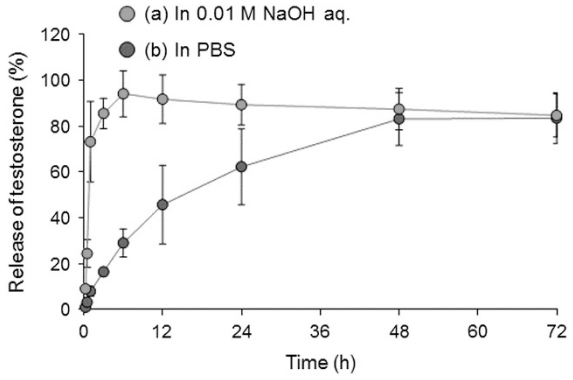


Figure 6 Testosterone release from vacuum-dried gels in 0.01 M aqueous NaOH (a) and in PBS (b) ($n=3$). A full color version of this figure is available at *Polymer Journal* online.

in water is 115 g l^{-1} at 20 °C (Material Safety Data Sheet of Ube Industries, Ltd., Japan)). When oil gels are immersed in PBS, the organic solvents tend to mix with water. Because DMSO is more soluble in water than DMC, DMSO tends to diffuse from the gels, which leads to testosterone release. However, it is desirable for testosterone to be released over a longer time period because testosterone-promoted callus formation occurs over approximately 14 days.[6]

It has been reported that drug diffusivity in gels decreases with decreasing gel porosity.[25,26] Thus, the release profile should be controlled by the oil gel porosity. The boiling points of DMSO and DMC are 189 °C and 90 °C, respectively. Therefore, it was assumed that DMC could be easily removed from the oil gels by vacuum-drying, which would decrease their porosity, because of its relatively low boiling point. Accordingly, vacuum-dried gels were prepared (DMC removal was evaluated based on the weight loss of the oil gels; data not shown), and their release profiles (Figure 3c) were determined. It was found that testosterone was released more slowly from the vacuum-dried gels than from the oil gels in DMC. To compare the release profiles, the time required for the oil gels to release 50% of the testosterone was determined. The release of approximately 50% of the testosterone required 6 h for the oil gels in DMSO, while it required 15 h for those in DMC.

The porosities of the gels were examined by scanning electron microscopy (Figure 4). In particular, the porosity of the vacuum-dried gels was compared with that of freeze-dried gels, which served as a reference. It was expected that the porosities of the freeze-dried gels and of the oil gels in DMC would be similar. The freeze-dried gels had pores of approximately 10 μm in diameter, indicating a porous structure.[20] On the other hand, no evidence of porosity was observed

on the vacuum-dried gel surfaces. On the basis of these results, it was concluded that the porosity of the oil gels in DMC can be reduced by vacuum-drying to obtain the desired slow-release profile. Moreover, vacuum-drying decreases the amount of organic solvent in the gels, resulting in a safer drug carrier.

Because the oil gels were composed of biodegradable poly(L-lactide) and poly(trimethylene carbonate) derivatives, the release profiles could also be controlled by changing their biodegradability. The degradation of the vacuum-dried gels in an alkali aqueous solution (0.01 M aqueous NaOH) and in PBS was studied. Figure 5 shows that the vacuum-dried gels were hydrolyzed in both 0.01 M aqueous NaOH and PBS. The hydrolysis occurred more readily in 0.01 M aqueous NaOH than in PBS. For example, approximately 40% of the vacuum-dried gels were hydrolyzed in 0.01 M aqueous NaOH (Figure 5a), whereas only 20% of the vacuum-dried gels were hydrolyzed in PBS (Figure 5b).

Next, the release profiles of the gels were measured in 0.01 M aqueous NaOH and in PBS. Figure 6 shows that testosterone was released more readily in 0.01 M aqueous NaOH (Figure 6a) than in PBS (Figure 6b), because the vacuum-dried gels were hydrolyzed more easily in 0.01 M aqueous NaOH than in PBS. From these results, it was concluded that the release profiles of the oil gels were related to the gel degradation. However, because the amount of testosterone released was not constant, testosterone must be released not only by the diffusion from the gel but also decomposition of the gel.[27] It is assumed that testosterone release from oil gels might be affected by several factors, such as the testosterone concentration gradient, oil gel degradation or organic solvent solubility in water.

CONCLUSIONS

Testosterone release from oil gels composed of poly(L-lactide) and a PTMC derivative was investigated. Using organic solvents with low toxicity, that is, DMSO and DMC, the testosterone release profiles of the oil gels were determined. The testosterone release from the oil gels in DMSO was faster than that from the oil gels in DMC. This difference in release profiles might be due to the different solubilities of the organic solvents in water. The release time was increased by vacuum-drying the oil gels in DMC.

This system can be widely employed for many lipophilic drugs, such as indomethacin[28] and hydrocortisone.[29] These vacuum-dried gels are good candidates for lipophilic drug carriers.

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