

Preparation of Water Dispersible Poly(methyl methacrylate)-based Vesicles for Facile Persistent Antibacterial Applications^{*}

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Abstract We report a facile strategy for incorporating persistent and effective antibacterial property into a widely used polymer, poly(methyl methacrylate) (PMMA), by copolymerizing methyl methacrylate (MMA) with 2-(*tert*-butylamino)ethyl methacrylate (TA) in one pot *via* atom transfer radical polymerization (ATRP). The subsequent self-assembly of the resultant poly(methyl methacrylate)-block-poly[(2-*tert*-butylamino)ethyl methacrylate] (PMMA₂₀-*b*-PTA₁₅) diblock copolymer affords well-defined water-dispersible vesicles, which can be facilely sprayed on the walls in hospitals for effective inhibition and killing of bacteria. ¹H-NMR and gel permeation chromatography (GPC) studies confirmed the successful synthesis of well-defined copolymer. Transmission electron microscopy (TEM), atomic force microscopy (AFM) and dynamic light scattering (DLS) studies proved the formation of vesicles with narrow size distribution. DLS studies revealed the excellent stability of vesicles at various temperatures. Antibacterial tests showed effective antibacterial activities of polymer vesicles against both Gram-positive and Gram-negative bacteria. Moreover, this strategy may be extended for preparing a wide range of polymeric materials for facile antibacterial applications in many fields.

Keywords: ATRP; Block Copolymer; Vesicle; Self-assembly; Antibacterial.

INTRODUCTION

Low molecular weight disinfectants have been widely used in places where people are susceptible to bacterial attack such as hospitals, public transportation vehicles, *etc*^[1–3]. However, they usually provide short-term antibacterial effect. In addition, the volatile disinfectants may make people uncomfortable, or allergic^[4, 5]. In contrast, antibacterial polymers can provide persistent antibacterial property and reduced bacterial resistance^[6–9]. However, the antibacterial activity of antibacterial polymers is usually not as effective as low molecular weight disinfectants. Recently, antibacterial polymeric nanostructures were confirmed to be able to significantly increase the antibacterial activity of polymers^[10–13]. The enhancement of the antibacterial activity is related to the much higher local concentration of antibacterial polymers in the self-assembled state than in bulk or un-self-assembled state.

Poly(methyl methacrylate) (PMMA) is a kind of massively used polymer in many fields, such as injectable powder^[14], monopolar surfaces^[15], organic/inorganic nanocomposites^[16] and nanoparticles^[17–20]. However, PMMA homopolymer doesn't have antibacterial ability. Therefore, efforts were made to reinvest the PMMA

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with antibacterial ability. For example, Jang *et al.* prepared the PMMA nanofiber containing silver nanoparticles whose antimicrobial activity is three-fold than that of AgNO_3 ^[9]. Sunintaboon and co-workers synthesized antibacterial nanoparticles with a PMMA core and a coated shell composed of chitosan, polyethyleneimine, and chitosan-mixed polyethyleneimine^[21]. Zuo *et al.* blended antibacterial poly[2-(*tert*-butylamino)ethyl methacrylate] (PTA) homopolymer with PMMA because PTA manifests a strong antibacterial property^[22]. As the PTA chains are partially hydrophilic and partially hydrophobic in neutral water (whose pK_a is relatively high)^[4], the polycationic side chains in PTA may adhere to the negatively charged bacterial membrane through electrostatic interaction^[23, 24], inducing the membrane rupture and subsequent death of the bacteria^[25, 26].

Herein, we report the preparation of a new water dispersible and antibacterial polymer vesicle based on $\text{PMMA}_{20}\text{-}b\text{-PTA}_{15}$ diblock copolymer (Fig. 1) for facile antibacterial applications in places where people are susceptible to bacterial attack. This vesicle can kill both Gram-negative *Escherichia coli* (*E. coli*) and Gram-positive *Staphylococcus aureus* (*S. aureus*). Moreover, this vesicle solution can be facily sprayed on the walls in hospitals and the surfaces of furniture for long-term bacterial inhibition and killing, which would be better than low molecular weight volatile disinfectants with short-term antibacterial effects. Please note that this antibacterial vesicle is different from our previously reported antibacterial nanostructures^[10], especially in this paper we introduced good antibacterial properties into the massively used PMMA for facile antibacterial applications by spraying on surfaces.

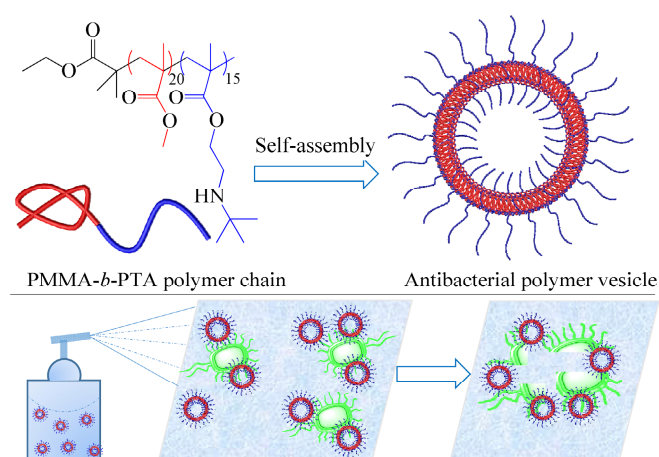


Fig. 1 Illustration of antibacterial polymer vesicles self-assembled from $\text{PMMA}_{20}\text{-}b\text{-PTA}_{15}$ diblock copolymer with potential antibacterial applications by spraying on surfaces

Hydrophilic PTA chains form coronas (blue). Hydrophobic PMMA chains and partial hydrophobic PTA chains form the membrane (red and blue). Vesicles can adhere to the negatively charged bacterial membrane through electrostatic interaction, thus inducing the membrane rupture. The vesicular solution can be used as a long-term antibacterial material by spraying in hospitals.

EXPERIMENTAL

Materials

2-(*tert*-Butylamino)ethyl methacrylate (TA) was purchased from F&K, dried over CaH_2 for 24 h and distilled under reduced pressure prior to use. Methyl methacrylate (MMA) and 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) were purchased from J&K and used as received. Toluene was dried by azeotropic distillation in the presence of sodium and benzophenone and distilled under reduced pressure prior to use. Cu(I)Br was washed with acetic acid and ethanol alternately five times and then dried in a vacuum. CH_2Cl_2 , CHCl_3 , *n*-hexane, DMF, LB Agar and LB broth were purchased from Aladdin and used as received. Gram-negative bacterium *E. coli* (ATCC35218) and Gram-positive bacterium *S. aureus* (ATCC29213) were purchased from Nanjing Bianzhen Biological Technology Co., Ltd. Other chemicals were used without further purification unless otherwise specified.

Synthesis of PMMA₂₀-b-PTA₁₅ Diblock Copolymer by ATRP

MMA (2.000 g), CuBr (0.0716 g), HMTETA (0.1150 g) and ethyl-2-bromoisobutanoate (0.0974 g) were added into a round flask with anisole as the solvent (25 mL). The solution was deoxygenated and stirred for 2 h in argon circumstance at 90 °C. TA (1.850 g) was dissolved in 2.0 mL of anisole, and was added into PMMA solution, the solution was stirred for another 24 h at 60 °C. After polymerization, the solution was cooled down and the copper catalyst was removed by an adsorption filtration through a neutral alumina column. The resulting solution was dialysis against deionized water for 2 days and finally freeze-dried.

Self-assembly of PMMA₂₀-b-PTA₁₅ Block Copolymer into Vesicles

PMMA₂₀-b-PTA₁₅ copolymer (6.0 mg) was dissolved in DMSO (2.0 mL). Then 4.0 mL of deionized water was dropped into the solution by a gas-tight syringe with a violent stirring for 12 h. Finally the solution was dialyzed against deionized water for 48 h. The actual concentration of polymer vesicles changes according to the types of characteristic methods.

Antibacterial Test

The antibacterial properties of polymer vesicles were obtained by two methods^[27]. First, all the wells of 96 well plates were filled with the LB Agar culture solution (100 µL), then each cells of the first line was injected with vesicles solution (~10.0 mg/mL, 100 µL). The mix solution of vesicles and LB was diluted into different concentrations (2-fold dilution in turn). Finally, every wells above was added with another 100 µL of bacterial solution. The MICs (minimal inhibitory concentrations) were recorded by measuring the optical density absorbance at 600 nm of UV light.

The second method to test antibacterial properties was conducted by counting the bacterial colony on the LB Agar. Vesicles (1.0 mg/mL) was divided and diluted into three types of concentrations of 0.25, 0.10 and 0.05 mg/mL. The vesicle solution (0.25, 0.10 and 0.05 mg/mL; 100 µL; pH 7.4; 37 °C) was extracted and distributed smoothly and evenly over a small piece of glass sheet within 1.0 cm². The water evaporated slowly at 37 °C. Bacteria cells were cultured in LB agar overnight and 1.8 mL of suspension was centrifuged and washed with physiological saline three times. The bacterial suspension was diluted to 1.8 mL with physiological saline. Then 10 µL of bacterial suspension was dropped onto the surface of each glass sheet and cultured in the centrifuge tubes at the humidity of > 90% for 2 h at 37 °C. The bacterial solution was diluted to certain concentrations by 10-fold dilution method. Finally, the solution of each concentration (100 µL) was added into the LB agar, which was incubated for 24 h at 37 °C. The bacterial colony was counted and set as an indicator of antibacterial activity.

Characterization**¹H-NMR**

The ¹H-NMR study was conducted by a Bruker AV 400 MHz spectrometer with CDCl₃ as solvent and TMS as standard at room temperature.

DLS

The DLS studies of vesicles in water were performed using a Nano-ZS 90 Nanosizer (Malvern Instruments Ltd., Worcestershire, UK) at a fixed scattering angle of 90°. Each reported measurement was conducted for three times. Glass cuvettes were used to analyze the aqueous aggregates solution. The data were processed by cumulative analysis of the experimental correlation function, and the diameter of vesicles was calculated from the computed diffusion coefficients using the Stokes-Einstein equation.

TEM

TEM images were taken with a JEOL JEM-2100F instrument at 200 kV equipped with a Gatan 894 Ultrascan 1 k CCD camera. The vesicle solution was diluted to 200 µg/mL and 7.0 µL of sample was dropped onto the carbon-coated copper grid and evaporated at ambient temperature. The sample was stained by 1.0 wt% phosphotungstic acid aqueous solution.

AFM

The vesicles solution was diluted into 50 $\mu\text{g/mL}$. The sample (10 μL) was dropped onto the silicon wafer (1 cm $\times$ 1 cm) and dried at ambient temperature. The observation was conducted on a Seiko (SPA-300HV) instrument operating in tapping mode at 200–400 kHz drive frequency.

UV-Vis spectroscopy

UV-vis studies were conducted using an UV-Vis spectrophotometer (UV-759S, Q/YXL270, Shanghai Precision & Scientific Instrument Co., Ltd) with a scan speed of 300 nm/min.

GPC

GPC analysis was carried out with a Waters Breeze 1525 GPC analysis system with two PL mix-D columns and a light scattering detector, using THF as eluent at a flow rate of 1.0 mL/min at 25 $^{\circ}\text{C}$.

RESULTS AND DISCUSSION

Synthesis of PMMA₂₀-*b*-PTA₁₅ Diblock Copolymer

The copolymer was synthesized by atom transfer radical polymerization (ATRP) in one pot by sequentially adding TA and MMA monomers. The $^1\text{H-NMR}$ spectrum of PMMA₂₀-*b*-PTA₁₅ is shown in Fig. 2. The degrees of polymerization of MMA and TA were 20 and 15, which were calculated by comparing peak a with peak f or peak j. GPC analysis revealed a relative molecular weight of 4400 (Fig. 3), which was close to the value calculated by $^1\text{H-NMR}$ (4907). Moreover, the M_w/M_n value of the block copolymer is 1.02, indicating the narrow molecular weight distribution.

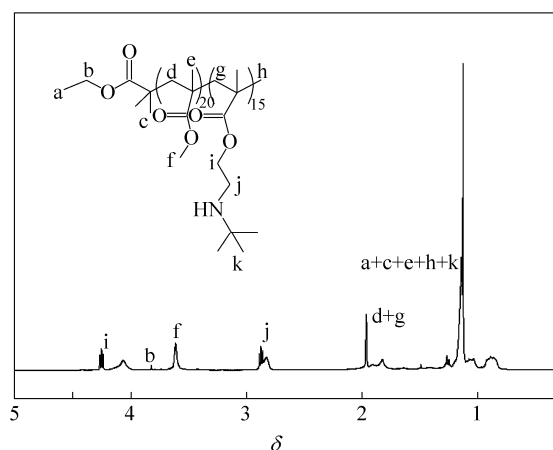


Fig. 2 $^1\text{H-NMR}$ spectrum of the PMMA₂₀-*b*-PTA₁₅ diblock polymer in CDCl_3

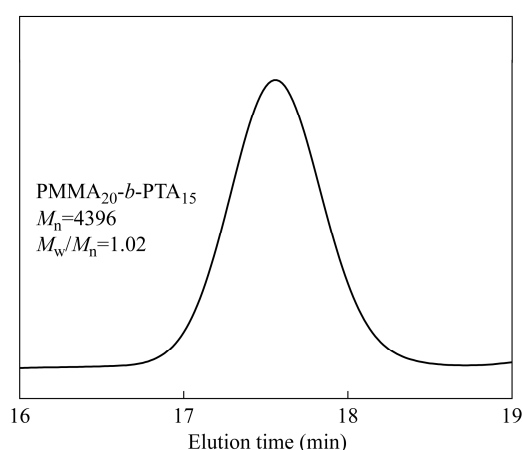


Fig. 3 GPC trace of PMMA₂₀-*b*-PTA₁₅ diblock polymer in DMF

Self-assembling Copolymer into Antibacterial Vesicles

Amphiphilic block copolymers can self-assemble into different nanostructures such as spherical micelles, cylinders, lamellae, and vesicles. Polymer vesicles can be used in drug carriers, biomedical coating, antioxidant agents, and nano-bioprobes, etc.^[28–35]. Polymer vesicles reported in this paper were prepared by dissolving PMMA₂₀-*b*-PTA₁₅ copolymer in DMSO and stirred with added water at ambient temperature (final DMSO/H₂O = 1/2; *V/V*). After dialysis, the vesicles were characterized by transmission electron microscopy (TEM), atomic force microscopy (AFM) and dynamic light scattering (DLS).

PMMA is hydrophobic while PTA is partially hydrophilic and partially hydrophobic in neutral water^[4]. Therefore, PMMA chains and a fraction of PTA chains form the membranes of vesicles while PTA chains form the coronas of vesicles.

TEM studies confirmed the vesicular structure (Fig. 4). The surface of the vesicle has some wrinkles, which

are caused by crispation and shrinkage of vesicles during the dryness. The mean diameter of vesicles is 250 nm. According to our recently developed protocol^[36, 37], the thickness of the membrane is about 16 nm.

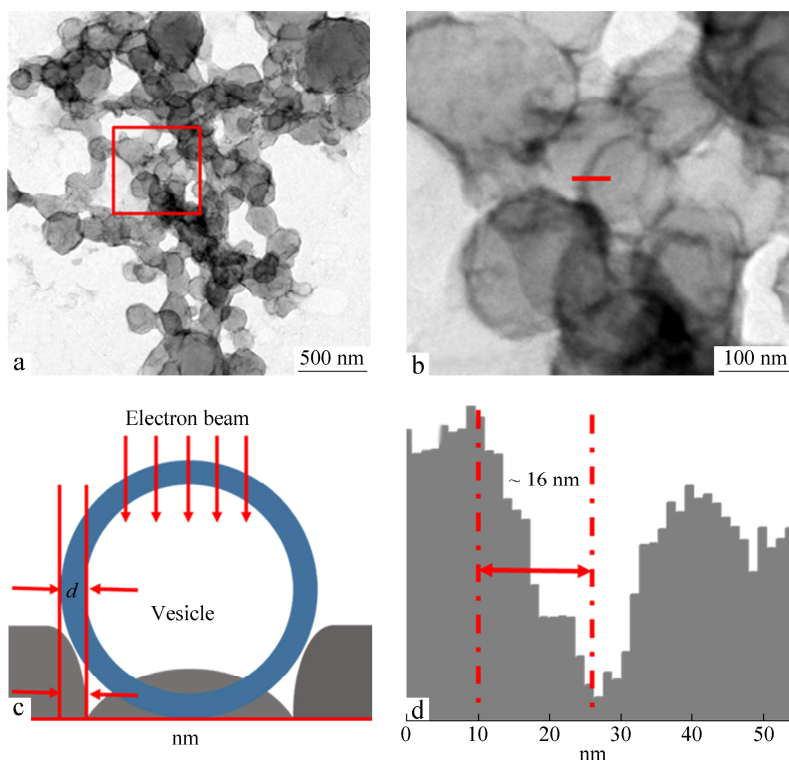


Fig. 4 TEM analysis of vesicles formed by PMMA₂₀-*b*-PTA₁₅ diblock copolymer
d is the electron transmittance chart related to the red scan-line in b, revealing a thickness of the membrane of 16 nm

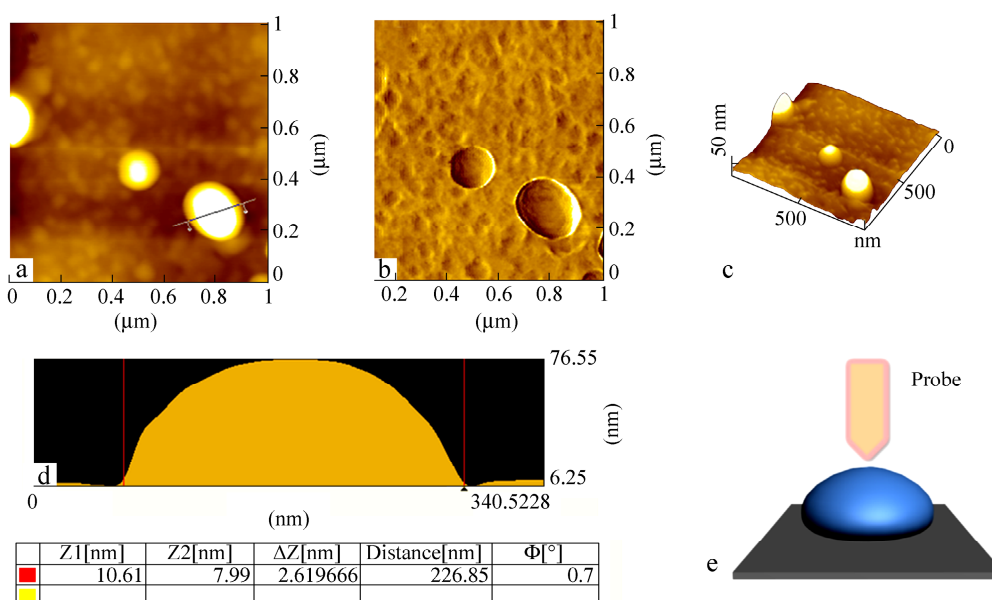


Fig. 5 AFM analysis of PMMA₂₀-*b*-PTA₁₅ vesicles: (a) height contrast; (b) phase contrast; (c) 3-dimensional image; (d) height profile along the scan line in panel A; (e) simulation of vesicles at the collapsed state

AFM analysis was used to further study the morphology of the vesicles (Figs. 5a–5d). The diameter and the height of the vesicle are 226 nm and 70 nm, respectively. After dropped on the silicon substrate, the deformable vesicles may collapse and flatten, leading to a high ratio of diameter to height (3.24, see Fig. 5e).

DLS study of vesicles revealed a hydrodynamic diameter of 157.7 nm and a very low PDI of 0.028 (Fig. 6a). The bigger size observed in the TEM and the AFM studies than in the DLS studies was originated from the collapse and flatten of vesicles on the copper grid or silicon substrate.

DLS studies were further performed at different temperatures to evaluate the stability of vesicles (Fig. 6b). From 20 °C to 60 °C, the hydrodynamic diameter of vesicles kept at ~150 nm, indicating their good stability.

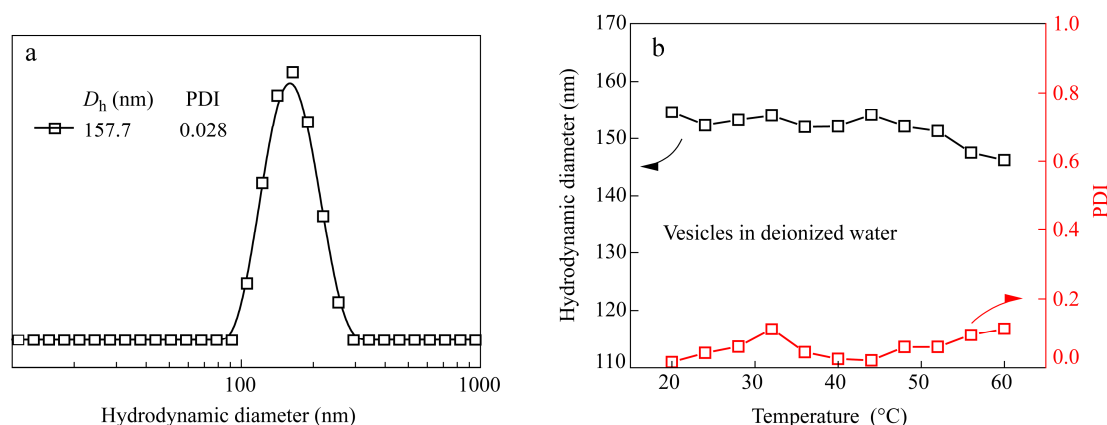


Fig. 6 (a) DLS study (by intensities) of PMMA₂₀-b-PTA₁₅ vesicles in water at 0.5 mg/mL and 25 °C; (b) Effect of temperature on the size and PDI of vesicles

Antibacterial Activities of PMMA₂₀-b-PTA₁₅ Vesicles

An enhancement in the antibacterial activities can be reached as the PTA segments carry positive charges in neutral water. Furthermore, the vesicular structure has a larger charge density than the individual polymer chains^[38]. Thus, the PTA-based polymer vesicles have excellent antibacterial properties without quaternization or loading external antibiotics^[26, 39].

In this study, *E. coli* and *S. aureus* were selected as the typical Gram-negative and Gram-positive bacteria to test the antibacterial properties of the PMMA₂₀-b-PTA₁₅ vesicles. Two different methods were conducted to evaluate the antibacterial activities of polymer vesicles, as illustrated below.

First, the minimum inhibitory concentration (MIC) of the antibacterial vesicles was measured. Normally, the MIC is defined as the critical concentration of vesicles that can inhibit the bacteria growth detected by UV lights. The vesicle solution was divided and diluted into different concentrations in the 96-well plate, after which equal volumes of *E. coli* and *S. aureus* were added into the well. The plate was cultured in the shaking bed, and the optical densities were detected and recorded after 8 h. The MIC values of the polymer vesicles are 0.505 and 0.252 mmol/L against *E. coli* and *S. aureus*, respectively (see Table 1). The antibacterial ability can be further proved with the accurate result in the Table 2.

The second method was used to specify the antibacterial efficiency. Vesicles (100 µL) with different concentration gradient at pH 7.4 were measured on the surfaces of glass sheets with an area of 1.0 cm². After evaporation of water, 10 µL of bacterial solution was added on the surface of the glass sheet. A glass without vesicles was used as the control. Finally, with another 2 h of incubation, the bacterial solution was diluted and dissolved in the LB agar. The agar was spread on the plate, whose digital pictures were taken after incubation for 48 h at 37 °C. In Fig. 7(a), many bacteria can be observed on the plates of control (without antibacterial vesicles). In contrast, in the presence of 0.05 mg/mL of vesicles, the colonies significantly decreased. Furthermore, only slightly scattered bacteria were found on the plates in the presence of higher vesicle concentrations (0.10 and 0.25 mg/mL). In Fig. 7(b), similar observations were found but the plate with 0.05 mg/mL of vesicles was smoother than that in Fig. 7(a), confirming that the vesicles has a better bacterial inhibition effect against *S. aureus* than against *E. coli*.

Table 1. MIC values of PMMA₂₀-*b*-PTA₁₅ polymer vesicles

<i>c</i> (mmol/L)	<i>E. coli</i>	<i>S. aureus</i>
2.01	–	–
1.01	–	–
0.505	–	–
0.252	+	–
0.126	+	+
0.063	+	+
0.032	+	+
0.016	+	+

“+” represents the growth of the bacteria and “–” represents no growth

Table 2. Antibacterial rate and Log reduction of the vesicles

Bacteria	<i>C</i> _{vesicles} (mg/mL)	Antibacterial rate (%)	Log reduction
<i>E. coli</i>	0.05	89.2	1.1
	0.1	93.2	1.3
	0.25	99.9	2.9
<i>S. aureus</i>	0.05	91.4	1.1
	0.1	95.3	1.5
	0.25	99.8	2.9

Antibacterial rate = $1 - B/A$; Log reduction = $\lg A - \lg B$ (*A* represents the surviving colonies number in the control and *B* the number in the vesicle sample.)

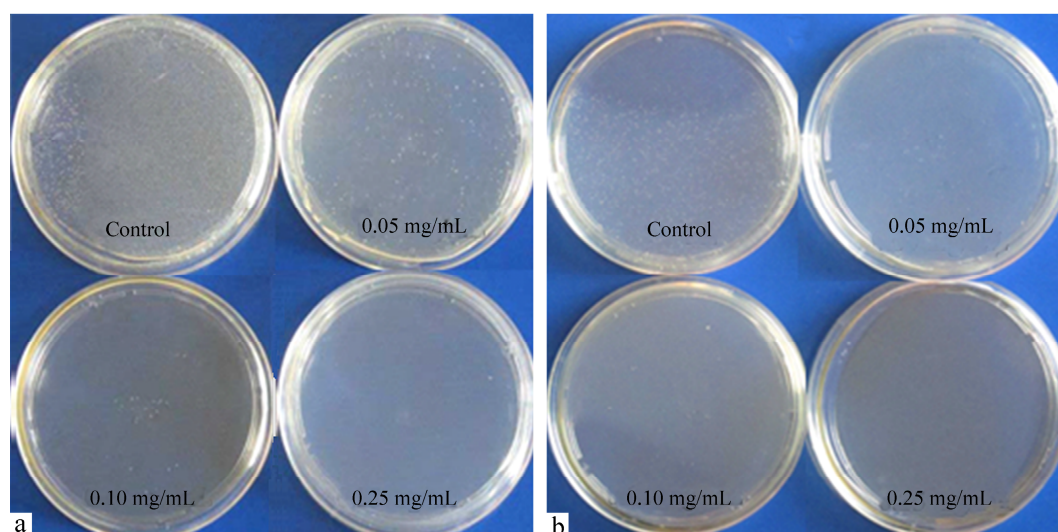


Fig. 7 Digital photographs of LB agar with different vesicles concentrations: (a) against *E. coli* and (b) against *S. aureus* (The visible white dot is the bacterial colonies.)

CONCLUSIONS

In summary, we have incorporated antibacterial PTA into PMMA by sequential ATRP of TA and MMA monomers to afford well-defined antibacterial PMMA-*b*-PTA diblock copolymer with a very narrow molecular weight distribution. This copolymer can self-assemble into vesicles in water. These stable vesicles showed good antibacterial properties against both Gram-positive and Gram-negative bacteria in the absence of external antibiotics or quaternary ammonium moieties, suggesting a wide range of antibacterial applications. This protocol also provides us with a new insight for preparing antibacterial polymeric formulations which may be facilely sprayed on the places (such as hospitals) where people are susceptible to bacterial attack for long-term antibacterial applications.

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