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The use of endogenous gaseous molecules (NO and CO_2) to regulate the self-assembly of a dual-responsive triblock copolymer

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Abstract

A nitric oxide (NO) and carbon dioxide (CO_2) dual-responsive block copolymer was self-assembled in aqueous solution upon gas stimuli to form nanostructures. Specifically, poly(oligo(ethylene glycol) methyl ether methacrylate)-*block*-poly(*N*,*N*-diethylaminoethyl methacrylate)-*block*-poly(2-(3-(2-aminophenyl)ureido)ethyl methacrylate) (POEGMA-*b*-PDEAEMA-*b*-PAPUEMA) (termed ODA) triblock copolymers, consisting of a CO_2-responsive PDEAEMA block and a NO-sensitive PAPUEMA block, were synthesized using sequential reversible addition-fragmentation chain transfer (RAFT) polymerization. The ODA triblock copolymer was found to self-assemble at pH 7.4 into three-layer micelles with PAPUEMA inner cores, PDEAEMA outer cores, and hydrophilic POEGMA coronas. Upon CO_2 addition, the collapsed PDEAEMA block became protonated, increasing water solubility following the generation of carbonic acid functionality, resulting in the formation of expanded micelles with PAPUEMA cores. Subsequent NO addition selectively transformed the interior PAPUEMA block into more hydrophobic benzotriazole moieties, driving a morphological transition from swollen micelles to nanorods. Importantly, the transformation from spherical nanoparticles to nanorods in this novel copolymer system is highly specific to NO exposure.

Introduction

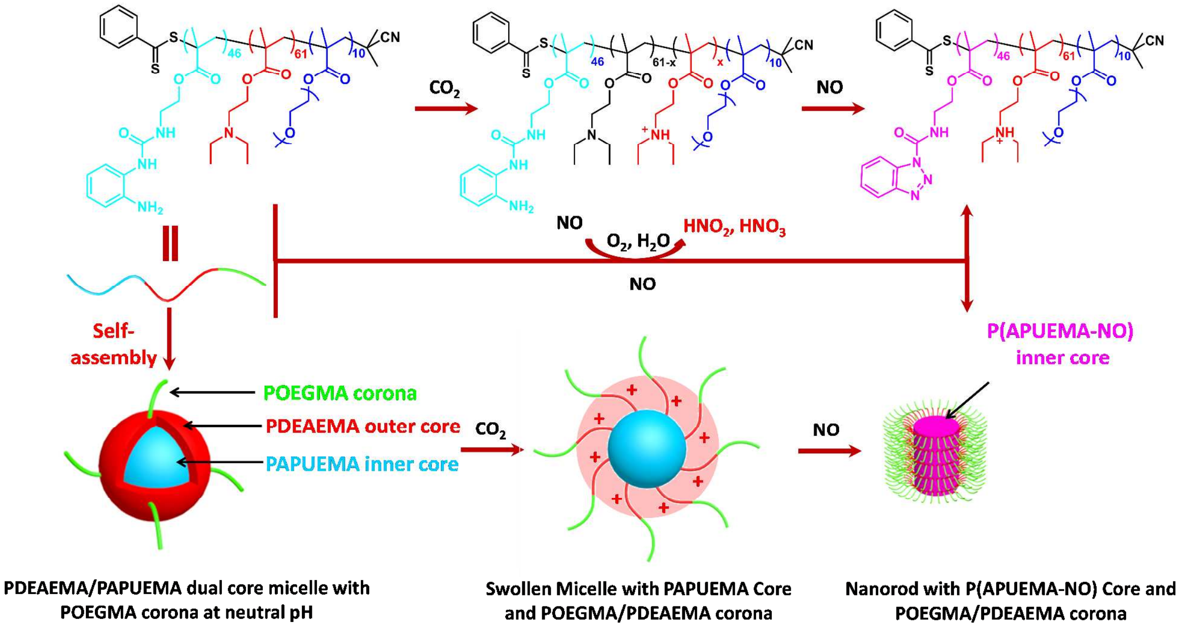
Stimuli-responsive polymers have received tremendous interest over the past few decades because of their applicability to a number of fields including gene and drug delivery,[1-4] sensing and imaging applications,[5] self-healing materials,[6] catalysis,[7] optical and electronic devices.[8, 9] In previous studies, numerous stimuli such as temperature,[10, 11] pH gradient,[12-14] light,[15-18] mechanical forces,[19-22] electric and magnetic fields[23, 24] have been applied to responsive polymers to trigger chemical and/or physical changes in chain conformation and solubility to induce self-assembly to higher-order structures. In our recent work, we explored the use of an endogenous signaling molecule, NO, to induce phase changes of stimuli-responsive polymers under biological conditions.[25] The use of intracellular stimuli and biologically relevant microenvironments has been explored in preceding work in conjunction with novel stimuli-responsive materials.[26-29]

The emerging use of endogenous gases as novel stimuli represents a recent research trend, in tandem with the design of truly biomimetic polymer systems for application in both the biomaterial and biomedical fields. The majority of attention has focused on carbon dioxide (CO_2)-responsive polymers.[30-39] CO_2, a key endogenous metabolite, has been used to self-assemble polymers via the formation of carbonic acid functionality. Amidine-[35, 38, 40-42] and tertiary amine-containing polymers,[43, 44] have been described as basic constituents of self-assembled nanostructures, e.g., micelles, nanotubes, and vesicles, regulated by a CO_2 stimulus. Yuan and co-workers[45] reported a CO_2-responsive polymersome by the introduction of amidine moieties. Zhao and co-workers[46] demonstrated that tertiary amine-containing polymers were inherently responsive to a CO_2 stimulus. CO_2-responsive materials are now well established.[30-39] In contrast, materials responsive to other common endogenous gases (carbon monoxide (CO) and nitric oxide (NO)) are extremely rare despite the opportunities presented for intracellular activity. In this paper we explore a highly novel approach by making materials dual-responsive to two endogenous molecules; viz, CO_2 and NO.

The integration of dual stimuli sensitivity into one system, to form a multi-responsive material, confers unique properties to the nanostructures with responses triggered by specialized biological environments (e.g., within hypoxic cells).[47, 48] The idea of making multi-gas responsive nanomaterials has received attention in very recent work by Jeong and coworkers,[49] who revealed that an aqueous solution of pentafluorophenyl end-capped poly(ethylene glycol) (PF-PEG-PF) exhibited a tunable lower critical solution temperature (LCST) when exposed to CO_2 and O_2 addition.

Zhu and coworkers[50, 51] also made CO_2- and O_2-responsive copolymers by combining a CO_2-responsive tertiary amine-containing monomer and an O_2-sensitive fluoro-containing monomer.

In our recent publication on NO-responsive polymers we exploited the high reactivity of *o*-phenylenediamine towards NO to form benzotriazole derivatives, thereby allowing us to tune the LCST behavior of thermoresponsive polymers using NO as a stimulus.[25] In this way we made truly biomimetic nanostructures responsive to intracellular concentration levels of NO. In the current paper we extend our synthetic design to incorporate additional sensitivity to CO_2 with the aim of creating nanostructures that are selectively responsive to increased CO_2 and NO, such as might possibly be found in the hypoxic regions of the tumour microenvironment.[52, 53] We fabricated NO-sensitive *o*-phenylenediamine moieties and CO_2-sensitive tertiary amine motifs within single chains. Herein, well-defined poly(oligo(ethylene glycol) methyl ether methacrylate)-*block*-poly(*N*,*N*-diethylaminoethyl methacrylate)-*block*-poly(2-(3- (2-aminophenyl) ureido)ethyl methacrylate) (POEGMA-*b*-PDEAEMA-*b*-PAPUEMA, termed as ODA) triblock copolymer, consisting of NO-responsive PAPUEMA block and CO_2-responsive PDEAEMA block, was synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization. The triblock copolymer spontaneously self-assembles into PDEAEMA/PAPUEMA dual core micelles at neutral pH. Upon CO_2 addition, the initially collapsed PDEAEMA block was swollen due to the CO_2-induced protonation, thereby expanding the original micellar nanoparticles; subsequent NO charging forms hydrophobic benzotriazole moieties in the interior PAPUEMA block, accompanied by morphological transition from swollen micelles to nanorods. Interestingly, the spherical nanoparticles-to-nanorods transition can be also realized solely on NO exposure without CO_2 charging (Scheme 1). To our knowledge, this work represents the first example where two endogenous gas species (i.e., NO and CO_2) synergistically regulate the morphology of polymer assemblies. The unique bio-relevant gas-responsive properties of the dual-responsive block copolymer provide potential benefits in applications such as chemotherapeutic drug delivery in the challenging milieu of the heterogeneous tumour microenvironment.



Scheme 1. Schematic representation of the gas-triggered self-assembly behavior of NO and CO_2 dual-responsive block copolymer. The ODA triblock copolymer self-assembles into PDEAEMA/PAPUEMA-core micelles at neutral pH without gas stimuli. Upon CO_2 exposure, the water solubility of PDEAEMA block is reversed due to the CO_2-triggered protonation process of PDEAEMA, resulting in the formation of PAPUEMA core micelles with swollen POEGMA/PDEAEMA coronas. The swollen micelles can then be transformed to nanorods on further NO addition by taking advantage of the formation of hydrophobic benzotriazole motifs. Moreover, the original ODA micellar aggregates can be directly transformed to nanorods with only NO stimulation.

Experimental Part

Materials. Oligo(ethylene glycol) methyl ether methacrylate (OEGMA, M_n = 300 g mol^{-1}) and *N*,*N′*-diethylaminoethyl methacrylate (DEAEMA) were purchased from Sigma-Aldrich. The OEGMA and DEAEMA monomers were purified by passing through a column filled with inhibitor remover (Aldrich) prior to use. 2-Cyanoprop-2-yl benzodithioate (CPBD) and 2,2′-azobis(2-methylpropionitrile) (AIBN) solution (0.2 M in toluene) were purchased from Sigma-Aldrich and used without further purification. All solvents (purchased from EMSURE

Reagents) were analytical grade and used without further purification unless otherwise noted. NO[54] and CO_2 were generated by slowly dropping saturated sodium nitrite ($NaNO_2$) solution and sodium bicarbonate ($NaHCO_3$) solution to sulfuric acid solutions (2 M), respectively. The generated NO and CO_2 gases were then charged into the polymer solution via a plastic tube.

Synthesis of 2-(3-(2-aminophenyl)ureido)ethyl methacrylate (APUEMA) monomer (Scheme S1). The APUEMA monomer was synthesized according to our reported protocol without protonation process.[25] Typically, *o*-phenylenediamine (2.7 g, 25 mmol) was dissolved in 100 mL anhydrous MeCN. 2-Isocyanatoethyl methacrylate (3.88 g, 25 mmol) was dissolved in 20 mL anhydrous MeCN and added dropwise. The mixture was further stirred at room temperature overnight. After that, MeCN was removed under vacuum and the residue was precipitated into an excess of diethyl ether to remove any unreacted *o*-phenylenediamine and 2-isocyantoethyl methacrylate. The insoluble solid (a mixture of the target monomer and dual-functionalized crosslinker) was collected and washed with diethyl ether three times. After purifying by column chromatography to remove dual-functional crosslinker, non-protonated APUEMA monomer was obtained as a white solid (4.9 g, 18.6 mmol). ^{1}H NMR ($CDCl_3$, δ, ppm, Figure S1a): 7.14 – 7.01 (m, 1H), 6.85 – 6.69 (m, 1H), 6.52 (s, 1H), 6.04 (dd, 1H), 5.60 – 5.50 (m, 1H), 5.20 (s, 1H), 4.27 – 4.16 (m, 1H), 4.16 – 3.93 (m, 1H), 3.59 – 3.38 (m, 1H), 1.95 – 1.81 (m, 3H). ^{13}C NMR ($CDCl_3$, δ, ppm, Figure S1b): 167.30, 157.44, 142.42, 136.02, 127.59, 127.39, 125.94, 123.31, 118.98, 116.68, 63.68, 39.20, 18.26.

*Treatment of APUEMA monomer with NO (Scheme S1)***.** APUEMA monomer (78 mg, 0.3 mmol) was dissolved in deionized water/MeCN (20 mL, v/v = 1:1). The solution was gently bubbled with NO gas for 1 h. Subsequently, MeCN was removed under vacuum. The aqueous solution was freeze-dried and the final product was purified by column chromatography using dichloromethane as the eluent. The product was obtained as a white solid (78 mg, yield: ~85%). ^{1}H NMR ($CDCl_3$, δ, ppm, Figure S2a): 8.29 – 8.13 (m, 1H), 8.04 (d, 1H), 7.57 (ddd, 2H), 7.48 – 7.35 (m, 1H), 6.10 (d, 1H), 5.55 (dd, 1H), 4.49 – 4.25 (m, 2H), 3.82 (dd, 2H), 1.94 – 1.82 (m, 3H). ^{13}C NMR ($CDCl_3$, δ, ppm, Figure S2b): 167.22, 149.38, 146.26, 135.78, 131.62, 130.03, 126.39, 125.50, 120.05, 113.87, 63.07, 39.65, 18.29.

Synthesis of POEGMA macroRAFT agent (Scheme 2). POEGMA macroRAFT was synthesized via RAFT polymerization of OEGMA monomer using CPBD as RAFT agent. In a typical experiment, OEGMA monomer (6.0 g, 20 mmol), CPBD (221 mg, 1 mmol), AIBN (500 μL, 0.1

mmol), and toluene (12 mL) were charged into a 20 mL polymerization vial with a stirring bar. The mixture was deoxygenated by sparging with nitrogen for 30 min and placed in a preheated oil-bath at 70 ^{o}C for 6 h. The polymerization was terminated by immersing the polymerization vial in an ice-water bath. POEGMA macroRAFT agent was purified by precipitation into an excess of petroleum ether, repeated three times. POEGMA macroRAFT agent was obtained as red viscous liquid (M_n = 4.2 kDa, M_w/M_n = 1.11, Figure S3a, Table 1). The degree of polymerization, DP, of the POEGMA macroRAFT agent was determined to be 10 by ^{1}H NMR analysis (Figure S4a). The POEGMA macroRAFT agent was thus denoted as POEGMA$_{10}$.

Synthesis of POEGMA-b-PDEAEMA diblock copolymer precursor (Scheme 2). POEGMA-*b*-PDEAEMA diblock precursor was synthesized via RAFT polymerization using POEGMA macroRAFT agent. Typically, POEGMA (320 mg, 0.1 mmol), DEAEMA (1.85 g, 10 mmol), AIBN (50 μL, 0.01 mmol), and 1,4-dioxane (4.0 mL) were charged into a 20 mL polymerization vial with a stirring bar. The mixture was deoxygenated by sparging with nitrogen for 10 min and placed in a preheated oil-bath at 70 ^{o}C for 12 h. The polymerization was quenched in an ice-water bath and the diblock copolymer precursor was purified by precipitation into an excess of petroleum ether (repeated three times). The POEGMA-*b*-PDEAEMA diblock precursor was obtained as a red solid (M_n = 14.1 kDa, M_w/M_n = 1.17, Figure S3b, Table 1). The DP of PDEAEMA block was determined to be ~61 by ^{1}H NMR analysis (Figure S4b). The diblock copolymer precursor was thus denoted as POEGMA$_{10}$-*b*-PDEAEMA$_{61}$ and abbreviated to OD.

Synthesis of POEGMA-b-PAPUEMA diblock copolymer (Table 1). POEGMA-*b*-PAPUEMA diblock copolymer was synthesized via RAFT polymerization using POEGMA macroRAFT agent. Typically, POEGMA (320 mg, 0.1 mmol), APUEMA (0.526 g, 2 mmol), AIBN (60 μL, 12 μmol), and DMF (4.0 ml) was charged to a 20 mL polymerization vial with a stirring bar. The mixture was deoxygenated by sparging with nitrogen for 10 min and placed in a preheated oil-bath at 70 ^{o}C for 16 h. The polymerization was quenched in an ice-water bath and the crude diblock copolymer was purified by dialysis against acetone. Fresh acetone was replaced every 4 h. The diblock copolymer was isolated via precipitation into an excess of diethyl ether. POEGMA$_{10}$-*b*-PAPUEMA$_{17}$ diblock copolymer was obtained as reddish solid and abbreviated to OA (M_n = 10.8 kDa, M_w/M_n = 1.18, Table 1).

Synthesis of POEGMA-b-PDEAEMA-b-PAPUEMA triblock copolymer (Scheme 2). POEGMA-*b*-

PDEAEMA-*b*-PAPUEMA triblock copolymer was synthesized via RAFT polymerization using OD diblock precursor as macroRAFT agent. Typically, OD diblock copolymer (725 mg, 0.05 mmol), APUEMA (658 mg, 2.5 mmol), AIBN (50 μL, 0.01 mmol), and DMF (3.0 mL) were charged into a 20 mL polymerization vial with a stirring bar. The mixture was deoxygenated by sparging with nitrogen for 10 min and placed in a preheated oil-bath at 70 ^{o}C for 12 h. The polymerization was quenched in an ice-water bath and the triblock copolymer was purified by precipitation into an excess of diethyl ether (three times). The POEGMA-*b*-PDEAEMA-*b*-PAPUEMA triblock copolymer was obtained as a pale red solid (M_n = 65.8 kDa, M_w/M_n = 1.22, Figure S3c, Table 1). The DP of PAPUEMA block was determined to be ~46 by ^{1}H NMR analysis (Figure S4c). The triblock copolymer was thus denoted as POEGMA$_{10}$-*b*-PDEAEMA$_{61}$-*b*-PAPUEMA$_{46}$ and abbreviated to ODA.

Self-assembly of POEGMA-b-PDEAEMA-b-PAPUEMA triblock copolymer. An ODA colloidal solution was prepared via a cosolvent method using 1,4-dioxane and DMF (*v*/*v* = 1:1) as cosolvent. Typically, 2 mg ODA triblock copolymer was dissolved into 2 mL 1.4-dioxane and DMF mixture. Under vigorous stirring, deionized water (2.0 mL) was then added at a rate of 1.0 mL/h by a syringe pump. After addition, the mixture was stirred for one more hour prior to the addition of water (16 mL) in one shot. The 1,4-dioxane was removed by dialysis against deionized water for 24 h and fresh water was replaced appropriately every 6 h.

Characterization.

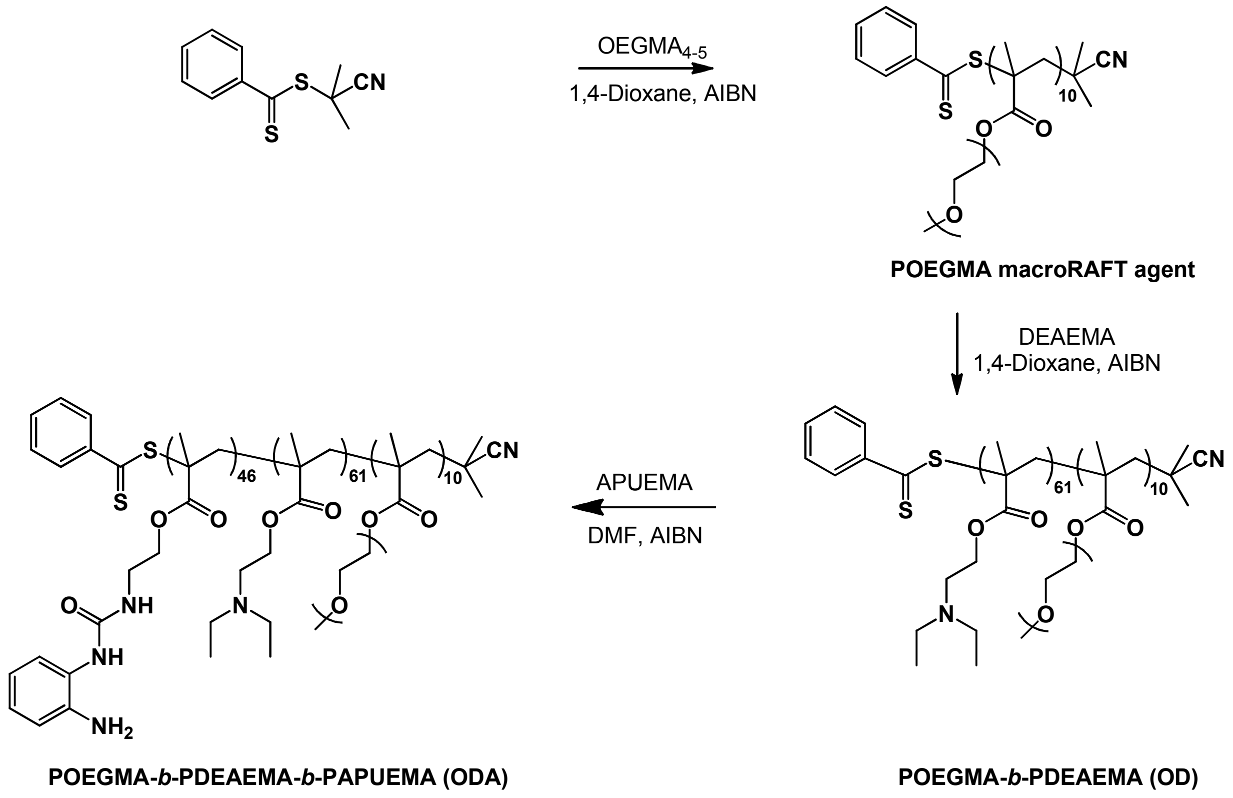
Nuclear Magnetic Resonance (NMR) Spectra. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC400F (400 MHz) spectrometer. Deuterium oxide (D_2O), deuterated chloroform ($CDCl_3$), and deuterated dimethylsulfoxide (DMSO-d_6) were used as the solvents.

Gel Permeation Chromatography (GPC). GPC analyses of polymer samples were performed in *N*,*N*-dimethylacetamide (DMAc with 0.03% *w*/*v* LiBr and 0.05% 2,6-dibutyl-4-methylphenol (BHT) using a Shimadzu modular system comprising a DGU-12A degasser, an SIL-10AD automatic injector, and a 5.0 μm bead-size guard column (50 × 7.8 mm) followed by four 300 × 7.8 mm linear Phenogel columns (bead size: a 5.0 μm; pore sizes: 105, 104, 103, and 500 Å) and an RID-10A differential refractive-index detector. The temperature of columns was maintained at 50 °C using a CTO-10A oven, and the flow rate was kept at 1 mL/min using a LC-10AT pump. A molecular weight calibration curve was produced using commercial narrow molecular weight distribution polystyrene standards with molecular weights ranging from 500 to 10^6 g/mol. Polymer solutions at 2–3 mg/mL were prepared in the eluent and filtered through 0.45 μm filters prior to injection.

Dynamic Light Scattering (DLS). DLS measurements were performed using a Malvern Zetasizer Nano Series running DTS software and using a 4 mW He–Ne laser operating at a wavelength of 633 nm and an avalanche photodiode (APD) detector. The scattered light was detected at an angle of 173°. The temperature was stabilized to ± 0.1 °C of the set temperature. Aqueous solutions of polymer samples were filtered through a 0.45 *μ*m pore size filter to remove dust prior to measurement. To assess size distributions, the autocorrelation function was fitted using the cumulants method. All data were averaged over three consecutive measurements.

Transmission Electron Microscopy (TEM). The sizes and morphologies of the self-assembled aggregates were observed using a FEI TECNAI G20 transmission electron microscope at an accelerating voltage of 200 kV. The aqueous solutions of the triblock polymer in varying conditions were dried on to 300 mesh, holey film, copper grid (ProSciTech) without staining.

UV-Vis absorption measurements. All UV-Vis spectra were acquired on a Shimadzu UV3600 spectrophotometer in quartz cuvettes of 10 mm path length.



Scheme 2. Schematic depicting the preparation of POEGMA-*b*-PDEAEMA-*b*-PAPUEMA (ODA) triblock via consecutive reversible addition-fragmentation chain transfer (RAFT) polymerization.

Results and Discussion

As detailed previously, tertiary amine-containing polymers are inherently sensitive to a CO_2 stimulus resulting in CO_2-induced protonation of tertiary amine moieties and thereby improved water-solubility.[30] In other recent work, our group demonstrated that polymers having an *o*-phenylenediamine group can react with NO to form benzotriazole derivates.[25] This chemistry was exploited to tune the thermoresponsive behavior of poly(*N*-isopropylacrylamide) copolymers by shifting the hydrophilic-hydrophobic balance of the polymer. In the present work we have extended these studies to prepare a novel dual gas-responsive polymer constructed from a tertiary-amine containing monomer and an *o*-phenylenediamine containing monomer. A well-defined double hydrophilic triblock copolymer, POEGMA-*b*-PDEAEMA-*b*-PAPUEMA (termed as ODA), was synthesized via sequential RAFT polymerization (Scheme 2). The target ODA triblock copolymer was measured to be nearly mono-disperse with an M_n of 65.8 kDa and a polydispersity (M_w/M_n) of 1.22 (Table 1 and Figure S3). The degrees of polymerization (DPs) of the POEGMA macroRAFT agent, diblock precursor as well as the target triblock copolymer were further identified by ^{1}H NMR analysis (Figure S4). It is noteworthy that the molecular weight determined by GPC proved to be larger than that obtained from NMR analyses; these inconsistent results can be attributed to the presence of free amine (i.e., APUEMA) in the polymer chains and to the non-size exclusion conditions imparted by the mismatch of analyte samples and the chromatography standards.

Table 1. Structural parameters of the macroRAFT agent, diblock precursor, and the NO- and CO_2-responsive triblock copolymer.

Entry	Sample	M_n/kDa^a	M_w/M_n^{a}	M_n/kDa^b
/	$POEGMA_{10}$	4.2	1.11	3.2
OD	$POEGMA_{10}$-*b*-$PDEAEMA_{61}$	14.1	1.17	14.5

ODA	POEGMA$_{10}$-*b*-PDEAEMA$_{61}$-*b*-PAPUEMA$_{46}$	65.8	1.22	26.6
ODA-NO	POEGMA$_{10}$-*b*-PDEAEMA$_{61}$-*b*-P(APUEMA-NO)$_{46}$	48.7	1.23	27.1
OA	POEGMA$_{10}$-*b*-PAPUEMA$_{17}$	10.8	1.18	7.7

^{a} determined by GPC using DMAc as eluent; ^{b} determined by ^{1}H NMR analysis.

We first examined the pH-responsiveness of the triblock copolymer by potentiometric titration. The pK_a of the OD diblock precursor was determined to be ~7.4; a value in accord with previous results (Figure S5a).[13] However, after introduction of the NO-responsive PAPUEMA block, the potentiometric titration curve exhibited significant differences when compared to the OD diblock precursor. Moreover, macroscopic precipitation was observed once the pH was above 7.5 for the ODA triblock copolymer, whereas the micellar solution of OD diblock precursor was relatively stable even when the pH was higher than the pK_a of PDEAEMA block (Figure S5b). This result was further confirmed by pH-dependent DLS measurements. As shown in Figure S6a, the OD diblock precursor was molecularly dissolved (i.e., present as unimers) in aqueous solution when the solution pH was lower than 7.4. In sharp contrast, micellar aggregates with PDEAEMA cores and hydrophilic POEGMA coronas were formed when the pH was higher than the pK_a of PDEAEMA block due to the deprotonation of the tertiary amine moieties. However, for the ODA triblock copolymer, unimers were only observed at pH values lower than 2, consistent with the pK_a of the PAPUEMA block (~ 2.1). Micellar nanoparticles were already formed when the pH was 4.6, lower than the pK_a of the PDEAEMA block, suggesting the formation of PAPUEMA-core micelles. Interestingly, the diameters of PAPUEMA-core micelles did not change dramatically when the solution pH was in the range of 4.6-7.0 due to the fact that the PDEAMEA block remained hydrophilic in this pH range (Figure S6b). Upon further pH increase, the micellar diameter rose from ~28 nm at pH 7.0 to ~40 nm at pH 7.5. Although the collapse of the PDEAEMA central block upon pH increase was expected to shrink the micellar nanoparticles, the current ODA triblock micelles may reorganize and form intermicellar aggregates when the hydrophilic-hydrophobic balance was shifted due to the deprotonation of PDEAEMA block.[55, 56] This assumption was also evidenced by appearance of macroscopic sediments observed during the pH titration experiments (Figure S5).

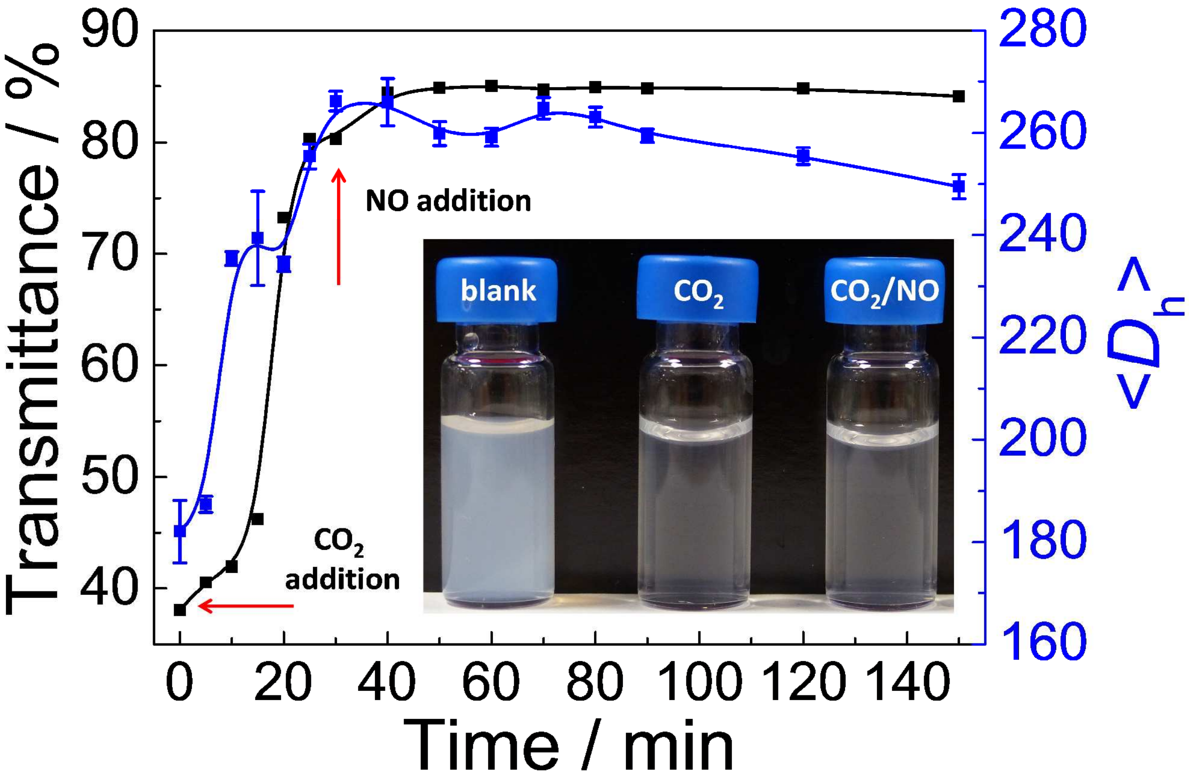


Figure 1. Transmittance and z-average hydrodynamic diameter, <D_h>, for an aqueous solution of ODA triblock copolymer (0.1 g/L) with time of exposure to CO_2 (0 - 30 min) and NO (40 - 150 min). The data represent a single experiment performed in triplicate with the error bars denoting the standard deviation.

It should be noted that the significant difference in the pK_a of the PDEAEMA and PAPUEMA blocks confers the opportunity to continuously tune the water-solubility of the ODA triblock copolymer by sequential CO_2 and NO addition. Consequently, the CO_2-responsive property of the ODA triblock copolymer was first investigated. Using 1,4-dioxane/DMF as a cosolvent, the ODA triblock copolymer self-assembled in aqueous media into spherical micelles consisting of PDEAEMA/PAUEMA cores and hydrophilic POEGMA coronas, with a diameter of ~ 180 nm (Figure 1). The formation of spherical nanoparticles was also evidenced by transmission electron microscopy (TEM) (Figure 2a). Upon sparging the micellar solution with CO_2 for 30 min (inducing a pH drop from 7.85 to 5.62), the initially opaque solution gradually became clear (the inset in Figure 1) and the solution transmittance increased from ~38% to ~81% (Figure 1). Concomitantly, the micellar hydrodynamic diameter, <D_h>, increased from ~180 to 260 nm after 30 min CO_2 bubbling. From the TEM images, it was observed that the initial smooth surfaces of the micellar nanoparticles became rough and increased micellar diameters were observed, consistent with DLS data (Figure 2b). We attributed these changes to a CO_2-induced protonation of the PDEAEMA block following the pH decrease in the colloidal solution. The cationic PDEAEMA block became stretched because of increased electrostatic repulsion, thus resulting in expanded <D_h>. Similar phenomena have been previously reported for CO_2-responsive systems.[41, 42, 44, 45]

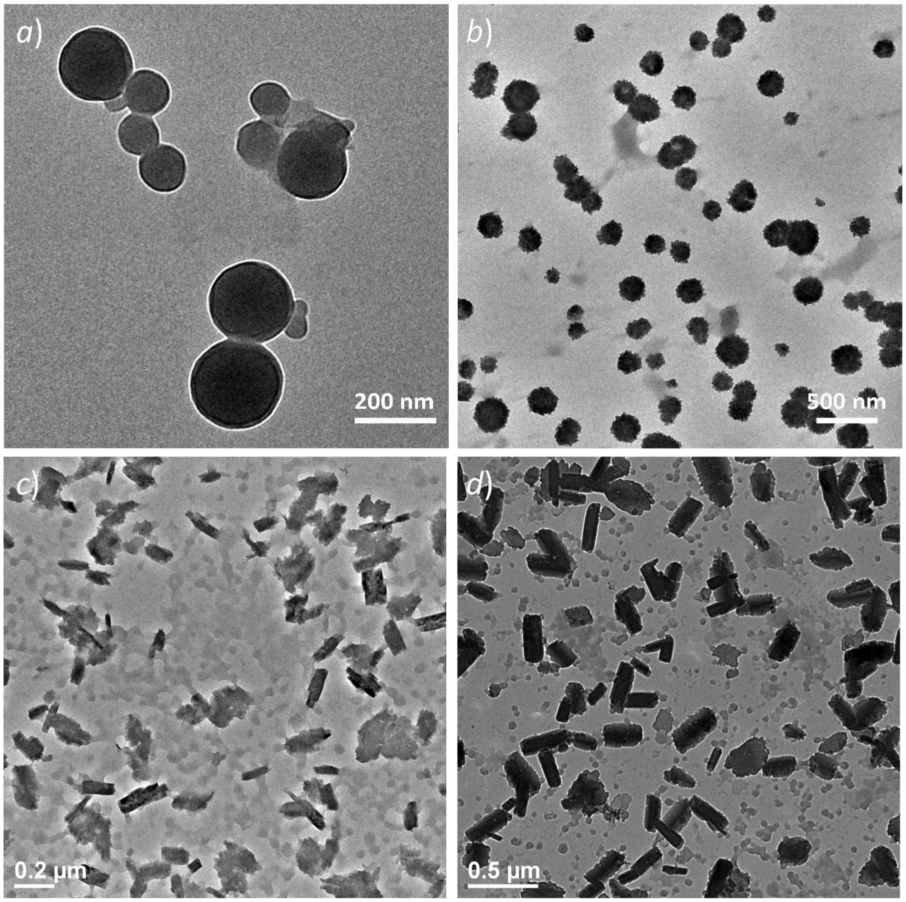


Figure 2. Typical transmission electron microscopy (TEM) images recorded for the colloidal solutions (0.1 g/L) of triblock copolymer (a) before gas treatment, (b) after 30 min CO_2 bubbling, (c) after 30 min CO_2 bubbling and a further 2 h NO bubbling and 24 h stirring at room temperature, and (d) after 2 h NO bubbling and 24 h stirring at room temperature.

While the OD diblock copolymer exhibited negligible absorbance in the range of 250-500 nm (Figure S7), the ODA triblock copolymer showed a similar absorbance to the OA diblock copolymer (Figures 3a and 3b). Although the UV-Vis spectra for the ODA triblock copolymer (Figure 3a) recorded a visible decrease in absorbance after CO_2 sparging, we could not find significant absorbance changes for OA diblock copolymer (Figure 3b). Therefore, this decreased absorbance with no peak shift or new absorbances was tentatively attributed to the increased transmittance upon CO_2 addition (Figure 1). These results indicate that the ODA triblock copolymer was sensitive to CO_2 stimulus and that CO_2-induced micellar expansion was successfully achieved. After 30 min of CO_2 addition, self-assembled aggregates rather than unimers were present in solution as confirmed by both DLS and TEM measurements (Figures 1 and 2b), indicating that although the acidic CO_2 solution can induce protonation of the PDEAEMA block, the reduction in pH is not sufficient to disassemble the NO-responsive PAPUEMA block – a process requiring a much lower pH (pK_a~2.1). These results are in accord with the potentiometric titration and DLS results (Figures S5 and S6). Importantly, the addition of CO_2 will only reverse the water solubility of PDEAEMA block, while the PAPUEMA block remains intact upon CO_2 sparging, enabling further switching the property of polymer solution by NO (Figure 3b).

In the next phase of work we investigated the effect of NO addition using both DLS and UV-Vis to study any phase changes which might be triggered by NO exposure. After 2 hour of NO addition, the transmittance and $\langle D_h \rangle$ of the ODA micellar solution did not show any significant changes (in contrast to CO_2 addition). We only observed a negligible transmittance increase from ~80% to ~84% and a $\langle D_h \rangle$ shift from ~280 nm to 250 nm (Figure 1). This transmittance transition could be directly visualized using macroscopic images of the colloidal solutions (the inset in Figure 1). Therefore it appeared that the ODA triblock copolymer was not sensitive to NO addition. However, on recovery of the ODA triblock copolymer after consecutive CO_2 and NO treatments, we observed that the ODA triblock copolymer exhibited an apparent reduction in molecular weight, which we attribute to the consumption of free amine groups (Figure S3d). NMR analyses revealed that the aromatic protons, shifted to downfield after exposure to both gases (Figure S4d). In addition, the previous CO_2 exposure led only to a slightly decreased absorbance (Figure 3a), whereas substantial absorbance changes were observed following NO addition (Figure 3c). Specifically, an emerging absorbance peak, centered at ~258 nm, was observed after NO exposure. These experimental observations corresponded with our previous APUEMA monomer/NO solution titration result indicating that the NO-responsive nature of the *o*-phenylenediamine motifs was retained even after polymerization.[25] Interestingly, after 2 h NO addition, TEM analyses confirmed a change in the nanoparticle morphology with the swollen micellar nanoparticles transformed into nanorod structures (Figure 2c). This significant morphological transition from micelles to nanorods, upon NO exposure, can be ascribed to the NO-induced formation of hydrophobic benzotriazole moieties (APUEMA-NO), resulting transforming the double hydrophilic block copolymer into an amphiphilic triblock copolymer, thereby increasing the packing parameter governing the self-assembly process thus driving the morphological transition.[57] In summary, the ODA triblock copolymer comprised of CO_2-responsive tertiary amine moieties (i.e., PDEAEMA) and NO-responsive *o*-phenylenediamine groups (i.e., PAPUEMA) was sensitive to both CO_2 and NO exposure. CO_2 triggered the expansion of micellar nanoparticles following protonation of the PDEAEMA block, while the further addition of NO induced a morphological transition from swollen micelles to nanorods following the formation of hydrophobic benzotriazole moieties.

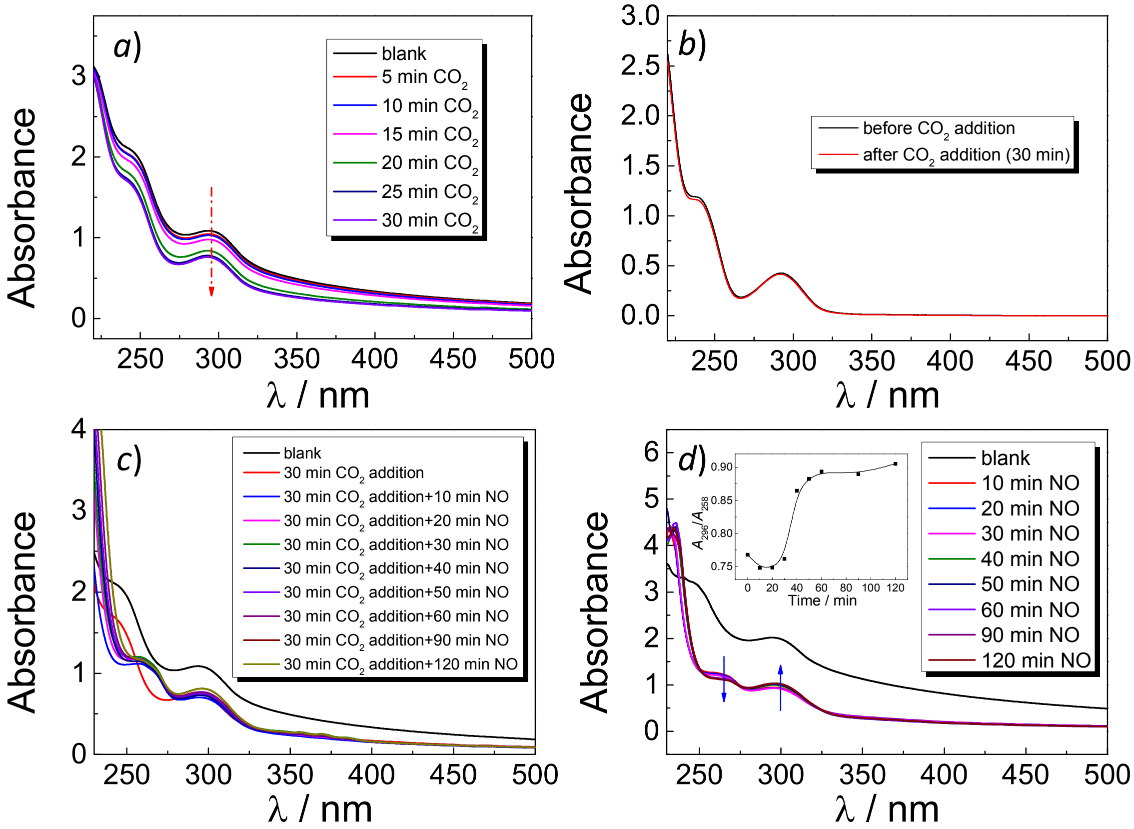


Figure 3. (a) Absorption spectra of an aqueous solution of ODA triblock copolymer (0.05 g/L) with variation of CO_2 exposure time. (b) Absorption spectra of an aqueous solution of OA diblock copolymer (0.05 g/L) before and after 30 min CO_2 addition. (c) Absorbance spectra of aqueous solutions of the ODA triblock copolymer (0.05 g/L) upon NO addition at defined time intervals following 30 min CO_2 pretreatment. (d) Time-dependent absorbance spectra of aqueous solutions of ODA triblock copolymer (0.05 g/L) upon NO addition at set time intervals. The inset shows absorbance ratio (A_{296}/A_{258}) as a function of time.

The ODA triblock copolymer proved sensitive to sequential CO_2 and NO addition, as confirmed by combined NMR, DLS, UV-Vis, and TEM analysis. Subsequently, we conducted further experiments in which we altered the sequence of gas exposure, exposing to NO first. Following NO gas exposure, the opaque colloidal solution became transparent within 10 min, (very similar to the CO_2 addition), and this was easily visualized (inset in Figure 4). This result was inconsistent with the previous transmittance changes (shown in Figure 1), that indicated only a slight increase in transmittance after NO addition. To clarify the reason for the contradictory observations, the pH, after NO charging, was examined. Surprisingly, the solution pH decreased from 7.8 to 2.9 after 10 min of NO charging and the final pH of the solution was determined to be ~ 1.0 after 2 h NO addition. Note that the reaction between NO and *o*-phenylenediamine moieties should be conducted under aerobic conditions.[58-60] Previous research revealed that NO mainly oxidized to nitrite rather than nitrate in an aerobic aqueous solution.[61] We inferred that, in the current case, some NO can also be oxidized by oxygen in air with the formation of NO_2, thereby facilitating the generation of nitric acid in aqueous solution. In turn, the concurrent generation of nitrous and nitric acid contributed to a significant pH drop, resulting in swelling of the PDEAEMA block and thereby increased transmittance.

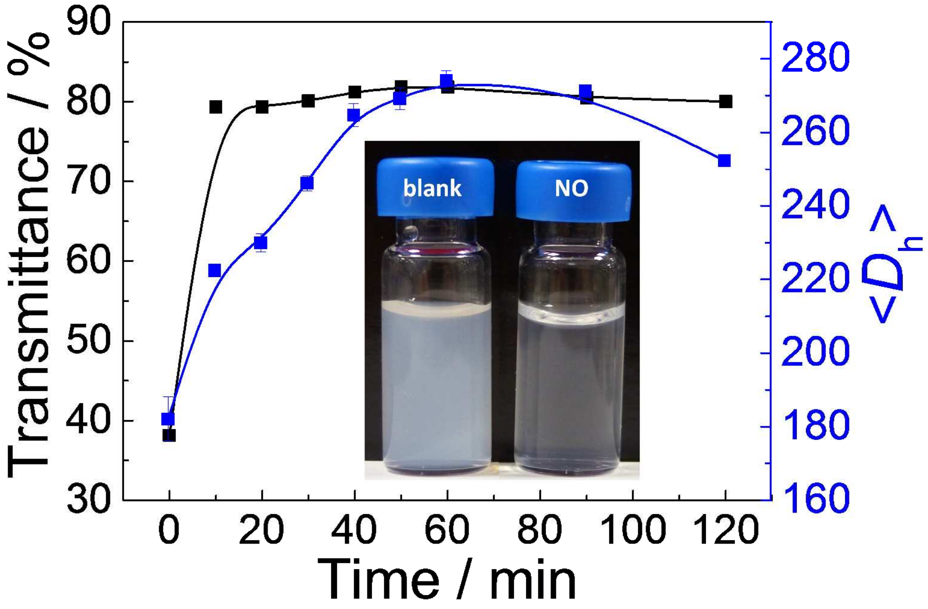


Figure 4. Transmittance and z-average hydrodynamic diameter, <D_h>, for an aqueous solution of ODA triblock copolymer (0.1 g/L) with increasing time of NO addition. The data represent a single experiment performed in triplicate with the error bars denoting the standard deviation.

As shown in Figure 4, during 2 h of NO charging, the transmittance of the colloidal solution increased from 38% to 80%. Simultaneously, the <D_h> of the assembled nanoparticles increased from ~180 nm to ~250 nm following NO addition. These results concurred with previous data obtained from experiments using combined CO_2 and NO stimuli (Figure 1). From the UV-Vis spectra, we observed new absorbance peaks located at 258 nm (Figure 3d). The TEM result revealed the formation of nanorods after NO treatment even without CO_2 addition (Figure 2d). The NO gas can synergistically swell the PDEAEMA block following formation of nitrous acid and nitric acid and also react with the PAPUEMA block to form benzotriazole moieties.

In summary, a novel NO and CO_2-responsive ODA triblock copolymer, consisting of hydrophilic POEGMA block, CO_2-responsive PDEAEMA block, and NO-sensitive PAPUEMA block, was successfully prepared via sequential RAFT polymerization. By taking advantage of the CO_2-induced protonation of tertiary amine moieties and NO-triggered transformation of *o*-phenylenediamine with the formation of hydrophobic benzotriazole derivates, the self-assembled morphologies of the ODA triblock copolymer can be continuously regulated by exposure to CO_2 and NO, to yield different morphologies from spherical micelles to swollen micelles and finally nanorods. In addition, the spherical nanoparticles could be directly transformed to nanorods via only NO stimuli without CO_2 addition which we ascribe to the spontaneous oxidation of NO to form nitric acid. This work describes for the first time a synthetic protocol to make gas-responsive polymers by combining different gas-sensitive components. We were careful to select polymer segments that reacted with both NO and CO_2; molecules ubiquitous in biological environments. NO and CO_2 dual responsive polymers represent a new class of truly biomimetic materials that can self-organise or respond to biological gasotransmitters auguring promising applications in both biomaterial and biomedical areas. It should be noted that both the CO_2 and NO concentrations used in this study were higher than known physiological concentrations. We are actively targeting future studies to improve the sensitivity of block copolymers toward gaseous stimuli to open up opportunities in biomedical applications.

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References

1. A. S. Hoffman, *Adv Drug Deliver Rev*, 2013, 65, 10-16.
2. M. S. Shim and Y. J. Kwon, *Adv Drug Deliver Rev*, 2012, 64, 1046-1058.
3. J. F. Mano, *Adv Eng Mater*, 2008, 10, 515-527.
4. M. A. C. Stuart, W. T. S. Huck, J. Genzer, M. Muller, C. Ober, M. Stamm, G. B. Sukhorukov, I. Szleifer, V. V. Tsukruk, M. Urban, F. Winnik, S. Zauscher, I. Luzinov and S. Minko, *Nat Mater*, 2010, 9, 101-113.
5. J. M. Hu and S. Y. Liu, *Macromolecules*, 2010, 43, 8315-8330.
6. J. M. Hu and S. Y. Liu, *Accounts Chem Res*, 2014, 47, 2084-2095.
7. J. L. Zhang, M. X. Zhang, K. J. Tang, F. Verpoort and T. L. Sun, *Small*, 2014, 10, 32-46.
8. R. Yerushalmi, A. Scherz, M. E. van der Boom and H. B. Kraatz, *J Mater Chem*, 2005, 15, 4480-4487.
9. D. Roy, J. N. Cambre and B. S. Sumerlin, *Prog Polym Sci*, 2010, 35, 278-301.
10. M. A. Ward and T. K. Georgiou, *Polymers-Basel*, 2011, 3, 1215-1242.
11. C. Weber, R. Hoogenboom and U. S. Schubert, *Prog Polym Sci*, 2012, 37, 686-714.
12. D. Schmaljohann, *Adv Drug Deliver Rev*, 2006, 58, 1655-1670.

13. J. M. Hu, G. Y. Zhang, Z. S. Ge and S. Y. Liu, *Prog Polym Sci*, 2014, 39, 1096-1143.
14. S. Dai, P. Ravi and K. C. Tam, *Soft Matter*, 2008, 4, 435-449.
15. X. R. Wang, G. H. Liu, J. M. Hu, G. Y. Zhang and S. Y. Liu, *Angew Chem Int Edit*, 2014, 53, 3138-3142.
16. H. Zhao, E. S. Sterner, E. B. Coughlin and P. Theato, *Macromolecules*, 2012, 45, 1723-1736.
17. Y. Zhao, *Macromolecules*, 2012, 45, 3647-3657.
18. J. S. Katz and J. A. Burdick, *Macromol Biosci*, 2010, 10, 339-348.
19. R. J. Wojtecki, M. A. Meador and S. J. Rowan, *Nat Mater*, 2011, 10, 14-27.
20. C. Weder, *Nature*, 2009, 459, 45-46.
21. D. A. Davis, A. Hamilton, J. L. Yang, L. D. Cremar, D. Van Gough, S. L. Potisek, M. T. Ong, P. V. Braun, T. J. Martinez, S. R. White, J. S. Moore and N. R. Sottos, *Nature*, 2009, 459, 68-72.
22. J. N. Brantley, C. B. Bailey, K. M. Wiggins, A. T. Keatinge-Clayth and C. W. Bielawski, *Polym Chem-Uk*, 2013, 4, 3916-3928.
23. H. Meng and J. L. Hu, *J Intel Mat Syst Str*, 2010, 21, 859-885.
24. J. Ge, E. Neofytou, T. J. Cahill, R. E. Beygui and R. N. Zare, *Acs Nano*, 2012, 6, 227-233.
25. J. M. Hu, M. R. Whittaker, H. Duong, Y. Li, C. Boyer and T. P. Davis, *Angew Chem Int Edit*, 2014, 53, 7779-7784.
26. M. Huo, J. Yuan, L. Tao and Y. Wei, *Polym Chem-Uk*, 2014, 5, 1519-1528.
27. R. L. McCarley, *Annu Rev Anal Chem*, 2012, 5, 391-411.
28. J. M. Hu, G. Q. Zhang and S. Y. Liu, *Chem Soc Rev*, 2012, 41, 5933-5949.
29. Z. S. Ge and S. Y. Liu, *Chem Soc Rev*, 2013, 42, 7289-7325.
30. S. J. Lin and P. Theato, *Macromol Rapid Comm*, 2013, 34, 1118-1133.
31. Y. F. Cai, W. M. Shen, R. Wang, W. B. Krantz, A. G. Faneb and X. Hu, *Chem Commun*, 2013, 49, 8377-8379.
32. J. Y. Quek, P. J. Roth, R. A. Evans, T. P. Davis and A. B. Lowe, *J Polym Sci Pol Chem*, 2013, 51, 394-404.
33. J. Pinaud, J. Vignolle, Y. Gnanou and D. Taton, *Macromolecules*, 2011, 44, 1900-1908.
34. A. C. Feng, C. B. Zhan, Q. Yan, B. W. Liu and J. Y. Yuan, *Chem Commun*, 2014, 50, 8958-8961.
35. T. Endo, D. Nagai, T. Monma, H. Yamaguchi and B. Ochiai, *Macromolecules*, 2004, 37, 2007-2009.
36. Q. Yan and Y. Zhao, *Chem Commun*, 2014, 50, 11631-11641.
37. H. B. Liu, Y. Zhao, C. A. Dreiss and Y. J. Feng, *Soft Matter*, 2014, 10, 6387-6391.
38. K. J. Zhou, J. F. Li, Y. J. Lu, G. Z. Zhang, Z. W. Xie and C. Wu, *Macromolecules*, 2009, 42, 7146-7154.
39. B. W. Liu, H. Zhou, S. T. Zhou, H. J. Zhang, A. C. Feng, C. M. Jian, J. Hu, W. P. Gao and J. Y. Yuan, *Macromolecules*, 2014, 47, 2938-2946.
40. Z. R. Guo, Y. J. Feng, Y. Wang, J. Y. Wang, Y. F. Wu and Y. M. Zhang, *Chem Commun*, 2011, 47, 9348-9350.
41. Q. Yan, J. B. Wang, Y. W. Yin and J. Y. Yuan, *Angew Chem Int Edit*, 2013, 52, 5070-5073.
42. Q. Yan and Y. Zhao, *Angew Chem Int Edit*, 2013, 52, 9948-9951.
43. S. S. Satav, S. Bhat and S. Thayumanavan, *Biomacromolecules*, 2010, 11, 1735-1740.
44. B. Yan, D. H. Han, O. Boissiere, P. Ayotte and Y. Zhao, *Soft Matter*, 2013, 9, 2011-2016.
45. Q. Yan, R. Zhou, C. K. Fu, H. J. Zhang, Y. W. Yin and J. Y. Yuan, *Angew Chem Int Edit*, 2011, 50, 4923-4927.
46. Q. Yan and Y. Zhao, *J Am Chem Soc*, 2013, 135, 16300-16303.

47. G. Pasparakis and M. Vamvakaki, *Polym Chem-Uk*, 2011, 2, 1234-1248.

48. J. M. Zhuang, M. R. Gordon, J. Ventura, L. Y. Li and S. Thayumanavan, *Chem Soc Rev*, 2013, 42, 7421-7435.

49. J. Y. Choi, J. Y. Kim, H. J. Moon, M. H. Park and B. Jeong, *Macromol Rapid Comm*, 2014, 35, 66-70.

50. Q. Zhang and S. P. Zhu, *Acs Macro Letters*, 2014, 3, 743-746.

51. Q. Zhang and S. P. Zhu, *Macromol Rapid Comm*, 2014, 35, 1692-1696.

52. M. Umbrello, A. Dyson, M. Feelisch and M. Singer, *Antioxid Redox Sign*, 2013, 19, 1690-1710.

53. Y. Kato, S. Ozawa, C. Miyamoto, Y. Maehata, A. Suzuki, T. Maeda and Y. Baba, *Cancer Cell Int*, 2013, 13.

54. T. W. Shiue, Y. H. Chen, C. M. Wu, G. Singh, H. Y. Chen, C. H. Hung, W. F. Liaw and Y. M. Wang, *Inorg Chem*, 2012, 51, 5400-5408.

55. Z. Y. Zhu, J. Xu, Y. M. Zhou, X. Z. Jiang, S. P. Armes and S. Y. Liu, *Macromolecules*, 2007, 40, 6393-6400.

56. H. Liu, C. H. Li, H. W. Liu and S. Y. Liu, *Langmuir*, 2009, 25, 4724-4734.

57. A. Blanazs, S. P. Armes and A. J. Ryan, *Macromol Rapid Comm*, 2009, 30, 267-277.

58. Y. Q. Sun, J. Liu, H. X. Zhang, Y. Y. Huo, X. Lv, Y. W. Shi and W. Guo, *J Am Chem Soc*, 2014, 136, 12520-12523.

59. H. Kojima, N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata and T. Nagano, *Anal Chem*, 1998, 70, 2446-2453.

60. T. Nagano and T. Yoshimura, *Chem Rev*, 2002, 102, 1235-1269.

61. L. J. Ignarro, J. M. Fukuto, J. M. Griscavage, N. E. Rogers and R. E. Byrns, *P Natl Acad Sci USA*, 1993, 90, 8103-8107.

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The use of endogenous gaseous molecules (NO and CO_2) to regulate the self-assembly of dual-responsive triblock copolymer

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