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Electronic Supporting Information

Biofilm Dispersal using Nitric Oxide Loaded Nanoparticles Fabricated by Photo-PISA: Influence of Morphology

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EXPERIMENTAL SECTION

Materials. Oligo(ethylene glycol) methyl ether methacrylate (300 g mol^{-1}, OEGMA, Sigma-Aldrich) and glycidyl methacrylate (GMA, Sigma-Aldrich) were deinhibited by passing them through a column of basic alumina. Tetrahydrofuran, ethanol, methanol, acetone (Chem-Supply) and 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl] pentanoic acid (CDTPA, Boron Molecular) were used as received. 2,2'-Azobisisobutyronitrile (AIBN) was purified by recrystallization from methanol and stored at -18°C before use. Deuterated solvents ($CDCl_3$ and acetone-d_6) were obtained from Cambridge Isotope Laboratories. All other chemicals were used as received unless otherwise specified.

Analytical instruments

^{1}H NMR spectroscopy was carried out using a Bruker Advance III HD 400 MHz spectrometer using deuterated solvents ($CDCl_3$-d and acetone-d_6) to analyze monomer conversions and polymer compositions.

Gel permeation chromatography (GPC) analyses of polymer samples were performed in *N,N*′-dimethylacetamide (DMAc) with 0.03% w/v LiBr and 0.05% 2,6-di-butyl-4-methylphenol (BHT)) at 50 °C (flow rate of 1 mL/min) with a Shimadzu modular system comprising and SIL-10AD automatic injector, a Polymer Laboratories 5.0 μL bead-size guard column (50×7.8 mm^2) followed by four linear PL (Styragel) columns (10^5, 10^4, 10^3 and 500 Å) and an RID-10A differential refractive-index detector. The GPC calibration was performed with narrow dispersity poly(methyl methacrylate) standards.

Dynamic light scattering (DLS) measurements were carried out at 25°C using a Malvern Zetasizer Nano ZS apparatus running DTS software (He–Ne laser, 4 mW, 633 nm, angle 173°). All samples were prepared at a concentration of approximately 0.2 mg mL^{-1} of polymer.

For transmission electron microscopy (TEM), NP sizes and morphologies were measured using a JEOL 1400 transmission electron microscope. The NP solutions were diluted with ethanol to a concentration of approximately 0.2 wt% before 10 μL was deposited onto formvar coated grids. Uranyl acetate staining was applied to all samples to increase contrast. A similar protocol was employed to image NONOate functionalized NPs except that NP solutions were diluted with acetonitrile.

UV-Vis spectra were recorded using a CARY 300 spectrophotometer (Varian) equipped with a temperature controller.

Tomographic microscopy images were obtained using a 3D Explorer, NanoLive, Lausanne, Switzerland. The laboratory strain *Pseudomonas aeruginosa* was grown in tissue culture dishes (FluoroDish, World Precision Instruments Inc.) at 37°C with shaking at 180 rpm in an orbital shaker (model OM11, Ratek, Boronia, Australia) for 6.5 h to obtain the desired biofilm cultures. The biofilm was subsequently treated with the desired compounds for a predetermined time before washing twice with PBS. The dishes were placed on the tomographic microscope and 3D tomographic images were taken with the best focus. All experiments were performed with two independent replicates.

Synthesis and Characterization

Synthesis of POEGMA Macro-CTA via RAFT polymerization

The synthesis of the POEGMA macro-CTA by RAFT polymerization was carried out as follows: OEGMA (6.0 g, 300 g mol^{-1}, 0.02 mol), CDTPA (0.1612 g, 4×10^{-4} mol), AIBN (8.21 mg, 5×10^{-5} mol) and 25 mL toluene were added to a 50 mL round flask, which was sealed with a rubber septum and metal wire and purged with nitrogen for 30 min in an ice bath. The polymerization was conducted for 4 h at 70 °C and then quenched in an ice bath with exposing to air. The crude polymer was analyzed by ^{1}H NMR and then purified by precipitation in a diethyl ether and petroleum spirit mixture (30:70, v/v). ^{1}H NMR indicated a monomer conversion of 49 % which was calculated using the following equation $\alpha = 100 \times [p / (p+m)]$, where $m = \int I^{5.8\text{-}5.5\ ppm}$ and $p = [(\int I^{4.5\text{-}4.5\ ppm} / 2)$. The theoretical molecular weight was determined to be $M_{n,theo}$ = 7 800 g/mol using the following equation: $M_{n,theo} = MW_{CDTPA} + [\alpha \times [OEGMA]_0/[CDTPA]_0 \times MW_{OEGMA}]$ where MW_{CDTPA} is the molecular weight of the RAFT agent, α is the monomer conversion, $[M]_0$ is the initial monomer concentration, $[CDTPA]_0$ is the initial concentration of the RAFT agent and MW_{OEGMA} is the molecular weight of OEGMA (300 g/mol).

Synthesis of PGMA homopolymers by PET-RAFT polymerization

PGMA homopolymers were synthesized by PET-RAFT polymerization in DMSO. Experiments were set up with [GMA]:[CDTPA]:[ZnTTP] = 200:1:0.02 at a solids content of 50 wt %. A typical experiment was set up as follows: GMA (1.1 g, 7.7×10^{-3} mol), CDTPA (15.6 mg, 39×10^{-6} mol), ZnTPP (0.53 mg, 7.8×10^{-7} mol) and DMSO (1.1 g, 1 mL) were added to a 4 mL glass vial, sealed with a rubber septum. The polymerization mixture was purged with nitrogen for 20 min and irradiated with red LED light (λ_{max}= 635 nm, 2.2 mW/cm^2) at room temperature. Samples were withdrawn at predetermined time intervals and quenched by exposure to air and storage in the dark. ^{1}H NMR and GPC were carried out to measure the monomer conversion and molecular weight distribution.

General procedure for synthesis of POEGMA-*b*-PGMA block copolymers by PET-RAFT dispersion polymerization

POEGMA-*b*-PGMA copolymers were synthesized by PET-RAFT dispersion polymerization in ethanol. Experiments were set up with various [GMA]:[POEGMA] ratios (150 and 200) with a [POEGMA]:[ZnTPP] = 1:0.002 and a total solids content of 10 wt%. A typical experiment with [GMA]:[POEGMA]=200:1 was set up as follows: POEGMA macro CTA ($M_{n,Theo.}$=7 800 g mol^{-1}, 30 mg, 3.8×10^{-6} mol), GMA (0.11 g, 7.7×10^{-4} mol), ZnTPP (5.1×10^{-6} g 7.6×10^{-9} mol) and ethanol (1.6 mL) were added to glass vials, sealed with a rubber septum and purged with nitrogen for 20 min in an ice bath. The polymerization mixture was irradiated with red LED light (λ_{max}= 635 nm, 2.2 mW/cm^2) at room temperature for a predetermined time before quenching by exposure to air and storage in the dark. ^{1}H NMR and GPC were carried out to measure the monomer conversion and molecular weight distribution. TEM and DLS were subsequently carried out to assess the size and morphology of the NPs.

Post-modification of POEGMA-*b*-PGMA NPs

POEGMA-*b*-PGMA copolymers were diluted in ethanol to a concentration of 30 mg mL^{-1} before the addition of an excess of benzyl amine ([amine]:[epoxy]=3:1). The mixtures were heated in an oil bath at 45°C overnight. After the solutions were cooled to room temperature, the NPs were purified by dialysis against acetonitrile before being analyzed by FTIR.

Post-modification of amine modified POEGMA-*b*-PGMA block copolymers with NO gas

A solution of the amine modified POEGMA-*b*-PGMA NPs (at a concentration of 20 mg mL^{-1} in acetonitrile) was placed in a Parr apparatus and clamped. The apparatus was purged and evacuated with N_2 three times, followed by charging with excess NO gas (25 °C, 5 atm). After 24 h, the excess NO was vented by purging with N_2 and the resultant NPs were stored at 4 °C.

***In vitro* NO release study**

NO released from the NONOate loaded NPs at specific time points was determined using a standard Griess reagent kit.[1-5] For each sample, 2 mL of a 2 mg mL^{-1} NP solution in acetonitrile was transferred to a dialysis membrane (Cellu-Sep 3500 MWCO). The dialysis membrane was immersed in 8 mL PBS buffer (pH 7.4) at 37°C in an incubator. At various time points, a 100 µL aliquot of the surrounding PBS solution was taken, mixed with 100 µL of Griess reagent and incubated at room temperature for 15 min. The sample was then diluted with 1.8 mL of PBS buffer and the UV-Vis absorbance of the resulting solution measured at 540 nm.

To determine the total NO loading in the samples, a NP solution at 0.1 mg mL^{-1} in water was used to minimise scattering of light. The concentration of NONOate was estimated using the following equation: $[NONOate] = (Abs^{250\ nm\ before\ release} - Abs^{250nm\ after\ release}) / (\varepsilon \times d)$ where $Abs^{250nm\ before\ release}$, $Abs^{250nm\ after\ release}$, ε and *d* correspond to the absorbance at 250 nm before and after NO release, the molar extinction coefficient of NONOates (ε = 8 500 $M^{-1}cm^{-1}$) and the cuvette pathlength respectively (1 cm).[6] Additionally, to confirm this data, the NONOate functionalized NPs were incubated in 0.5 M sodium hydroxide (NaOH) at 37°C for

3 hours to rapidly decompose the NONOates following an established protocol.[1] The amount of NO in solution was then determined using the Griess assay.

Biofilm dispersal

The laboratory strain *P. aeruginosa* PAO1 was used to characterize the effects of NO on planktonic growth and biofilm dispersal. To grow biofilms, in all assays, overnight cultures in Luria Bertani medium were diluted 1:200 in freshly prepared M9 minimal medium (containing 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, 20 mM glucose and 100 mM $CaCl_2$, pH 7.0). The suspension was then aliquoted (1 mL per well) into tissue-culture treated 24-well plates (Costar, Corning®). The plates were incubated at 37 ^oC with shaking and the biofilms were allowed to grow for 6.5 h without any disruption.[7, 8] Then, diluted NONOate functionalized polymeric nanoparticles (S-NO and W-NO) in M9 and spermine NONOate (Cayman Chemical, USA) were added to the wells to yield final treatment concentrations of 30-120 μg mL^{-1} of NPs-NO and 6-24 μg mL^{-1} of Sper-NO , while control wells were left untreated. The plates were then incubated for a further 30 or 60 min before quantification. Biofilm biomass was quantified by crystal violet (CV) staining which is a highly reproducible method.[9] The biofilm on the well surfaces was first washed once with 1 mL of PBS to remove loosely attached biofilm cells, before staining with 0.03 % CV solution of 1: 10 Gram crystal violet (BD®, USA) in PBS for 20 min. Stained biofilm was washed twice with PBS and the amount of remaining CV stained biofilm was measured by first diluting with 1 mL absolute ethanol and measuring the OD_{595} of the homogenized suspension using a microtitre plate reader (Wallac Victor2, Perkin-Elmer). OD measurements of control wells were deducted from all values.

SUPLLEMENTARY FIGURES

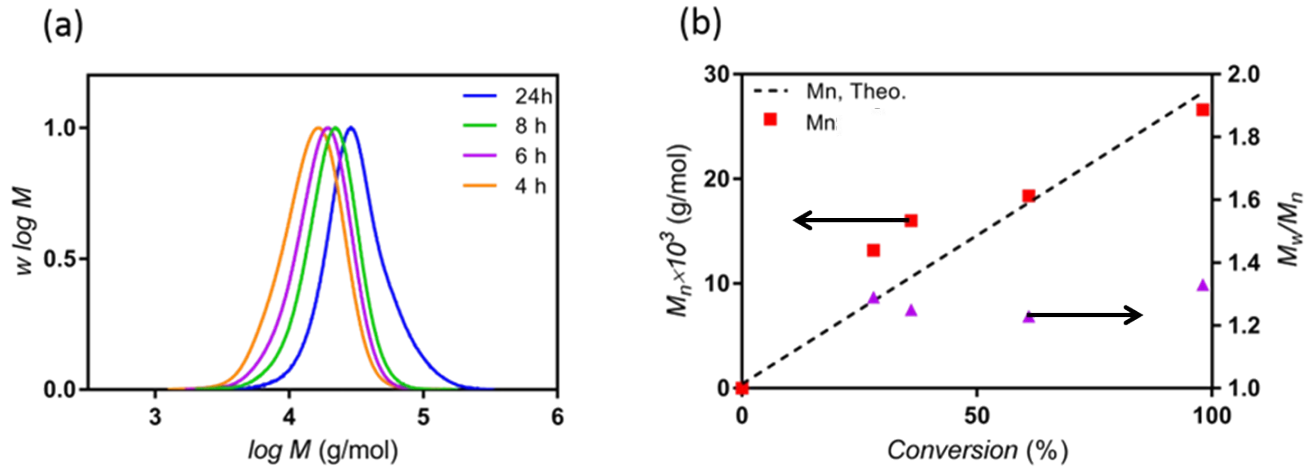

Fig. S1. Characterization data for PGMA homopolymers synthesized using PET-RAFT polymerization showing the evolution of: (A) molecular weight chromatograms and (B) number-average molecular weight and dispersity with monomer conversion. Polymerizations were performed under red light (λ_{max} = 635 nm, 2.2 mW/cm^2) using [GMA]:[CDTPA]:[ZnTTP]= 200:1:0.02 at a total solids content of 50 wt% in DMSO

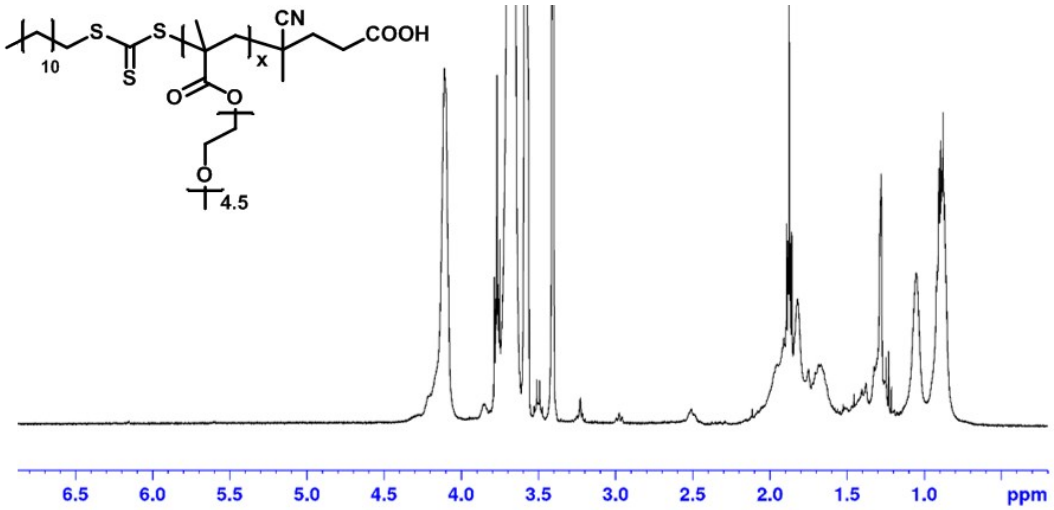

Fig. S2. ^{1}H NMR spectra of purified POEGMA macro-CTA used in this study ($M_{n,GPC}$ = 8 600 g mol^{-1}, $M_{n,Theo.}$= 7 800 g mol^{-1}, Đ = 1.13).

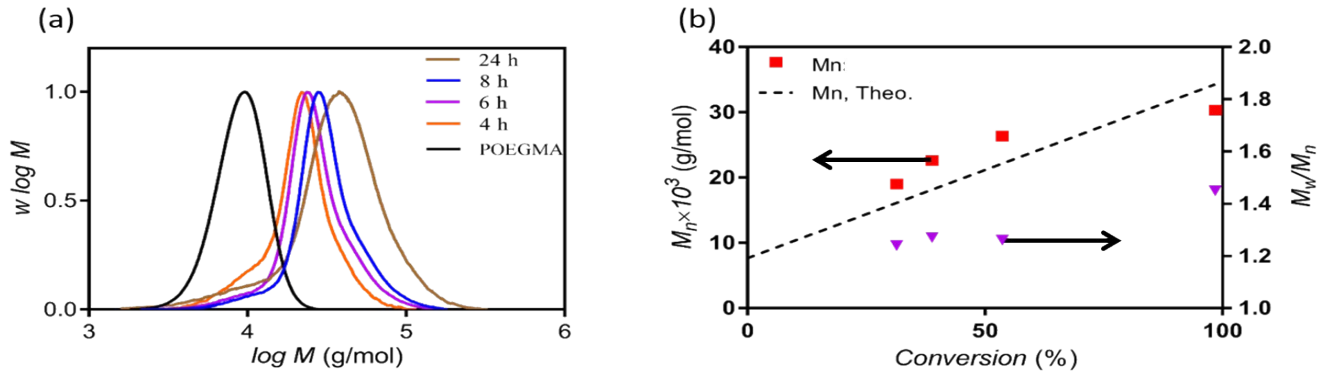

Fig. S3. Model kinetic study of the PET-RAFT dispersion polymerization of GMA in ethanol showing the evolution of (a) GPC molecular weight distribution and (b) number average molecular weight and dispersity with monomer conversion. Polymerization was performed using a [GMA]: [POEGMA]:[ZnTPP]= 200:1:0.002 under red light (λ_{max} = 635 nm, 2.2 mW/cm^2) and at a total solids content of 10 wt% in ethanol.

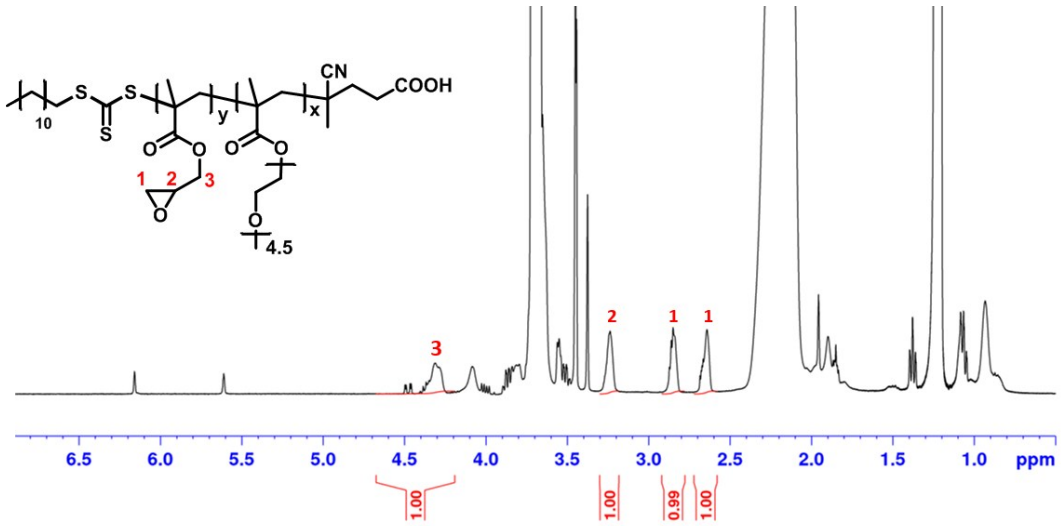

Fig. S4. Typical ^{1}H NMR spectra of crude POEGMA-*b*-PGMA copolymers synthesized by PET-RAFT dispersion polymerization.

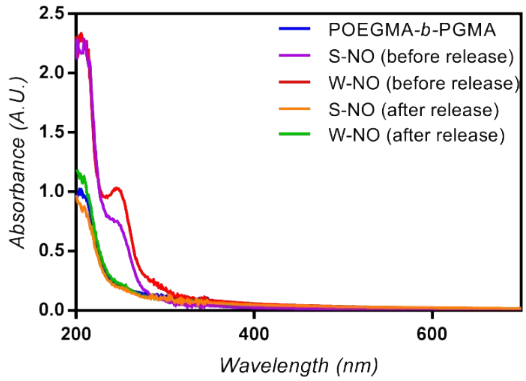

Fig. S5. UV−Vis absorption spectrum of POEGMA-*b*-PGMA (blue line), NONOate loaded NPs (S-NO/purple line and W-NO/red line) before release and corresponding NPs after two months incubation in PBS (S-NO/orange line and W-NO/green line) demonstrating successful NONOate conjugation. All spectra were acquired at a polymer concentration 0.1 mg mL^{-1} in water.

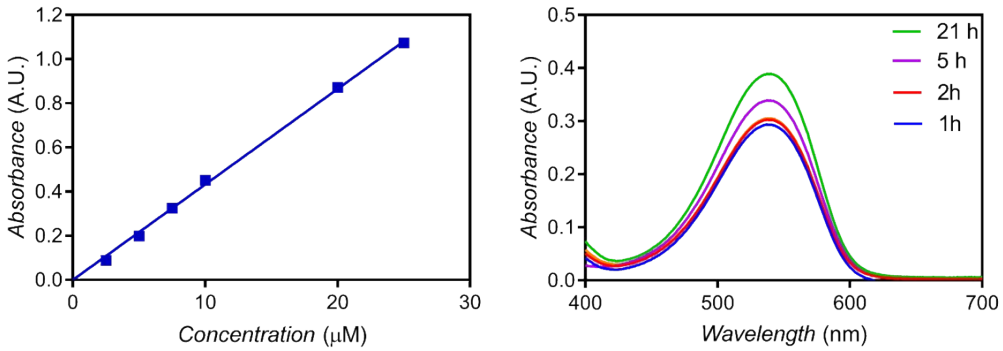

Fig. S6. Calibration curve (left) and a typical UV-Vis absorption spectrum of the azo dye (right) generated in the Griess assay at different concentrations of nitric oxide.

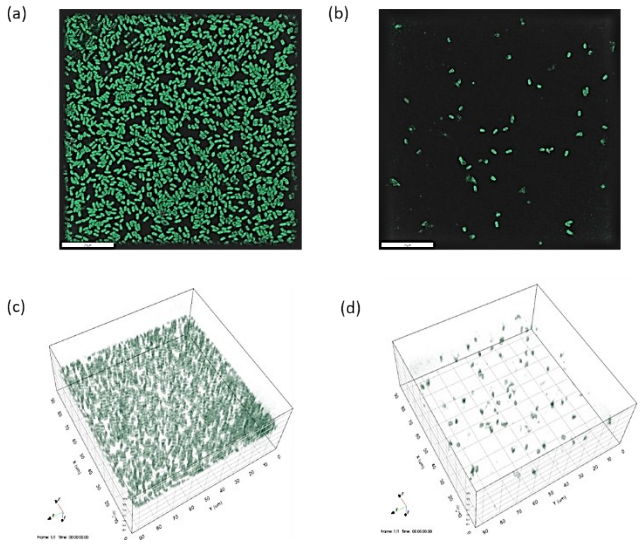

Fig. S7. Tomographic microscope images of (a) untreated and (b) W-NO NPs treated 2D images, (c) untreated and (b) W-NO NPs 3D images induced biofilm dispersal. *P. aeruginosa* biofilms were grown for 6.5 h before being treated for a further 1 hour with 60 μg mL^{-1} of W-NO NPs. Biofilms biomass were analysed by 3D cell explorer.

ADDITIONAL REFERENCES

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