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Self-Assembled Poly(butadiene)-b-Poly(ethylene oxide) Polymersomes as Paclitaxel Carriers

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Abstract

In this work, self-assembled poly(butadiene)-b-poly(ethylene oxide) (PB-PEO) polymersomes (polymer vesicles) and worm micelles were evaluated as paclitaxel carriers. Paclitaxel was successfully incorporated into PB-PEO polymersomes and worm micelles. The loading capacity of paclitaxel inside PB-PEO colloids ranged from 6.7-13.7% w/w, depending on the morphology of copolymer colloids and the molecular weight of diblock copolymer. Paclitaxel loaded OB4 (PB_{219}-PEO_{121}) polymersome formulations were colloidally stable for 4 months at 4 °C, and exhibited slow steady release of paclitaxel over a 5 week period at 37 °C. Evaluation of the *in vitro* cytotoxicity of paclitaxel-polymersome formulations showed that the ability of paclitaxel-loaded polymersomes to inhibit proliferation of MCF-7 human breast cancer cells was less compared to paclitaxel alone. By increasing the concentration of paclitaxel in polymersomes from 0.02 μg/mL to 0.2 μg/mL, paclitaxel-polymersome formulations showed comparable activity in inhibiting the growth of MCF-7 cells. Taken together, these results demonstrate that paclitaxel-polymersomes have desirable restrained release profile and exhibit long-term stability.

Introduction

Paclitaxel is an anti-cancer therapeutic for the clinical treatment of advanced breast cancer, colon cancer, non-small cell lung cancer and ovarian cancer.(1) The therapeutic efficacy of paclitaxel has been hindered by its low solubility in water (~0.4 μg/mL). (2) Currently, paclitaxel is formulated in a 50:50 mixture of Cremophor EL (polyoxyethylated castor oil) and dehydrated ethanol for clinical administration.(3) Cremophor EL has been reported to elicit various side effects in patients, including hypersensitivity, nephrotoxicity, and neurotoxicity. (3) In addition, the use of Cremophor EL as a paclitaxel delivery vehicle was also observed to alter the biochemical properties of high density lipoproteins (HDL), and decrease the electrophoretic mobility of serum lipoproteins in blood.(4) Various aqueous based formulations of paclitaxel, such as polymeric micro/nanospheres (5), liposomes (6,7), polymer micelles (4,8-12), core-cross-linked polymer micelles (13,14), cyclodextrin (2), have been developed to increase the solubility of paclitaxel, minimize its side effects, and decrease its systemic clearance.

Amphiphilic block copolymers composed of a hydrophobic domain and a hydrophilic domain are thermodynamically driven to self-assemble into various ordered structures such as spherical

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micelles, worm-like micelles, and closed bilayer structures referred as polymersomes, depending on the volume/mass fraction of the hydrophilic to hydrophobic blocks. (15-17) Recently, diblock copolymer micelles have been extensively investigated as drug delivery vehicles to improve the therapeutic efficacy of many lipophilic drugs, such as FK506, which is used in organ transplant recipients for immunosuppression(18,19); L-685,818, a structural analogue of FK506(19); dihydrotestosterone(20); and paclitaxel(4,8-11,21). In all cases, the hydrophobic drug partitions into the hydrophobic micelle core, while the hydrophilic shell maintains contact with the bulk aqueous environment. When the micelle diameter is under 200 nm, the micelle-incorporated drug will selectively accumulate in solid tumors due to the enhanced permeability and retention (EPR) effect.(22)

Lee et al.(10) successfully loaded paclitaxel into poly(2-ethyl-2-oxazolin)-b-poly(ε-caprolactone) (PEtOz-b-PCL) micelles. PEtOz-b-PCL micelles exhibited maximum paclitaxel loading capacities approaching 7.6 wt% of polymer, and maximum paclitaxel solubility in aqueous solution reaching 0.76 mg/mL. Star-branched PEO-PLA copolymers were also investigated as a micellar carrier for paclitaxel, in this case, the release of paclitaxel from PEO-PLA micelles was controlled over 2 week period (23). Using poly(DL-lactide)-b-methoxy poly (ethylene glycol) (PDLLA-MePEG) micelles as paclitaxel carriers, Zhang et al. (11,24-26) successfully incorporated 25 wt% of paclitaxel into PDLLA cores, which increased the solubility of paclitaxel in water to 50 mg/mL.(11,24,26) However, these micellar paclitaxel formulations were typically stable for several days upon storage at 4°C before paclitaxel precipitated out of solution. *In vivo* studies showed that paclitaxel rapidly dissociated from PDLLA-MePEG micelles in blood and was eliminated as unencapsulated paclitaxel, while PDLLA-MePEG micelles were rapidly eliminated through the urine with a circulatory half-life of less than 3 hours.(8) Fast drug diffusion out of the polymeric micelles or micelle dissociation in the circulation system may have accounted for the resultant quick release of paclitaxel from the micellar formulation.(8) In a recent clinical trial with paclitaxel loaded PDLLA-PEG micelles (Genexol-PM, Samyang Corporation, Seoul, Korea) a lower AUC was observed, along with a shorter plasma half-life, at the highest paclitaxel concentration in the tumor (27-29). Loading paclitaxel into polymer micelles permits the delivery of a higher paclitaxel dose without additional toxicity.

In order to increase the stability and solubility of paclitaxel in micellar formulations, we developed polymersomes (polymer vesicles) as paclitaxel carriers using amphiphilic diblock copolymers. The self-assembly mechanism of amphiphilic diblock copolymers to form polymersomes is similar to that of lipids which self-assemble to form liposomes. In fact, the hydrophobic block of amphiphilic diblock copolymers can self-assemble into a bilayer structure that can incorporate hydrophobic drugs, whereas the hydrophilic block maintains contact with the bulk aqueous environment. The advantage of using polymersome-based drug delivery systems versus liposomal-based drug delivery systems is that the bilayer of polymersomes can be engineered to be much thicker (up to 40 nm) than that of liposomes (~4 nm), which could, in theory, improve both the hydrophobic drug solubility and mechanical stability.

To the best of our knowledge, polymersome incorporated paclitaxel formulations have not been studied, although polymer micelles(4,8-11) have been extensively investigated as paclitaxel carriers. Various biodegradable polymers, including poly(lactic acid) (PLA)(11, 14,24-26) and poly(ε-caprolactone) (PCL)(10,13), have been used as the hydrophobic core block in amphiphilic diblock copolymers to form polymeric micelles that function as paclitaxel carriers. However, these micelles are physically unstable in the blood circulation due to disintegration of the micelles, which results in fast dissociation of paclitaxel from the micelles before it reaches targeted tissues.(8) In order to increase the stability of amphiphilic diblock copolymer paclitaxel formulations, an inert and biocompatible diblock copolymer, poly

(butadiene)-b-poly(ethylene oxide) (PB-PEO), was used to form into polymersomes or worm micelles, as the paclitaxel carrier. The biocompatibility of PB has been established, and hence it is commonly used as the hydrophobic block in triblock copolymers.(30,31) Biodegradable diblock copolymer can also be blended with PB-PEO polymersomes to increase its biodegradability. In this study, we demonstrated the feasibility of PB-PEO polymersomes and worm-like micelles as paclitaxel carriers. The effect of the PB-PEO molecular weight and the morphology of PB-PEO colloids on paclitaxel loading capacity, colloidal stability, release profile, and *in vitro* cytotoxicity were investigated.

Materials and Methods

Materials

Diblock copolymers poly(butadiene)-b-poly(ethylene oxide) with various molecular weights were purchased from Polymer Source (Montreal, QC, Canada). The physical properties of all diblock copolymers used in this study are listed in Table 1. Polycarbonate membranes were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). All water was taken from a Barnstead E-Pure® (Barnstead/Thermolyne, Dubuque, IA) ultra pure deionization water system with a resistivity of 18.1 MΩ.cm. All other chemicals (reagent grade) were purchased from Sigma-Aldrich. (St. Louis, MO) and used without further purification.

Preparation of paclitaxel loaded polymersomes

Polymersome incorporated paclitaxel formulations were prepared via membrane extrusion. (32-37) In brief, 1 mL of diblock copolymers (5 mg/mL dissolved in chloroform) and different amounts of paclitaxel (dissolved in acetonitrile) were mixed together and dried in a round-bottom flask by rotary evaporation of the solvents at 37°C for 5 hours to ensure complete removal of chloroform and acetonitrile. Addition of 1 mL of PBS to the resultant dried copolymer/paclitaxel film led to spontaneous formation of vesicles off the round-bottom flask glass surface into the bulk solution. Rehydrated copolymer/paclitaxel solutions were then sonicated at 40 Hz for 5 min (Branson 2510, Branson Ultrasonic, Danbury, CT) and further incubated in a water bath at 60°C to promote vesicle self-assembly.(38) The vesicles were subsequently extruded 25 times through 200 nm pore diameter membrane to form polymersomes or worm-like micelles.(39) Light microscopy was employed to determine if there is paclitaxel crystal precipitate out of the colloidal solution.

Dynamic light scattering and zeta potential of polymersomes

The hydrodynamic diameter, polydispersity, and zeta-potential of paclitaxel loaded polymersomes and empty polymersomes was measured at 25°C using a Brookhaven Zeta Sizer (Zeta Plus, Brookhaven Instruments Corp., Holtsville, NY). All paclitaxel loaded polymersomes were stored at 4 °C. The colloidal stability was determined by measuring the size distribution every day over the study period until larger aggregates were detected.

Static light scattering of polymersomes

The size distribution of empty polymersome and paclitaxel loaded polymersome dispersions was measured using an Eclipse asymmetric flow field-flow fractionator (AFFFF) coupled in series to a 18-angle Dawn EOS multi-angle static light scattering photometer, equipped with a linearly polarized 30-mW gallium-arsenide laser operating at 690 nm, and an Optilab DSP differential interferometric refractometer (Wyatt Technology Corp., Santa Barbara, CA). The resulting light scattering spectra was analyzed using the ASTRA software (Wyatt Technology Corp.).(36,40,41) The mobile phase for all experiments consisted of PBS at pH 7.4 filtered through 0.2 μm filters.

HPLC analysis of paclitaxel loading and releasing from polymersomes

The drug loading content is defined as the ratio of the mass of encapsulated drug (paclitaxel) to the mass of diblock copolymers used for polymersome preparation. Free paclitaxel was removed by filtration through 0.45 μm membrane. Loaded paclitaxel in polymersomes and released paclitaxel levels in PBS buffer were determined with a high-performance liquid chromatography (HPLC) system (Waters Corporation, Milford, MA) using n-octylbenzamide as an internal standard.(7) Paclitaxel was extracted from polymersomes by adding 4.0 mL of tert-butyl methyl ether to the polymersome dispersion and mixing the sample for 30s.(42) The mixture was then centrifuged for 15 min at 3000 ×*g*, and 3.0 mL of the organic layer was then transferred into a 5 mL vial and evaporated under nitrogen. The residue was reconstituted with acetonitrile (65:35). Consequently, 30 μL of the solution was injected into a Symmetry C18 column equipped with a C18 column guard (Waters Corporation, Milford, MA). The column was eluted with acetonitrile/water (65:35) to separate paclitaxel from the diblock components. The flow rate was set at 1.0 mL/min, and the detection wavelength was set at 230 nm. The recovery efficiency of paclitaxel was determined by constructing a calibration plot of paclitaxel absorbance at 230 nm versus solutions of known paclitaxel concentrations. This was accomplished by dissolving known amounts of paclitaxel and polymer in acetonitrile, and measuring the paclitaxel absorbance as described previously.(42) The paclitaxel loading content of polymersomes was determined in triplicate for each sample. The extraction efficiency was then used as a correction factor for all determinations of paclitaxel concentration.(5)

In vitro release of paclitaxel from polymersomes

The *in vitro* release of paclitaxel was characterized as described previously.(5) Briefly, 0.4 mL of paclitaxel loaded polymersomes dispersion, which contained 5 mg/mL copolymer and 0.05 mg/mL paclitaxel, was incubated in 10 mL of PBS (pH 7.4) at 37°C under agitation. The amount of paclitaxel released was determined by measuring paclitaxel concentration in the supernatant. No free paclitaxel was observed during the release study by light microscopy. After 1, 7, 14, 21, 28, and 35 days, the polymer colloid suspension was centrifuged at 10,000 ×g for 15 min and the supernatant (10 mL) was taken out and replaced with 10 mL of fresh PBS buffer. The paclitaxel was extracted using 4 mL of tert-butyl methyl ether and analyzed by HPLC as described above.

Cytotoxicity of polymersomes

The cell line used to test the cytotoxicity of paclitaxel incorporated polymersome dispersions was the MCF-7 cell line, a human breast cancer cell line obtained from American Type Culture Collection (Manassas, VA). The MCF-7 cell line was cultured in α-MEM (Invitrogen, Carlsbad, CA) medium containing 25 mM HEPES, 5% fetal bovine serum (Life Technology Inc.), and 0.1% penicillin/streptomycin antibiotics (Invitrogen, Carlsbad, CA) in a 5% CO_2 humidified atmosphere at 37°C. Cells were plated in 96-well plates. After a 24-hour recovery period, MCF-7 cells were subjected to various concentrations of free paclitaxel in ethanol or paclitaxel incorporated polymersome dispersions. Cell viability was assessed by using the MTS cell proliferation assay kit which contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) (Promega, Madison, WI). After 72 hours, 20 μL of assay reagent was added to the treated MCF-7 cells and incubated for 2 hrs, the absorbance at 490 nm was recorded which is directly proportional to the number of living cells in culture.

Results and discussion

Particle size distribution, paclitaxel loading capacity, and colloidal stability of polymersomes

All diblock copolymers used in this study are labeled as OB1 through OB5, and listed in Table 1. PEO-based diblock copolymers, in the range of ~20-42% PEO volume fraction, self-assemble into polymer vesicles with a closed bilayer structure in preference to other structures.(38) All the amphiphilic diblock copolymers investigated in this study except OB3, which self-assembled into worm-like micelles, self-assembled into polymersomes with a bilayer structure or polymersomes coexisting with spherical micelles as corroborated by our previous atomic force microscopy (AFM) study.(32) The molecular structures of OB1 through OB5 are shown in Figure 1. The hydrodynamic diameter, polydispersity, and zeta potential of various diblock copolymer colloids (without paclitaxel) are listed in Table 2. The paclitaxel loading content, zeta potential, colloidal stability, and size of various diblock copolymer-paclitaxel formulations are listed in Table 3. Initially, all diblock copolymer-paclitaxel formulations exhibit narrow size distributions (half-width <20 nm). Paclitaxel loaded OB5 and OB2 polymersomes exhibit the highest paclitaxel loading capacities (13.76% and 13.72%, respectively). The order of decreasing paclitaxel loading capacity is OB4>OB1>OB3 after OB5 and OB2. Formulations of OB4 possess long term colloidal stability (~4 months) and sufficiently high loading capacity (11.06%).

We also evaluated the effect of paclitaxel concentration on the PB-PEO colloids' loading capacity. Various amounts of paclitaxel in acetonitrile were mixed with 5 mg of PB-PEO diblock copolymers in chloroform and subsequently evaporated under vacuum. The resultant polymer-paclitaxel films were then rehydrated with 1 mL of PBS buffer. The amount of paclitaxel solubilized in PB-PEO colloids was then analyzed by HPLC. All paclitaxel loading capacity studies were based on 5 mg/mL of diblock copolymers. The amount of paclitaxel initially mixed with polymers in organic solution and solubilized in PB-PEO colloids are denoted as $[Paclitaxel]_{solution}$ and $[Paclitaxel]_{PB\text{-}PEO}$. All the Figure 2 shows that as the initial paclitaxel concentration increases, the paclitaxel loaded in polymersomes or worm-like micelles (OB3) increases, reaches a maximum, and levels off at 1.5 mg/mL of paclitaxel concentration in the bulk. It is interesting to note that after the encapsulated paclitaxel in PB-PEO colloids reached a maximum, further increasing the paclitaxel concentration in solution caused the concentration of paclitaxel solubilized in the PB-PEO colloids to drop. A rapid release of paclitaxel from the PB-PEO colloids occurred immediately after the polymer-paclitaxel film was rehydrated and self-assembled into polymersomes or worm-like micelles. Paclitaxel crystals were observed using optical microscopy. The hydrophobic paclitaxel molecules may have altered the packing parameter, thereby destabilizing the hydrophobic bilayer of PB-PEO polymersomes, which resulted in the observed decrease of paclitaxel solubilized in the PB-PEO colloids. Figure 2 also shows that as the initial paclitaxel concentration was further increased, the amount of paclitaxel incorporated in PB-PEO colloids no longer increased, which implies that for certain mass fractions of diblock copolymer, paclitaxel reaches its saturated level in the hydrophobic bilayers of PB-PEO diblock copolymer vesicles.

The colloidal stability of amphiphilic diblock copolymer vesicles was analyzed by measuring the zeta potential and the size distribution changes of polymersomes over the study period in buffer (0.05 mM, pH 7.4). Colloidal stability is defined as complete discreteness with no aggregates present, which can be achieved by mutual repulsion due to a high zeta potential, adsorption of strongly hydrated hydrophilic protective colloids, or steric hindrance due to a nonionic polymer with sufficient chain length.(43) The paclitaxel loaded PB-PEO polymersomes showed similar zeta potentials around -5 mV as seen in Table 3. In high ionic strength PBS buffer, the Debye length of a colloidal particle is very small, therefore all PB-PEO colloids are slightly negatively charged. The negative zeta potential probably comes from

Cl^- adsorption to the hydrophobic PB block. Initially, the size distribution of all of paclitaxel loaded PB-PEO colloids (maximum paclitaxel concentration in 5 mg/mL of PB-PEO copolymer) exhibited a single peak around 200 nm. The size distributions of paclitaxel loaded PB-PEO colloids were measured daily until aggregates were detected by dynamic light scattering. After 1 and 3 weeks of storage at 4 °C, paclitaxel loaded OB2 and OB1 colloids, began to aggregate as seen in Figure 3 by the presence of 2 distinct peaks for the PB-PEO polymerosomes (~200 nm) and PB-PEO aggregates (~800 to 1000 nm). Similarly, after 2 weeks, aggregates were detected by dynamic light scattering for the OB5 polymersomes paclitaxel formulation, and the peak for paclitaxel loaded OB5 colloids (Figure 3 C) was split into two peaks, ~100 nm (PB-PEO micelles) and ~1000 nm (PB-PEO aggregates). During the 4 month study period, the size distribution of paclitaxel loaded OB4 polymersome retained a single peak and narrow size distribution as seen in the size distribution measured by static light scattering (Figure 4). DLS experiments also confirmed the presence of single peak of OB4 polymersomes at 200 nm in diameter. Given the small magnitude of the zeta potential, long-term colloidal stability of OB4 polymersome dispersions do not originate from electrostatic repulsion, but from steric repulsion derived from the long PEO corona on the surface of the colloids.

The loading capacity of polymer vesicles is dependent on the molecular weight of the hydrophobic block, since copolymers with larger hydrophobic molecular weight formed into thicker hydrophobic bilayers. OB5 possesses the largest molecular weight hydrophobic block, hence polymersomes self-assembled from OB5 possess higher paclitaxel loading capacity. However, OB2 also exhibited a very high paclitaxel loading capacity, despite the low molecular weight of its hydrophobic block. The branched structure of the poly(butadiene) block of OB2 confers more encapsulation volume to the hydrophobic bilayer of the resultant polymersome, which could potentially accommodate more hydrophobic paclitaxel molecules.

Our previous AFM study showed that OB3 formed into worm-like micelles exhibiting spaghetti-like patterns and tubular structures.(32) Various colloid morphologies will likely have different loading capacities and release kinetics.(44) For example, worm-like micelles are expected to have prolonged circulatory half-lives.(45,46) However, in this study OB3 worm-like micelles only incorporated 6.72% of paclitaxel, and were only stable for a week in aqueous solution. After a week, paclitaxel crystals were observed by optical microscopy for this sample. The short term stability of OB3 worm-like micelles may be attributed to the hydrophobic paclitaxel binding to the hydrophobic PB block, which alters the curvature of the hydrophobic chains and thereby the chain packing parameter, eventually resulting in disintegration of the micelle.(44)

OB5 has a larger molecular weight hydrophobic block, and therefore, higher paclitaxel loading capacity compared to OB4. However, the colloidal stability of the OB5-paclitaxel formulation is shorter compared to OB4-paclitaxel formulations. In fact the zeta potential of both formulations are similar in magnitude and both copolymers have long PEO chains, hence the colloidal stabilities are expected to behave similarly. Our previous AFM study show that OB5 has the ability to self-assemble into polymer micelles or polymersomes.(32) We hypothesize that at higher paclitaxel concentrations, the morphology of OB5 switched to polymer micelles over time. It is likely that the high loading content of paclitaxel results in thermodynamically unfavorable stretching of the hydrophobic chains, which results in micellization of the polymersomes(47,48) Hence, some paclitaxel will gradually be released from OB5 polymersome formulations. Our hypothesis is corroborated by dynamic light scattering measurements (Figure 3 C), in which the size of OB5-paclitaxel polymersome formulations decreased to the size of micelles during storage at 4°C after 2 weeks.

Zhang et al. (24) used poly(DL-lactide)-b-methoxy polyethylene glycol as a micellar paclitaxel carrier. In their study, up to 25% of paclitaxel (paclitaxel/polymer w/w loading ratio) was loaded into the matrices of PDLLA-MePEG micelles. However, the micellar paclitaxel solution typically remained stable for several days upon storage at 4°C.(9) In our study, 11.06% paclitaxel (paclitaxel/polymer w/w loading ratio) was loaded into OB4 polymersome bilayers and the formulations were colloidally stable for 4 months. This evidence of the stability of these formulations leads to the inference that paclitaxel in the hydrophobic core of OB4 polymersomes may be either molecularly bound to the hydrophobic polymer chains or distributed in an amorphous state in the hydrophobic block.

Paclitaxel loaded OB4 polymersomes exhibited high paclitaxel loading capacities and long-term colloidal stabilities. The loading ratio of paclitaxel/amphiphilic diblock copolymer is much higher compared to liposomes (~2%), (42) since polymersomes possess thicker hydrophobic bilayers with the potential of incorporating more paclitaxel. All paclitaxel loaded polymer vesicles investigated in this study were stored in the hydrated state using PBS buffer at 4°C. Using optical microscopy, we found no evidence of paclitaxel recrystallization in paclitaxel loaded OB4 polymersome formulations after 4 months. Our results thus show that paclitaxel loaded OB4 polymersome formulations are promising candidates for further *in vitro* and *in vivo* studies. The long-term stability of OB4 polymersome-paclitaxel formulations is desirable, since this provides an avenue in which paclitaxel could be effectively delivered to the targeted tumor site without releasing paclitaxel into the blood plasma or to health tissue.

The effect of paclitaxel concentration on size of OB5 polymersome

Initially, paclitaxel loaded OB5 polymersome formulations (for paclitaxel concentration below saturated level) displayed average diameters of ~200 nm. Figure 5 shows the size distribution of paclitaxel loaded OB5 polymersomes at different paclitaxel concentrations (initial paclitaxel concentration solubilized in 5 mg/mL of OB5 copolymer) stored at 4°C for 2 weeks measured by static light scattering. At lower paclitaxel concentrations (0, 0.2, 0.4 mg/mL), the size of these polymersomes were similar in magnitude compared to empty polymersomes. As the paclitaxel concentration increases (0.6, 0.7 mg/mL) the size of paclitaxel-loaded polymersomes decreases. The presence of smaller sized particles (~100 nm) indicates disintegration of OB5 polymersomes, while the presence of larger sized particles (~900 nm) indicates aggregation of OB5 polymersomes which were detected by dynamic light scattering (see Figure 3C). From previous AFM studies, we know that OB5 can self-assemble into polymersomes and spherical micelles under different conditions. (32) The size of OB5 polymersomes is ~200 nm in diameter, whereas OB5 polymer micelles is ~100 nm in diameter.(32) The incorporation of hydrophobic molecules in the hydrophobic bilayers of polymersomes increases the degree of stretching of the hydrophobic chains in the amphiphilic diblocks in order to accommodate more hydrophobic molecules in the hydrophobic core. (47,48) As the degree of stretching increases, the high stretching entropic energy penalty of the hydrophobic chains in the core of polymersomes will lead to precipitation of paclitaxel in order to decrease the total free energy of micellization.(49) Hence, polymersome-paclitaxel colloids will eventually transform into micelles and the size of the OB5 colloids will decrease. The interesting possibility that block copolymer vesicles can transform into cylindrical or spherical micelles by increasing the amount of solubilized hydrophobic molecules has been theoretically predicted based on a molecular thermodynamic analysis.(47,48) The morphology transition induced by solubilized hydrophobic molecules has to be further investigated by AFM or Cryo-TEM imaging.

As evidenced by dynamic light scattering measurements (Figure 3A and B), the peaks representing OB2 and OB1 polymersomes (~200 nm) did not change upon storage, whereas the peaks representing OB5 polymers colloids shifted to the size of polymer micelles (~100 nm). The effect of paclitaxel concentration on the size of polymersomes was not examined for

other formulations, since we don't expect morphology changes for other paclitaxel formulations.

In vitro release of paclitaxel from polymersomes

In vitro release profiles of paclitaxel loaded PB-PEO diblock copolymer colloids in PBS buffer at 37°C are shown in Figure 6. The release of paclitaxel from diblock copolymer formulations is expressed as the cumulative weight fraction of paclitaxel released from PB-PEO colloids into PBS buffer at a specific time point compared to the initial paclitaxel concentration in the hydrophobic core at the beginning of the time course study. Paclitaxel-polymersome OB4 dispersions show a slow steady release of paclitaxel, which continued over a 5-week period. 17%of the initial paclitaxel content was released from the hydrophobic bilayers of OB4 polymersomes over the time course of the study. Initially, OB3 dispersions showed a faster paclitaxel release followed by a steady slow paclitaxel release, with more than 84% of the initially loaded paclitaxel being released from the hydrophobic core of OB3 worm micelles after 5 weeks. Paclitaxel loaded OB1, OB5, OB2 polymersomes showed a fast release profile at the beginning of 2 weeks. After 2 weeks, slow steady release profiles were observed for these polymersome formulations.

The release of drugs from the hydrophobic core of diblock copolymer colloids depends upon the rate of diffusion of the drug from the colloids, the colloidal stability, and the rate of biodegradation of the copolymer. Polymer-drug interactions, localization of the drug within the colloid, and the glass transition temperature of the hydrophobic blocks are major factors that influence drug release kinetics.(44) The slow release profile of OB4 indicated that paclitaxel interacts strongly with the hydrophobic PB block. However, OB5-paclitaxel formulations were found to exhibit a fast release of paclitaxel at the beginning of the study, followed by a steady slow release of paclitaxel after two weeks of incubation in PBS. It is hypothesized that the localization of paclitaxel within the outer corona region or at the interface of OB5 polymersomes will account to some extent for the faster rate of release of paclitaxel at the beginning of the release study.(44) Because of the fairly high molecular weight of the PEO block (12.6 kDa) for the OB5 copolymer, it is possible that some paclitaxel is trapped in the hydrophilic corona region of OB5 polymersomes. Since the outer corona region of the polymersomes is quite mobile, paclitaxel release from this area will be rapid.(44) For OB3 formulations, the faster paclitaxel release profile most likely originates from disintegration of the worm-like micelles as hydrophobic paclitaxel binds to the PB block, thus destabilizing the worm-like micelle structure. Hence, paclitaxel rapidly dissociates from the hydrophobic core of worm-like micelles.

In vitro assay of paclitaxel loaded polymersomes for their ability to inhibit MCF-7 cell proliferation

Paclitaxel cytotoxicity stems from its ability to promote polymerization of tubulin dimers into microtubules. The resultant microtubules are stabilized by paclitaxel which prevents depolymerization, thereby inhibiting cell proliferation in the late G2 or M phase of the cell cycle.(50-52) In our study, the cytotoxicity of various polymersomes and polymersome-paclitaxel formulations was tested on human breast adenocarcinoma (MCF-7) cells using MTS proliferation assay. The percentage of viable cells was expressed as the ratio of the absorbance of the control experiment (no copolymer or paclitaxel added), which was taken to be 100% survival for the incubation periods (72 hrs) studied, to the absorbance of MCF-7 cells treated with various paclitaxel-polymer colloids formulations. MCF-7 cells treated with various PB-PEO polymer colloids (without paclitaxel) exhibited no cytotoxicity at 2 μg/mL of diblock copolymer as shown in Figure 7a. Further increasing the diblock copolymer concentration to 100 μg/mL did not elicit cytotoxicity for the 72 hr cell culture regardless of the type of diblock copolymer utilized. Paclitaxel loaded polymersomes were incubated with MCF7 cells for 3

days. The final paclitaxel concentration in medium was kept at 0.02 ug/mL for all formulations. As shown in Figure 7 B, paclitaxel loaded polymersome formulations exhibited similar cytotoxicity compared to paclitaxel alone dissolved in ethanol. In fact, >80% of MCF-7 cells were killed by OB1, OB3 paclitaxel formulations at 0.02 μg/mL of paclitaxel, whereas more than 90% of MCF-7 cells were killed by paclitaxel alone at the same paclitaxel concentration.

The cytotoxicity of PB-PEO formulated paclitaxel is correlated to the observed paclitaxel release profile. The cell viability of paclitaxel containing PB-PEO colloids showed that free paclitaxel alone had the highest level of cytotoxicity. The order of decreasing cytotoxicity of PB-PEO colloids formulated paclitaxel then follows: OB3>OB1>OB2≈OB5>OB4. The release rate of paclitaxel from PB-PEO colloids showed the following order OB3>OB5>OB1>OB2>OB4. OB4 polymersome-paclitaxel formulations showed the least cytotoxicity in the 3-day incubation period compared to free paclitaxel. This could be attributed to the slower release of paclitaxel from OB4 polymersomes to the cell culture medium as shown before (Figure 6). Release of paclitaxel into the medium is one of two mechanisms that are involved in transporting paclitaxel to the cell cytoplasm. The released paclitaxel then passively diffuse through the cell membrane and kill the cells. The second mechanism is internalization of paclitaxel-polymer colloid particles via endocytosis into the cytoplasm where paclitaxel is released.(20) The discrepancy between the position of OB5-paclitaxel cytotoxicity and release profile among 5 polymeric paclitaxel formulations could be explained by the much longer PEO corona on OB5 polymersomes due to the higher molecular weight of OB5 compared to OB1 and OB2 which may have hindered endocytosis of OB5 polymersomes into MCF-7 cells. Smaller degree of internalization of OB4 polymersomes due to the longer PEO corona on OB4 polymersomes may also explain the lower cytotoxicity of OB4 towards MCF-7 cells. Therefore, in a short term study, OB4-paclitaxel formulation showed the least cytotoxicity.

The cytotoxicity study conducted above does not consider the effect of dosage on cell viability. Therefore, cell viability was examined as a function of dosage to show that all formulations could achieve the same cytotoxicity as free paclitaxel alone by increasing paclitaxel concentration in the polymer colloids. As shown in Figure 8, paclitaxel loaded polymersome formulations killed MCF-7 cells in a dose-dependent manner. At 2 ng/mL of paclitaxel, 76% of cell proliferation was inhibited in the absence of copolymer, whereas for most of the other paclitaxel-polymersomes complexes, at the same paclitaxel concentration (OB4, OB2, and OB5), more than 50% of MCF-7 cells remained alive. In a 3-day cytotoxicity study, paclitaxel released from OB1, OB2, OB3, OB4, OB5 polymersomes corresponded to 12%, 3%, 32%, 15%, and 20%. Paclitaxel in the medium was much lower than its lethal dose (~2 ng/mL). Thus lower cytotoxicity was observed at 2 ng/mL. When the paclitaxel concentration increased to 20 ng/mL, paclitaxel released from the polymer complex is close to its lethal dose (except for OB4) and comparable cytotoxicity was observed.

Further increasing the paclitaxel concentration in polymersomes increased the cytotoxicity (i. e. decreased cell viability) of paclitaxel-polymersome formulations. However, OB4 polymersome-paclitaxel formulations showed much less cytotoxicity. By increasing paclitaxel dosage to 400 ng/mL in paclitaxel-polymersome, MCF-7 cell proliferation was significantly inhibited (>80%). The low level of cytotoxicity of OB4 formulation could be attributed to the restrained release profile of paclitaxel in OB4 formulations or small degree of endocytosis as discussed above. For paclitaxel-OB4 formulations, about 20% of the initially incorporated paclitaxel was released into PBS in 5 weeks. The restrained release profiles of paclitaxel and the dose dependent cytotoxicity profiles of OB4 formulations are desirable for paclitaxel delivery. In this way, the local concentration of paclitaxel at the tumor site *in vivo* will be retained at sufficiently high therapeutic levels to kill tumor cells.

Conclusions

Paclitaxel was successfully solubilized into the hydrophobic bilayer of PB-PEO polymersomes. The paclitaxel loading capacity of PB-PEO ranged from 6.7%-13.7%. The solubility of paclitaxel was greatly increased from 0.4 μg/mL in water, to 0.7 mg/mL in OB4 polymersomes. OB4 polymersome-paclitaxel formulations were colloidally stable over 4 months in the hydrated state at 4°C, and retained ~80% of the initially incorporated paclitaxel over the study period. OB4 polymersomes paclitaxel formulation showed less cytotoxicity compared to other formulations and paclitaxel alone due to the slow release of paclitaxel from OB4 polymersome bilayers. Further studies are required to elucidate the pathway of paclitaxel-polymersome complex internalization into MCF-7 cells, and in vivo biodistribution of paclitaxel loaded polymersomes.

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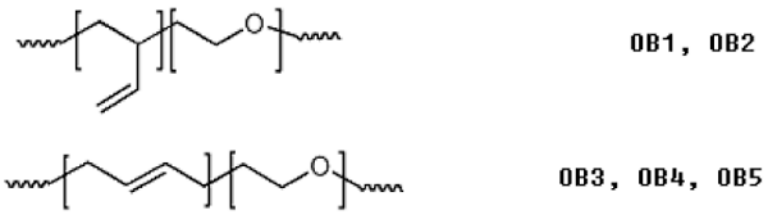

Figure 1.
Chemical structure of poly(butadiene(1,2 addition)-b-ethylene oxide) (OB1, OB2) and poly (butadiene(1,4 addition)-b-ethylene oxide (OB3, OB4, OB5).

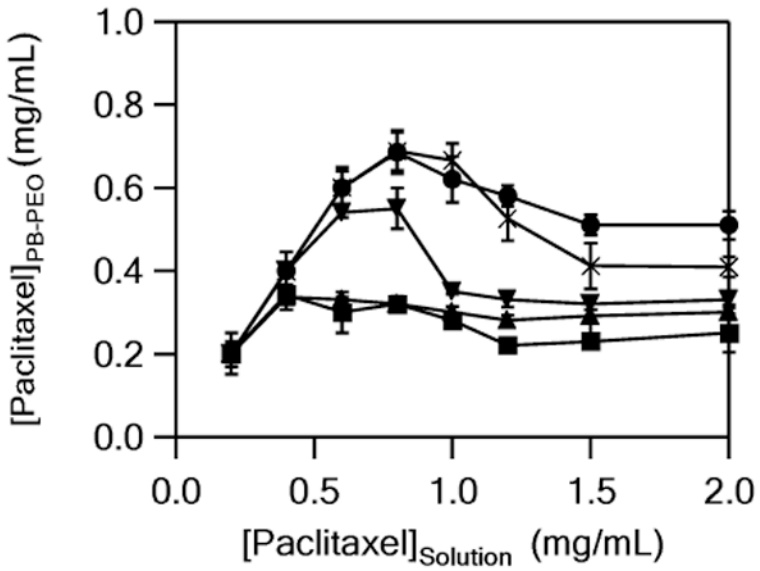

Figure 2.
Paclitaxel concentration loaded in OB1 (■), OB2 (×), OB3 (▲), OB4 (▼), OB5 (●) diblock copolymer colloids as a function of paclitaxel concentration initially in solution. All paclitaxel loading experiments were based on 5 mg/mL of diblock copolymers.

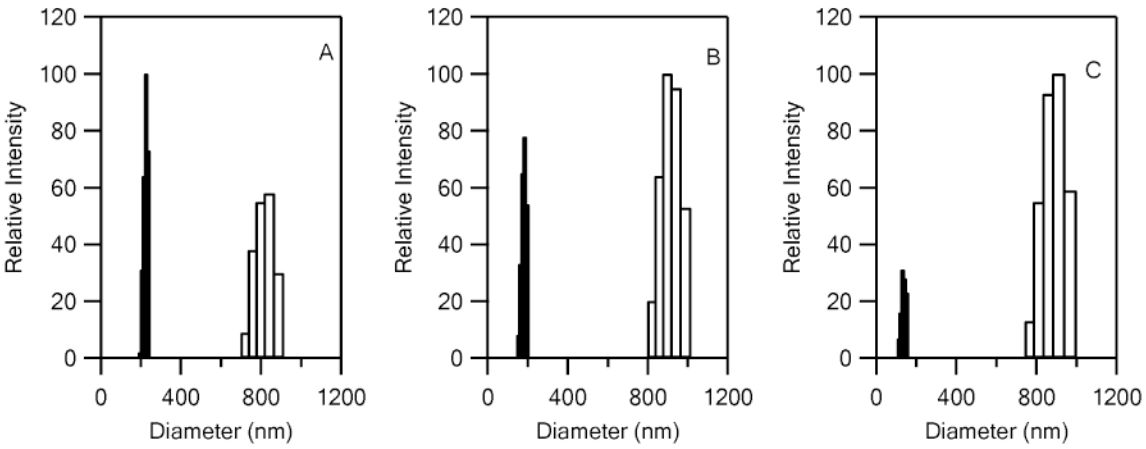

Figure 3.
Size distributions of paclitaxel loaded (A) OB2 polymersomes after 1 week and (B) OB1 polymersomes after 3 weeks (C) OB5 after 2 weeks. Polymersomes started forming into larger aggregates around 800 to 1000 nm.

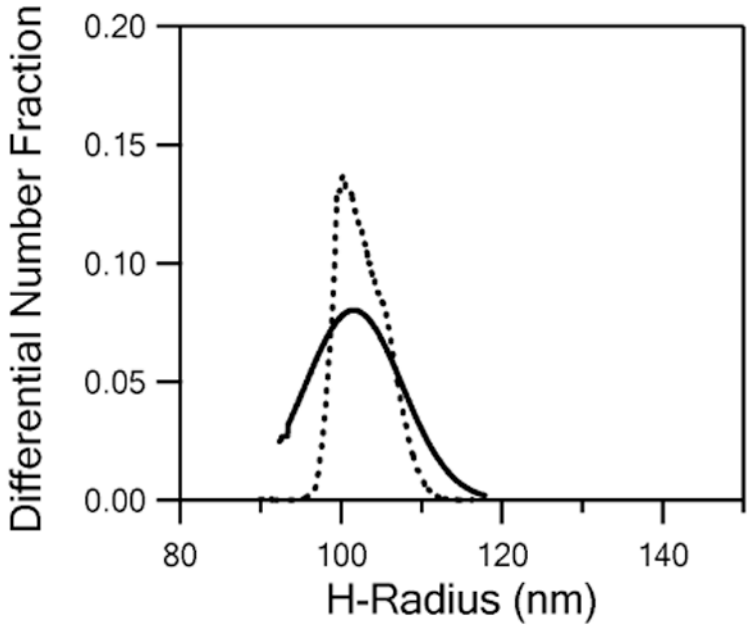

Figure 4.
Size distribution of paclitaxel loaded OB4 polymersome dispersions. Solid lines represent freshly prepared dispersions, while dashed lines represent dispersions after 4 months of storage at 4°C.

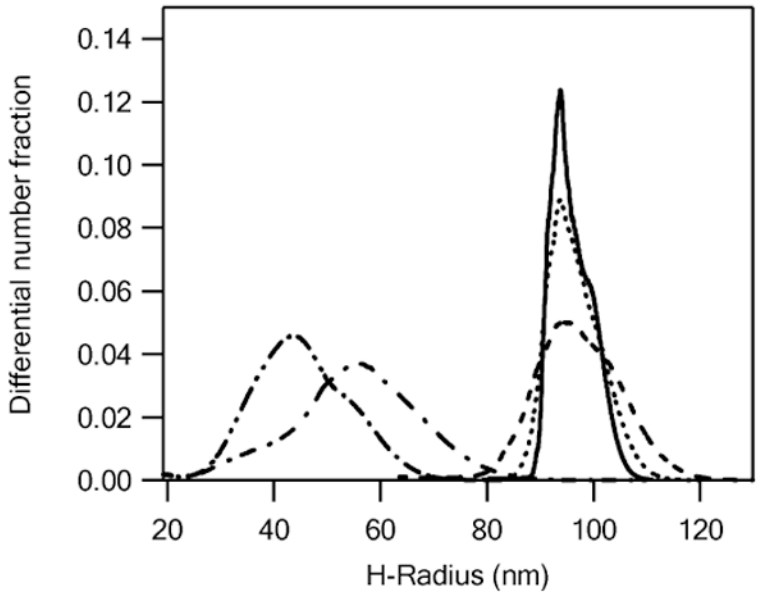

Figure 5.
Size distribution of OB5 polymersomes (——) and OB5 polymersomes incorporating 0.2 mg/mL (•••••), 0.4 mg/mL (– – –), 0.6 mg/mL (–• –• –•), and 0.7 mg/mL (–••–••–••–) of paclitaxel upon storage at 4°C for 2 weeks.

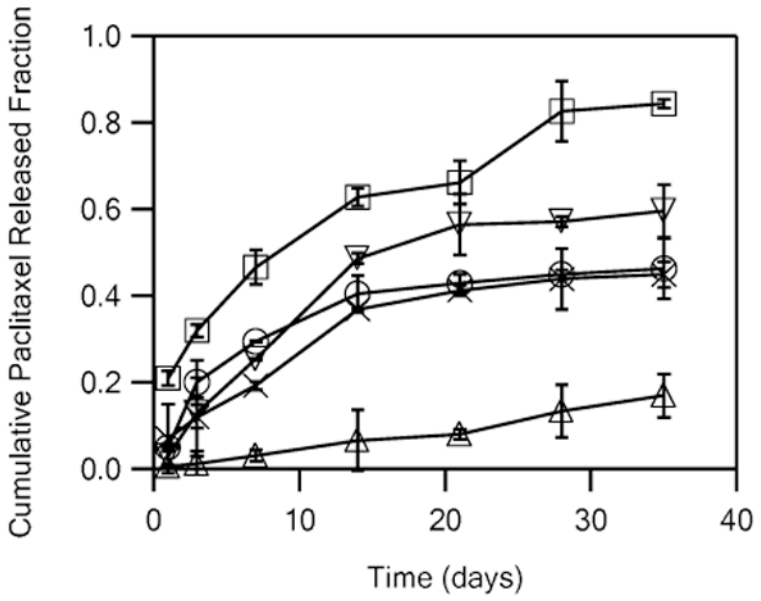

Figure 6.
Release profiles of paclitaxel loaded polymersome dispersions in PBS buffer at 37°C. OB1 (×),OB2 (▽),OB3(□), OB5(○),OB4 (△), and All paclitaxel releasing experiments were based on 5 mg/mL of diblock copolymers and 1% paclitaxel loading content.

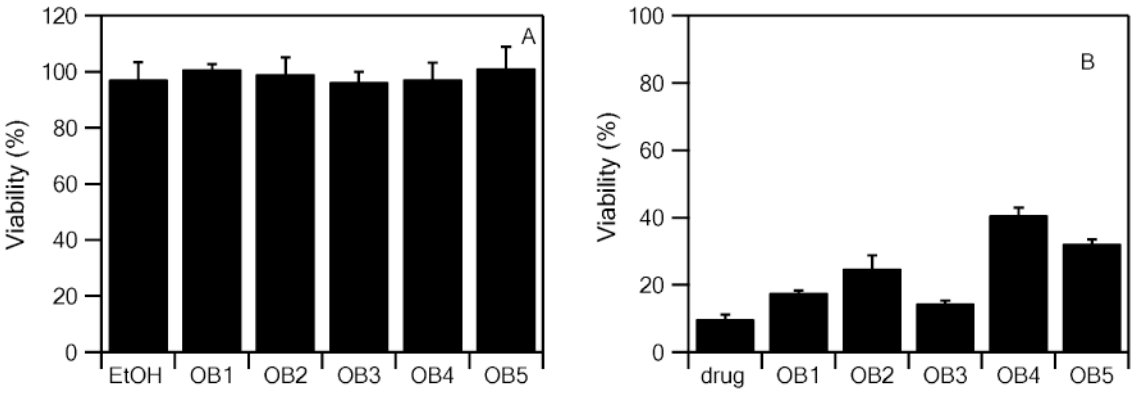

Figure 7.

MCF-7 cell viability after incubation with polymersomes (without paclitaxel) and paclitaxel loaded polymersomes for 3 days. (A) MCF-7 cells treated with polymersomes at 2 μg/mL. (B) MCF-7 cells treated with paclitaxel loaded polymersomes at 2 μg/mL of polymersomes incorporated with 0.02 μg/mL paclitaxel.

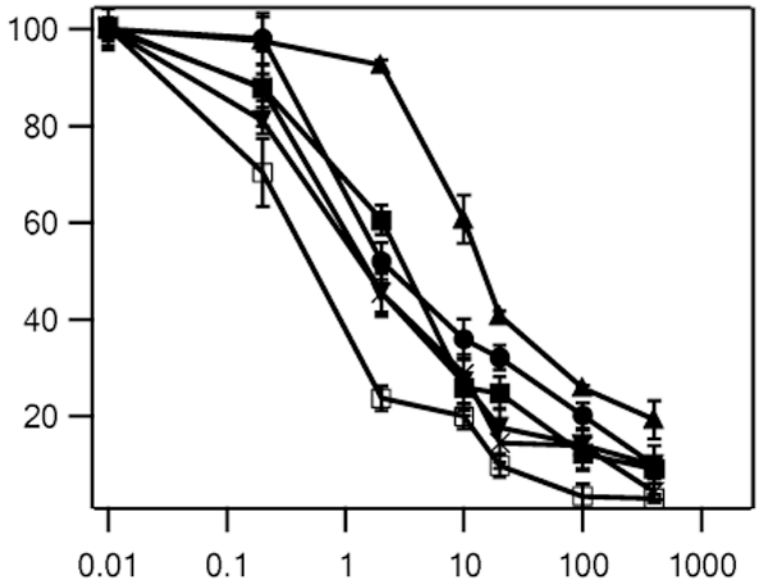

Figure 8.
Dose dependent cytotoxicity of paclitaxel loaded polymer colloid dispersions of OB1 (▼), OB2 (■), OB3 (×) OB4 (▲), OB5 (●), paclitaxel alone (□).

Table 1

Physical properties of amphiphilic diblock copolymers used in this study.

Copolymer name	Formula PB_m-PEO_n	M_n (kg/mol)	N_{PB}	N_{PEO}	w_{PEO}	PDI	Morphology (32)
OB1	PB_{33}-PEO_{21}	2.7	33	21	0.33	1.09	B
OB2	PB_{46}-PEO_{30}	3.8	46	31	0.34	1.04	B
OB3	PB_{96}-PEO_{52}	7.3	96	52	0.32	1.06	N, C, Cy
OB4	PB_{219}-PEO_{121}	17.1	219	121	0.31	1.04	B
OB5	PB_{407}-PEO_{286}	34.6	407	286	0.36	1.06	B+S

M_n: Number averaged molecular weight.

N_{PB}: Number of monomer repeat units in PB block

N_{PEO}: Number of monomer repeat units in PEO block

w_{PEO}: Weight fraction of PEO block in diblock copolymer

PDI: Polydispersity index

Morphologies determined by AFM: B=bilayers; C=Cylindrical worms, S=spherical micelles, Cy=cylindrical worms with Y-junctions, and N=Networks.

Table 2

Hydrodynamic diameter, polydispersity, and zeta potential of empty diblock copolymer colloids.

Polymer composition	Zeta Potential (mV)	Size Diameter (nm)	Polydispersity
OB1	-5.09±0.56	189.5±17.9	0.094
OB2	-4.22±0.98	193.4±13.5	0.079
OB3	-4.78±0.74	--	--
OB4	-4.67±0.38	199.9±6.8	0.059
OB5	-5.81±0.87	201.4±2.8	0.007

Table 3

Paclitaxel loading capacity, size, colloidal stability, and zeta-potential of polymersomes (or micelles for OB3). All loading experiments were based on 5 mg/mL of diblock copolymers.

Polymer composition	Paclitaxel: polymer ratio (w/w %)	Paclitaxel solubilized in polymer (mg/mL)	Stability (days)	Zeta Potential (mV)	Size Diameter (nm)
OB1	6.85	0.34±0.05	22	-4.67±0.74	193.5±17.9
OB2	13.72	0.69±0.09	7	-3.74±0.52	199.8±15.2
OB3	6.72	0.34±0.01	6	-4.14±0.66	--
OB4	11.06	0.55±0.01	>120	-5.39±0.69	184.3±13.2
OB5	13.76	0.69±0.05	14	-5.83±0.38	184.0±9.8