NO-Responsive Polymers

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Biomimetic Polymers Responsive to a Biological Signaling Molecule: Nitric Oxide Triggered Reversible Self-assembly of Single Macromolecular Chains into Nanoparticles**

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Abstract: *Novel nitric oxide (NO) responsive monomers (NAPMA and APUEMA) containing o-phenylenediamine functional groups have been polymerized to form NO-responsive macromolecular chains as truly biomimetic polymers. Upon exposure to NO—a ubiquitous cellular signaling molecule—the NAPMA- and APUEMA-labeled thermoresponsive copolymers exhibited substantial changes in solubility, clearly characterized by tuneable LCST behavior, thereby inducing self-assembly into nanoparticulate structures. Moreover, the NO-triggered self-assembly process in combination with environmentally sensitive fluorescence dyes could be employed to detect and image endogenous NO.*

Nitric oxide (NO), known as an atmospheric pollutant and a potential health hazard, is recognized as an endothelium-derived relaxing factor (EDRF) and a broad-spectrum biological signaling molecule that operates at both systemic and specific cellular levels.[1] NO is a gaseous radical species with a half-life of several seconds, biosynthesized in living tissues, and functions as a ubiquitous messenger molecule in the cardiovascular, nervous, and immune systems in animals (and also within plant life).[2] NO exerts influence over critically important physiological activities (i.e. as an EDRF in blood vessels, a neurotransmitter in the central nervous system, and a mediator in the immune system).[3] Misregulation of NO is thought to be a factor in numerous human diseases including cardiovascular disorders, gastrointestinal distress, neurodegeneration, and hypertension.[4] In a drive to emulate natural systems and processes, synthetic polymers have been designed to react to stimuli in diverse applications such as artificial muscles, smart membranes, and triggered delivery vectors.[5] The most widely published triggers used to induce polymer responsiveness have been temperature, pH value, ionic strength, external fields, and mechanistic forces, inducing phase changes by manipulating the polymer–solvent versus polymer–polymer interactions.[6] However, harnessing biological signaling molecules to trigger responsiveness in synthetic polymer systems is relatively rare, despite an imperative to design polymers to emulate natural systems (biomimetic polymers).

Stimuli-responsive polymers that are responsive to intracellular pH gradients, thiols, hydrogen peroxide (H_2O_2), and enzymes[7] have been elegantly explored, targeting applications including intracellular imaging and diagnostic and drug/gene delivery. However, studies on biomimetic polymers responsive to intracellular signaling molecules (i.e. NO, carbon monoxide (CO), and hydrogen sulfide (H_2S)) are rare, despite the widespread cellular presence of these messenger molecules. In recent years, there have been some reports describing the use of carbon dioxide (CO_2) sensitive polymers, mostly exploiting amidine and tertiary amine functionalities, and this has become a topic of growing international interest, as reflected by a steady increase in publications.[8] CO_2-responsive polymers presage a new field of stimuli-responsive polymers, triggered by simple gaseous molecules present physiologically. We therefore decided to exploit NO concentration as a trigger for assembling macromolecules, and to our knowledge this is the first example where NO is reported as a molecular trigger that induces responsiveness in synthetic polymer chains, thereby driving or influencing self-assembly, in a truly biomimetic process.

Published research on novel fluorescence sensors for in vitro and in vivo sensing and also imaging of NO provided some knowledge on potentially useful NO reactive chemistry.[9] Various sensing mechanisms including selective NO-triggered reactions and NO-mediated displacements or reduction of metal ions have all been explored.[10] In previous studies describing the fabrication of NO-sensitive probes, the electron-rich *o*-phenylenediamine group has been frequently utilized as a fluorescence quencher moiety through a photo-induced electron-transfer (PET) mechanism.[11] *o*-Phenylenediamine groups are reactive towards NO, producing benzotriazole moieties in a highly efficient and highly selective manner in the presence of O_2. Partially inspired by previous studies, we envisioned that novel NO-responsive polymer chains could be designed by exploiting highly reactive *o*-phenylenediamine moieties. Herein, we demonstrate the synthesis of NO-responsive monomers and their incorpo-

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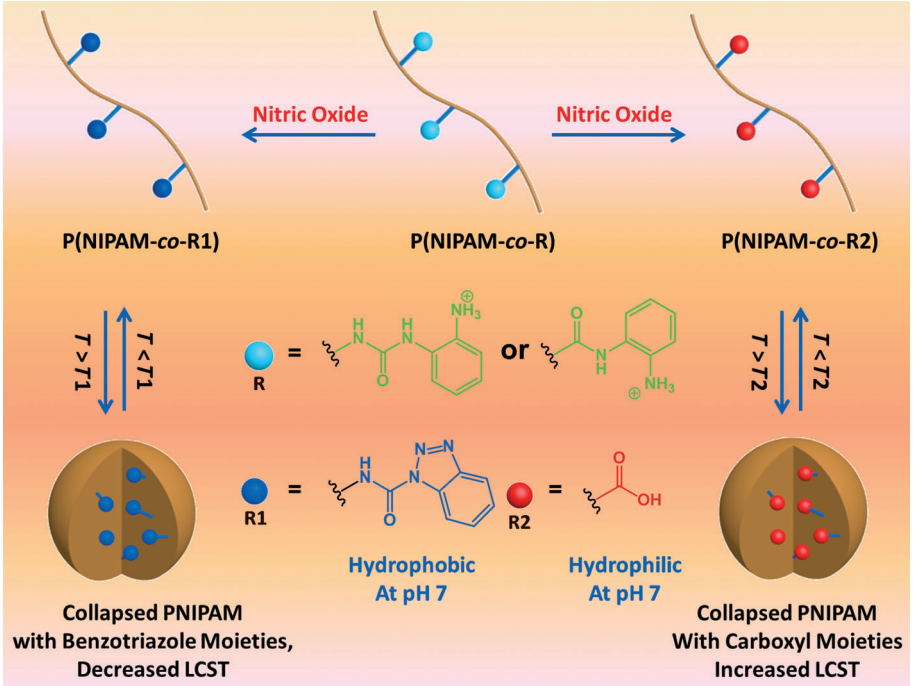
ration into thermoresponsive copolymers, thereby providing the opportunity to delicately mediate the thermoresponsive behavior of polymers by using an NO stimulus (Scheme 1).



Scheme 1. Schematic representation showing the reversible regulation of the lower critical solution temperatures (LCSTs) of NO-responsive copolymers, P(NIPAM-*co*-NAPMA) and P(NIPAM-*co*-APUEMA), using NO as a molecular trigger.

The NO-responsive monomers *N*-(2-aminophenyl) methacrylamide hydrochloride (NAPMA) and 2-(3-(2-aminophenyl)ureido)ethyl methacrylate hydrochloride (APUEMA) were easily synthesized by treating equivalent amounts of *o*-phenylenediamine with methacryloyl chloride or 2-isocyanatoethyl methacrylate, respectively, (see Scheme S1a and S1b in the Supporting Information) and the chemical structures of NAPMA and APUEMA monomers were confirmed by NMR spectroscopic analyses (see Figures S1–S3 in the Supporting Information).

To investigate the NAPMA/NO and APUEMA/NO reaction kinetics we titrated NO into aqueous solutions of NAPMA and APUEMA monomers and monitored the ensuing reaction by UV/Vis spectroscopy. The NAPMA and APUEMA monomers exhibited absorptions centered at about 288 and 286 nm, respectively (Figure 1 a and c). Upon addition of NO (0–48 μM), the absorption band of NAPMA decreased slightly and then red-shifted to 302 nm, whereas the absorption band of APUEMA gradually increased in intensity, which was associated with the co-appearance of two bands, located at about 258 nm and 295 nm (Figure 1 a and c). If we arbitrarily defined the NO detection limit as the concentration at which a 10 % change in absorbance could be measured at 302 nm or at 258 nm, the NO detection limits were then determined to be about 2.5 μM or 2.4 μM, respectively, from the two characteristic absorption bands (Figure 1 b and d). The final products, after reaction with NO, were separated and characterized by NMR spectroscopic analyses (see Figures S4 and S5 in the Supporting Information), thereby providing evidence for the formation of an amide-substituted benzotriazole derivative and a urea-functionalized benzotriazole residual, respectively. Our titration results clearly indicate that the NAPMA and APUEMA monomers are highly reactive with NO under extremely mild conditions (aqueous solution and room temperature). Since the effect of NO in biological environments is highly concentration-dependent,[12] the current highly sensitive NAPMA and APUEMA monomer structures may augur promising intracellular applications.

Next, we further investigated the stabilities of the newly formed products in aqueous solution by UV/Vis spectroscopy. Interestingly, the aqueous solution of the amide-substituted benzotriazole exhibited an absorption centered at 302 nm, in accord with the titration data (see Figure 1 a). Upon extending the incubation time, the amide-substituted benzotriazole gradually hydrolyzed, as evident by a slowly decreasing absorption at 302 nm. Typically, the hydrolysis of the amide-substituted benzotriazole was complete within 2 h (Figure 2 a,b). A similar hydrolysis phenomenon was also observed in *o*-phenylenediamine-containing NO probes.[11b,d] By sharp contrast, the absorption spectra of the urea-

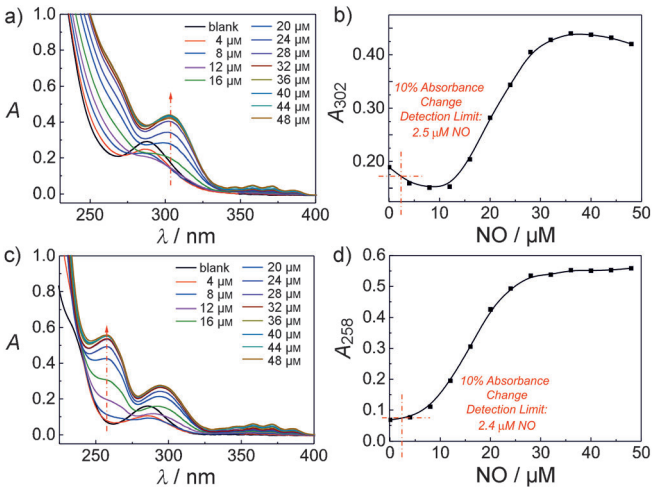


Figure 1. a,c) UV/Vis spectra and b,d) absorption intensity changes recorded for aqueous solutions of (a,b) the NAPMA monomer and (c,d) the APUEMA monomer (20 μM) upon addition of various NO concentrations.

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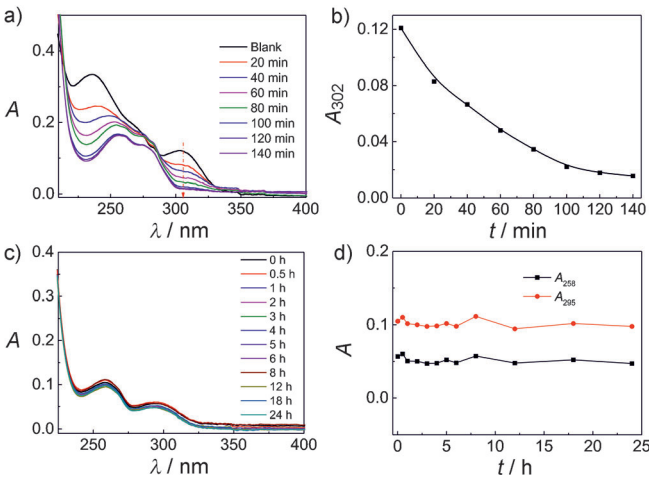
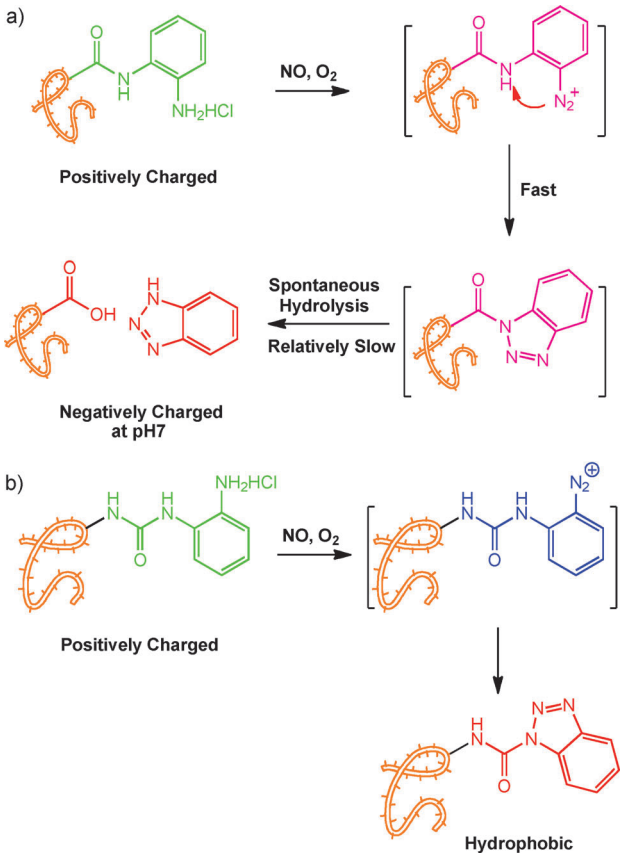


Figure 2. a,c) UV/Vis spectra and b,d) changes in the absorption intensity of aqueous solutions of (a and b) the NAPMA and (c,d) APUEMA monomer after reaction with NO.



Scheme 2. Schematic illustration of the proposed NO-triggered mechanisms of copolymers bearing NAPMA and APUEMA moieties.

functionalized benzotriazole derivative remained unchanged after a long time (e.g. 24 h), thus indicating that the urea-functionalized benzotriazole is relatively stable in aqueous solution (Figure 2 c,d).

The hydrolysis products of the NAPMA monomer following reaction with NO revealed the coexistence of benzotriazole and methacrylic acid (MAA) groups, as evident by ^{1}H NMR spectroscopic analysis (see Figure S6 in the Supporting Information). After purification, the final hydrolysis product was identified by NMR spectroscopy, thereby confirming the formation of benzotriazole (see Figure S7 in the Supporting Information). The chemical conversion of the NAPMA monomer in the presence of NO was further characterized by FTIR spectroscopy (see Figure S8 in the Supporting Information), which showed that, after hydrolysis, absorption band corresponding to the amide group (at 1670 cm^{-1}) disappeared. Moreover, the absorption spectrum of the final hydrolysis product is relatively similar to that of the benzotriazole (see Figure S9 in the Supporting Information), further confirming our proposed mechanism (Scheme 2 a).

From our preliminary results, we thus ascertained that the NAPMA monomer could react with NO under extremely mild conditions to yield an amide-substituted benzotriazole intermediate that is sensitive to hydrolysis in an aqueous solution to form benzotriazole and residual carboxyl groups (Scheme 2 a). The APUEMA monomer could also respond to NO under the same conditions, as confirmed by NMR and FTIR spectroscopic analyses (see Figures S3 and S10 in the Supporting Information), thereby resulting in a relatively stable urea-functionalized benzotriazole unsusceptible to hydrolysis in aqueous solution (Scheme 2 b). We therefore envisaged that NO-triggered processes could be exploited to induce unique compositional transitions in macromolecular chains (incorporating NAPMA and APUEMA monomer units) for potentially manipulating polymer phase transitions and inducing self-assembly.

Consequently, we synthesized well-defined NAPMA-containing and APUEMA-labeled thermoresponsive copolymers, P(NIPAM-*co*-NAPMA) and P(NIPAM-*co*-APUEMA), through reversible addition-fragmentation chain transfer (RAFT) polymerization. The copolymers exhibited symmetrical molecular weight distributions with relatively low polydispersities (PDI < 1.1), as evident by size-exclusion chromatography (SEC) analyses (see Figure S11 in the Supporting Information). This result demonstrated that the NAPMA and APUEMA monomers can be successfully copolymerized using RAFT polymerization. The degrees of polymerization of the copolymers and molar contents of NAPMA and APUEMA were determined by ^{1}H NMR spectroscopy (see Figures S12a and S13a in the Supporting Information; the measured structural parameters of the thermoresponsive copolymers are summarized in Table S1).

Provided that there are free amine groups in the monomers, they can influence the thermoresponsive properties of the copolymers. Subsequent pH-titration experiments with the NAPMA and APUEMA monomers gave pK_a values of 3.46 and 3.36, respectively (see Figure S14 in the Supporting Information), thus confirming that the amino groups are in a neutral state at pH 7.0.

It is well-documented that the PNIPAM homopolymer has a lower critical solution temperature (LCST) at around 32 °C in aqueous solution.[13] The P(NIPAM-*co*-NAPMA) and P(NIPAM-*co*-APUEMA) copolymers also exhibited LCST

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behaviors in aqueous solution, as evident by dynamic light scattering (DLS) analyses. Typically, the copolymers formed aggregates with intensity-average hydrodynamic diameters $\langle D_h \rangle$ between 600 and 800 nm at 40 °C without NO (see Table S1 in the Supporting Information). Temperature-dependent turbidity measurements revealed that the LCST values of the P(NIPAM-*co*-NAPMA) and P(NIPAM-*co*-APUEMA) copolymers were 34 °C and 28 °C for copolymers with about 5 mol % NAPMA and APUEMA monomer, respectively, thus showing that the NAPMA and APUEMA groups have a detectable impact on of the overall phase transition behavior of the NIPAM-rich chains (see Table S1 and Figure S15 in the Supporting Information).

Subsequently, we explored the thermoresponsive behavior of the copolymers following addition of NO. The final copolymers, after NO treatment, were characterized by SEC measurements (see Figure S11 in the Supporting Information) and ^{1}H NMR spectroscopy (see Figures S12b and S13b in the Supporting Information). From the SEC traces, we discerned that after reaction with NO, the P(NIPAM-*co*-NAPMA) copolymers exhibited slightly decreased elution times while P(NIPAM-*co*-APUEMA) copolymers had slightly increased elution times, which is indicative of compositional changes (see Figure S11 in the Supporting Information). A successful reaction of NAPMA with NO was confirmed by the absence of the NMR signals between 7–8 ppm assigned to the phenyl rings of the NAPMA moieties (see Figure S12b in the Supporting Information). Although the P(NIPAM-*co*-APUEMA) copolymers do not dissolve in D_2O at room temperature after reaction with NO, the ^{1}H NMR spectrum in [D_6]DMSO revealed that the protons ascribed to the phenyl ring of APUEMA moieties were shifted downfield, in accord with the monomer transition after reaction with NO (see Figure S3 in the Supporting Information). Our results confirmed that the copolymerization of NAPMA and APUEMA monomers with NIPAM did not compromise (or affect) the NO-responsive behavior.

The mechanisms of the reaction of the NAPMA and APUEMA moieties with NO are given in Scheme 2, where thermoresponsive P(NIPAM-*co*-NAPMA) copolymers transform to P(NIPAM-*co*-MAA) copolymers following treatment with NO, while the P(NIPAM-*co*-APUEMA) copolymers form urea-functionalized benzotriazole moieties with long-term stability in aqueous solution. The measured LCST of P(NIPAM-*co*-NAPMA) copolymers with 5 mol % NAPMA, after reaction with NO, was determined to be 38 °C at pH 7, while that of P(NIPAM-*co*-APUEMA) copolymers with 5 mol % APUEMA drops to 7.5 °C. The increased LCST values of P(NIPAM-*co*-NAPMA) are in accord with the expected reaction mechanism following formation of negatively charged carboxyl groups at pH 7.0, which results in increased LCST values (Scheme 2a). We speculate that the phenylamine moieties of the APUEMA monomer units transform to hydrophobic urea-derived benzotriazole motifs, thereby leading to decreased LCST values (Scheme 2b). Moreover, we find that the LCST changes of the copolymers are highly dependent on the NO concentration. Typically, the LCST values of P(NIPAM-*co*-NAPMA) gradually increased with increasing NO concentrations, while the LCST values for the P(NIPAM-*co*-APUEMA) copolymers, in contrast, decreased upon the gradual addition of NO (see Figure S15 in the Supporting Information).

The P(NIPAM-*co*-APUEMA) copolymer could react with NO thereby decreasing the LCST and presumably resulting in the formation of aggregates. The sizes of the P(NIPAM-*co*-APUEMA) copolymers, after reaction with NO, were found by DLS to have an intensity-averaged hydrodynamic diameter $\langle D_h \rangle$ of about 37.8 nm at 20 °C (Figure 3a), while TEM showed the formation of spherical micelles (Figure 3b). These results suggest that the self-assembly of the P(NIPAM-*co*-APUEMA) copolymer could be efficiently regulated by exposure to NO. It is intriguing that we may be able to manipulate the self-assembly behavior of the copolymers by using a relatively low NO concentration that falls in the biological/physiological concentration range. Subsequently, the self-assembly behavior of P(NIPAM-*co*-APUEMA) copolymer in the presence of a low NO concentration was examined. To eliminate any possible side reactions between the RAFT terminal group and NO, which would potentially consume NO (see Figure S16 in the Supporting Information), the RAFT terminal group was removed in the presence of an excess of azoisobutyronitrile (AIBN). The P(NIPAM-*co*-APUEMA) copolymer exhibited an extremely low scattering intensity and small diameter (i.e. 5.9 nm) at 20 °C, which suggests that the copolymer was in a unimer state without NO (Figure 3c,d), in line with the temperature-dependent changes in turbidity (see Figure S15 in the Supporting Information). However, aggregates with prominently increased scattering intensities and diameters were observed upon gradual addition of NO. Specifically, in the presence of 0–40 μM NO, the diameters increased from 5.9 nm (unimers) to 38 nm (micelles). Meanwhile, the corresponding scattering intensity exhibited a cumulative 10.4-fold

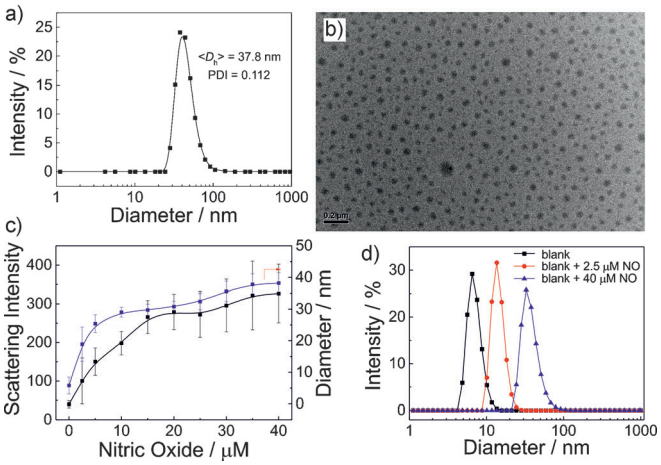

Figure 3. a) Typical DLS profile and b) transition electron microscopy (TEM) image of an aqueous solution (0.05 g L^{-1}) of P(NIPAM-*co*-APUEMA) ([APUEMA] = 20 μM) after treatment with an excess of NO at 20 °C. c) Scattering intensity and diameter changes in an aqueous solution (0.05 g L^{-1}) of P(NIPAM-*co*-APUEMA) upon addition of various amounts of NO at 20 °C. d) Changes in the size of P(NIPAM-*co*-APUEMA) in aqueous solution (0.05 g L^{-1}) in the presence of 0, 2.5, and 40 μM NO.

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increase (Figure 3c). Intriguingly, the minimally detectable size change by DLS was determined to be approximately 2.5 μM NO, which resulted in the formation of micellar aggregates with a diameter 18 nm (Figure 3d) and implying the copolymer could also be employed to detect NO by inducing assembly.

To further confirm the NO-triggered self-assembly process, we subsequently introduced a polarity-sensitive fluorescent dye (4-(2-methylacryloyloxyethylamino)-7-nitro-2,1,3-benzoxadiazole, NBD) to the P(NIPAM-*co*-APUEMA) copolymers to afford P(NIPAM-*co*-APUEMA-*co*-NBD). The fluorescence of the P(NIPAM-*co*-APUEMA-*co*-NBD) copolymer was very weak at 20 °C, which could be ascribed to two synergistic effects: 1) the NBD dye being located in a hydrophilic environment as the copolymer was in a unimer state; 2) the deprotonated APUEMA poses as a fluorescence quencher through a PET mechanism. In contrast, the copolymer exhibited a drastic increase in fluorescence upon addition of NO, leveling off in the presence of 30 μM NO, but exhibiting a cumulative 5.2-fold increase. This was accompanied by macroscopic turbidity (see inset in Figure 4a) and scattering intensity changes (see Figure S17 in the Supporting Information), which suggests the formation of aggregates. In contrast, the fluorescence of P(NIPAM-*co*-NBD) without APUEMA moieties displayed negligible changes at the same NO concentration, thereby indicating an inert fluorescence property of NBD against NO. The above results confirmed that the NO-triggered self-assembly yielded decreased environmental polarity, thereby resulting in an enhanced fluorescence of the NBD moieties (Figure 4a,b).[6b,14]

Considering the temperature-responsive nature of the P(NIPAM-*co*-APUEMA-*co*-NBD) copolymer, we further examined the temperature-dependent fluorescence changes. Upon gradual addition of NO, the fluorescence intensity underwent a 7.1-fold increase at 37 °C at the same NO concentration (see Figure S18 in the Supporting Information). The further increased fluorescence, compared to the fluorescence at 20 °C, could be ascribed to the thermoinduced collapse of NIPAM chains, thus leading to a further decreased environmental polarity that improved the quantum yield of NBD. This result is significant, as it indicates that NO-responsive copolymers could be employed as temperature sensors.[6b,14]

Finally, the performance of the NO-responsive copolymer in living cells was assessed. The P(NIPAM-*co*-APUEMA-*co*-NBD) copolymer was nontoxic even at concentrations of 1 mg mL^{-1}, as confirmed by assessment of the in vitro cytotoxicity against the MRC-5 cell line (see Figure S19 in the Supporting Information). After internalization by MRC-5, it is intriguing that the initially quenched NBD fluorescence could now be observed. This enhanced fluorescence is likely due to the scavenging of APUEMA moieties by endogenous NO, thereby resulting in the detection of fluorescence inside the MRC-5 cells (Figure 4).

In conclusion, novel NO-responsive monomers, NAPMA and APUEMA bearing *o*-phenylenediamine groups, were synthesized and found to react effectively with NO under extremely mild conditions to generate amide-substituted benzotriazole intermediates and urea-functionalized benzotriazole derivatives, respectively. Moreover, the amide-substituted benzotriazole intermediate could be spontaneously hydrolyzed in aqueous solution to yield carboxyl moieties with the liberation of a benzotriazole motif, while the resultant urea-functionalized benzotriazole derivates proved relatively stable. The LCST values of the thermoresponsive copolymers P(NIPAM-*co*-NAPMA) and P(NIPAM-*co*-APUEMA) could be significantly regulated by exposure to NO stimuli, with drastic increases and decreases in the LCST value, respectively. The self-assembly of P(NIPAM-*co*-APUEMA) could be delicately regulated at extremely low NO concentrations by taking advantage of the NO-triggered formation of a hydrophobic motif. Our preliminary intracellular results revealed that the NO-responsive copolymer is noncytotoxic and could respond to endogenous NO concentrations through fluorescence changes. This study, for the first time, demonstrates the manipulation of thermoresponsive copolymers using a biological signal molecule (NO) to induce significant LCST changes, which leads to self-assembly and fluorescence changes. Further studies of structural parameters that control the size and shape of self-assembled aggregates and the exploration of potential applications, such as NO-triggered release and in vivo imaging, are currently underway.

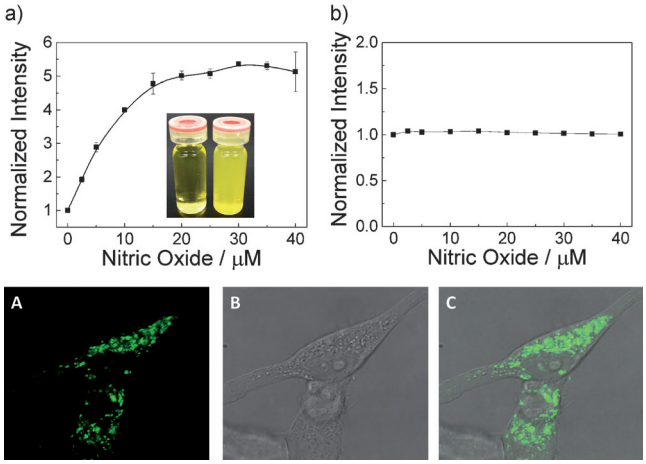

Figure 4. Changes in the fluorescence intensity (λ_{ex} = 470 nm, λ_{em} = 530 nm) of aqueous solutions (0.05 g L^{-1}) of a) P(NIPAM-*co*-APUEMA-*co*-NBD) and b) P(NIPAM-*co*-NBD) at pH 7.0 with various amounts of NO at 20 °C. The inset macroscopic image in (a) shows the aqueous solution of P(NIPAM-*co*-APUEMA-*co*-NBD) (1.0 g L^{-1}) in the absence (left) and presence (right) of NO at 20 °C. Confocal laser scanning microscopy images (bottom) after incubation of P(NIPAM-*co*-APUEMA-*co*-NBD) with MRC-5 cells for 5 h (A: fluorescent channel, B: bright-field channel, C: merged bright-field and fluorescent channel).

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