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The effect of poly(trimethylene carbonate) molecular weight on macrophage behavior and enzyme adsorption and conformation

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ABSTRACT

Poly(trimethylene carbonate) (PTMC) with molecular weights greater than 100 kg/mol is known to degrade readily *in vivo* while PTMC of less than 70 kg/mol is resistant to degradation. The reason for the molecular weight dependent degradation rate of PTMC is unclear, and may be due to differences in macrophage behavior or enzyme adsorption or activity. Macrophage number and production of reactive oxygen species (ROS) and esterase were measured when cultured on 60 and 100 kg/mol PTMC. Cholesterol esterase and lipase were adsorbed to 60 and 100 kg/mol PTMC and mass and viscoelastic properties of the adsorbed enzyme layers were measured. No significant differences were observed in macrophage number or production of degradative species. Significant differences were measured in mass, shear modulus and viscosity of the adsorbed cholesterol esterase layer, suggesting that the cholesterol esterase is adsorbing in a different conformation on the 60 and 100 kg/mol PTMC. Despite similar bulk moduli, the surface modulus of 60 kg/mol PTMC was significantly lower than 100 kg/mol. It is proposed that the difference in surface stiffness and polymer chain flexibility affect the arrangement of water bound to and freed from the polymer chains during adsorption, thus affecting enzymatic adsorption, conformation, and activity.

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1. Introduction

High molecular weight poly(trimethylene carbonate) (PTMC) is increasingly being investigated as a biomaterial for soft tissue regeneration [1–3], surgical devices [4–6], and drug delivery applications [7–9]. This interest is due, in part, to its degradation mechanism. PTMC degrades *in vivo* but, unlike the commonly used poly(hydroxy acids) such as poly(lactide-*co*-glycolide), does not do so via hydrolysis and does not yield acidic degradation products [10,11]. Rather, PTMC degrades *in vivo* via macrophage mediated enzymatic and/or oxidative degradation as a result of frustrated phagocytosis [12,13]. Upon failure to engulf the material, macrophages attach to the surface and secrete hydrolytic enzymes and reactive oxygen species to attempt to degrade the surface [14].

Interestingly, the *in vivo* degradation behavior of PTMC is molecular weight dependent. PTMC with molecular weight greater than 100 kg/mol has been reported to degrade faster *in vivo* than lower molecular weight (<70 kg/mol) PTMC [11]. Similar degradation behavior has been reported for other poly(alkylene carbonates) such as poly(ethylene carbonate) (PEC) [15].

The reason for the molecular weight dependent degradation rate of PTMC is, as yet, unclear and there is evidence for different mechanisms. PTMC is known to be susceptible to enzymatic degradation by cholesterol esterase [16] and lipase [11]. Zhang et al. observed that *in vitro* incubation in lipase alone resulted in faster degradation of 291 kg/mol PTMC compared with 69 kg/mol PTMC. This result was proposed to be caused by the greater hydrophilicity of the lower molecular weight surface after aqueous conditioning, which could alter the conformation of the enzyme when adsorbed at the PTMC interface, thereby reducing its activity [17].

It has also been suggested that the primary degradation mechanism of both PTMC and PEC is oxidation [12,18,19]. Oxidative degradation occurs *in vivo* as part of frustrated phagocytosis when macrophages attach to the material surface and secrete reactive oxygen species such as nitrates/nitrites, hydroxyl radicals and superoxide anions [20]. Of these, the superoxide anion is believed to be responsible for the *in vivo* oxidative degradation of PTMC [12,13,18,21].

A third explanation for the difference in degradation rate may be that the macrophages preferentially attach to, or are more active

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on, PTMC with molecular weights greater than 100 kg/mol PTMC as compared to PTMC with molecular weights lower than 70 kg/mol [22]. For example, the molecular weight of PTMC may influence the polymer surface properties, such as chain mobility or orientation of hydrophilic regions of the polymer chain, which, in turn, may influence the adsorption of adhesive or antibody proteins such as fibronectin and immunoglobulins [23–26]. A difference in quantity or conformation of proteins may then affect macrophage attachment, secretion of reactive oxygen species, or production of degradative enzymes [22].

In this study, we attempted to ascertain the reason for the molecular weight dependence of degradation rate observed with PTMC. Macrophage number and the secretion of both reactive oxygen species and esterase were measured when cultured on the surfaces of 60 kg/mol PTMC and 100 kg/mol PTMC *in vitro*. To determine whether there were differences in enzyme conformation following adsorption to the 60PTMC and 100PTMC surfaces, we also examined the quantity and viscoelastic properties of adsorbed cholesterol esterase and lipase, two enzymes implicated in the macrophage mediated degradation of poly(alkylene carbonates) [11,13,27].

2. Materials and methods

2.1. PTMC synthesis

Poly(trimethylene carbonate) was synthesized from trimethylene carbonate (TMC) monomer by ring-opening melt polymerization. TMC monomer (Biomatrik, China), initiator and stannous 2-ethylhexanoate (Fisher Scientific, Canada) catalyst were placed in a flame-dried glass ampoule and flame-sealed under vacuum. Molecular weights were targeted at 60 kg/mol (60PTMC) and 100 kg/mol (100PTMC). 1-pentanol (Sigma Aldrich, Canada) was used as an initiator for the preparation of 60PTMC. For 100PTMC, no initiator was added and residual water served as the acting initiator. The polymerization proceeded for 15 h at 130 °C. After polymerization was completed, the PTMC was dissolved in dichloromethane (DCM, Fisher Scientific, Canada), filtered through a 20 μm filter (P8, Fisherbrand) to remove glass particles, and purified by precipitation in methanol at −20 °C. Molecular weight was measured using gel permeation chromatography (GPC, Viscotek GPCmax VE 2001) at 25 °C using tetrahydrofuran (Fisher Scientific, Canada) as the eluent (1 mL/min). Molecular weight was determined by a universal calibration using polystyrene standards.

2.2. Contact angle measurements

Sessile drop water contact angles were measured on films of 60PTMC and 100PTMC. To form the films, a 0.3 g/mL PTMC in DCM solution was pipetted onto glass coverslips (22 mm × 22 mm), covered with aluminum foil to prevent the formation of bubbles, and dried overnight. Even layers were confirmed by caliper measurement of the edge thickness of the coated coverslips. Coating thickness was approximately 0.7 mm. Contact angles were measured for both dry samples and samples that had been conditioned for 24 h and 7 days in distilled water. For these measurements, 1 μL of distilled water was deposited on the surface of the flat, coated coverslip (VCA Optima XE, AST Products Inc.). Measurements were taken after 30 s of equilibration. Contact angles are reported as the average ± standard deviation of 3 samples with 5 drops measured at random locations per sample.

2.3. Glass transition temperature measurements

Glass transition temperatures (T_g) of 60PTMC and 100PTMC were measured using differential scanning calorimetry (DSC 1, Mettler Toledo). The samples were cooled to −45 °C and run using a heating-cooling-heating cycle from −45 to 25 °C. Glass transition temperatures were determined for both dry samples and samples that had been conditioned in distilled water for 24 h ($n = 2$) and 7 days ($n = 2$). Glass transition temperatures were measured from the second heating cycle.

2.4. Water uptake measurement

Water uptake was determined by gravimetric analysis. Dry samples ($n = 2$) of 60PTMC and 100PTMC were weighed and conditioned in distilled water for 24 h and 7 days. Surface water was removed by blotting with Kimwipes™ before weighing the samples a second time. Water content is reported as average percent mass gained after conditioning in water.

2.5. Enzymatic degradation of PTMC

Discs (6 mm diameter, 0.3 mm thick) were punched from films of 60PTMC and 100PTMC and incubated in solutions of porcine pancreas cholesterol esterase [28] (1 U/mL, phosphate buffered saline (PBS), Worthington) and lipase [29] (Thermomyces languinosus, >100,000 U/mL, Sigma, used as received). The discs were dried and weighed prior to incubation. Enzyme solutions were replaced every other day. Samples were removed from the solutions at 1, 5, 7 and 9 weeks. At each time point, the PTMC samples were removed from the enzyme solution, rinsed in distilled water, lyophilized for 48 h and weighed. Degradation is reported as percent of original mass lost ($n = 3$).

2.6. Macrophage culture

Macrophages were cultured on 60PTMC and 100PTMC surfaces to determine differences in cell number, esterase production and ROS/superoxide anion production. 60PTMC and 100PTMC were dissolved in DCM (0.3 g/mL). The PTMC solutions were pipetted into the wells of 96-well TCPS plates (50 μL/well). Coated well plates were dried for 16 h, sterilized under UV irradiation in a laminar flow hood for 30 min and conditioned in media for 12 h prior to seeding. RAW 264.7 immortalized murine monocyte derived macrophages were seeded in the coated wells, as well as into uncoated TCPS wells to act as a comparison surface, at a density of 30,000 cells/cm^2. The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic. The medium was replaced every other day.

2.7. Macrophage culture analysis

Adherent macrophages were removed from the wells prior to analysis. 200 μL of non-enzymatic cell dissociation media (Sigma) was added to the wells. Macrophages were then removed from the wells by scraping followed by sonication for 30 s. Macrophage number was determined by a fluorescent DNA binding assay (Quantifluor, Promega, $n = 6$) and ROS/superoxide production was measured using a ROS/superoxide fluorescent detection kit (Enzo, $n = 6$). Macrophage esterase production was determined by measuring the change in absorbance of *p*-nitrophenol (pNP) which is generated when active esterases hydrolyze *p*-nitrophenyl acetate (pNPA) [30] ($n = 6$). Briefly, 50 μL of sample cell suspension and 50 μL of phosphate buffer (50 mM, pH 7.4) were pipetted into the wells of a black 96-well assay plate. 20 μL of pNPA (10 mM, DMSO) were added to the wells with a multichannel pipette. Absorbance of pNP (410 nm) was immediately measured for 90 s with one measurement per second. Activity of esterase was measured as change in absorbance of pNP per second.

2.8. Scanning electron microscopy

To acquire images of PTMC degradation by macrophages, the bottoms of cell culture inserts (PET, Falcon, 12 well format) were coated with 60PTMC and 100PTMC (0.3 g/mL in DCM), covered in aluminum foil and allowed to dry overnight. Coated cell culture inserts were sterilized by UV irradiation in a laminar flow hood and conditioned in media for 12 h prior to cell seeding. Cells were seeded at 30,000 cells/cm^2. At days 1 and 14, the cells were removed from the PTMC surfaces by incubation in non-enzymatic cell dissociation solution (Sigma) for 5 min. The surfaces were dehydrated in a graduated ethanol series and chemically dried overnight using hexamethyldisilazane. After drying, the cell culture insert membranes were cut out of the inserts using a scalpel, affixed to SEM stubs, sputter coated with gold (10 mA, 4 min), and imaged using a Hitachi S-2300 scanning electron microscope at an accelerating voltage of 15 kV.

2.9. QCM-D sensor coating

Thickness, modulus and viscosity of enzyme layers adsorbed to 60PTMC and 100PTMC were quantified using a quartz crystal microbalance with dissipation (QCM-D E1, Q-Sense). Gold QCM-D sensors (4.95 MHz, 14 mm diameter, Q-Sense) were spin-coated (WS-400, Laurell Technologies) with a 1% solution of PTMC in chloroform. 50 μL of the solution was pipetted onto the stationary sensor before spinning (1400 rpm, 5 min). The coated sensors were dried overnight at room temperature and stored in a desiccator until used. The thickness and surface roughness of the polymer coating were determined using QCM-D measurements and atomic force microscopy, described below, respectively.

2.10. QCM-D protein adsorption

Dry PTMC coated sensors were inserted into the QCM-D chamber. Phosphate buffered saline (pH 7.4, Sigma) was flowed over the sensor (50 μL/min) for 8 h until stable frequency and dissipation baselines were measured. Enzyme solution (1 mg/mL) cholesterol esterase in PBS or 1 mg/mL lipase (porcine pancreas, Sigma in PBS) was then flowed into the sensor chamber (50 μL/min) for 10 min until the chamber was filled. The flow of the enzyme solution was then halted and the enzyme allowed to adsorb statically for 24 h, approximating the adsorption that takes place in a static degradation study ($n = 3$).

2.11. Surface plasmon resonance

Surface plasmon resonance was used to quantify mass of enzyme adsorbed from a solution of cholesterol esterase (1 mg/mL) or lipase (1 mg/mL). Unmodified gold SPR sensor chips (Au, Biacore) were coated with 60PTMC and 100PTMC. Briefly, 20 μL of a 0.1 mg/mL solution of PTMC in chloroform was pipetted onto the surface of a SPR sensor. The coated SPR sensors were dried for 8 h before the experiment. The

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