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Micelles for Delivery of Nitric Oxide

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Abstract: We designed block copolymer pro-amphiphiles and amphiphiles for providing very long-term release of nitric oxide (NO). A block copolymer of *N*-acryloylmorpholine (AM, as a hydrophile) and *N*-acryloyl-2,5-dimethylpiperazine (AZd, as a hydrophilic precursor) was synthesized. The poly(*N*-acryloyl-2,5-dimethylpiperazine) (PAZd) is water-soluble, but chemical reaction of the secondary amines with NO to form a *N*-diazeniumdiolate (NONOate) converts the hydrophilic PAZd into a hydrophobic poly(sodium-1-(*N*-acryloyl-2,5-dimethylpiperazin-1-yl)diazen-1-ium-1,2-diolate) (PAZd·NONOate), driving aggregation. The PAM block guides this process toward micellization, rather than precipitation, yielding ca. 50 nm spherical micelles. The hydrophobic core of the micelle shielded the NONOate from the presence of water, and thus protons, which are required for NO liberation, delaying release to a remarkable 7 d half-life. Release of the NO returned the original soluble polymer. The very small NO-loaded micelles were able to penetrate complex tissue structures, such as the arterial media, opening up a number of tissue targets to NO-based therapy.

1. Introduction

Nitric oxide (NO), the molecule of the year in 1992, is terribly interesting in the context of a number of cardiovascular and other tissue responses,[1,2] yet its therapeutic use is complicated by its short lifetime, motivating researchers to develop NO donors as pro-drugs, including *N*-diazeniumdiolates (NONOates) formed by chemical reaction of NO with a secondary amine, hereafter termed as NONOation. In spite of pioneering work of investigators such as Keefer and colleagues on development of long-acting NONOates,[3−5] the impact of NONOates has been hampered by their rapid release characteristics. In the context of cardiovascular medicine, coronary arterial atherosclerosis is closely related to endothelial dysfunction and pathophysiologically altered vascular homeostasis.[6,7] Interventionally, percutaneous transluminal coronary angioplasty (PTCA) is performed to restore blood flow in occluded lesions but is hampered by post-PTCA restenosis due to the mechanical stimuli of dilation and interactions with the stent.[8,9] To prevent post-PTCA restenosis, much focus has been placed on local drug delivery with inhibitors of cell migration and proliferation.[10,11] Endogenous NO generated from endothelial nitric oxide synthase (eNOS) physiologically induces endothelial-dependent relaxation of blood vessels and modulates the tone of arterial vascular smooth muscle cells (VSMCs).[12,13] Thus, NO-based drugs could be interesting in prevention of post-PTCA restenosis.

NO is difficult to deliver directly, so it is frequently administered as a prodrug, referred to as a NO donor; yet most NO donors are decomposed much too fast to be useful as an antirestenotic drug, which may require NO release over a few weeks after deployment of the stent.[11,14] Furthermore, the lifetime of NO in tissues is a mere 4−15 s, corresponding to a diffusion distance of approximately 150−500 μm, rendering stent-based delivery to the thick arterial media problematic.[15,16] Taking these requirements into consideration, we conceived NO-releasing micelles, which (1) release NO at a sufficiently slow rate, (2) are sufficiently small (e.g., less than 100 nm) to penetrate tissues under mild pressure, and (3) are self-assembled

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structures that can be eventually dissembled after all NO has been delivered.

2. Experimental Section

2.1. Syntheses. Monomers were synthesized as described elsewhere[17,18] with modifications. Homo- and copolymerization were carried out as described elsewhere.[17]

2.2. *In situ* Micelle Formation (Micellization). PAM-PAZd was dissolved in Milli-Q water with adequate amount of base. After degassing with Ar, NO was pressurized to 80−150 psi in an autoclave. This reaction was continued for 5 d. Micelles were dialyzed for 2 h and lyophilized or frozen and kept at −20 C° until used.

2.3. NO Analysis. At 25 °C, dissociation of NONOate in PBS (10 mM, pH 7.4) was monitored by UV spectrometry at 250 nm. At 37 °C, NO radicals generated from poly(NONOate)s in PBS (10 mM, pH 7.4) were recorded by a NO analyzer.

2.4. Ex vivo Infusion of Micelles in the Rabbit Carotid Artery. Carotid arteries from male New Zealand white rabbits weighing 3 to 3.5 kg were obtained from a local slaughterhouse immediately upon sacrifice. Vessels were stored in a PBS solution and put on ice for transport. Excess tissue and adventitia were removed, and a 2 cm-long arterial segment was mounted on 2.5 mm diameter cannula. The arteries were then stretched longitudinally to their *in vivo* length, submerged in a Krebs buffer solution and kept at 37 °C for 1 h. Following this, a Krebs solution filling the artery was replaced by approximately 1 mL of fluorescent-labeled micelle solution. A Millar Mikro-Tip Catheter Transducer was inserted through one cannula while a 20 mL syringe full of air was attached to the other cannula. Experimental conditions were achieved by depressing the plunger of the syringe and fixing the transmural pressure at 1 atm (for 1 min) or repeatedly depressing and releasing the plunger of the syringe, creating a pulsating pressure ranging from ambient to 1 atm every 10 s (×10). After the experiment the artery was rinsed for one minute in Krebs solution, fixed in tissue freezing medium (Tissure-Tek O.C.T.) and kept at −20 °C.

3. Results and Discussion

We first hypothesized that the hydrophobic microenvironment within a micelle core can protect a reservoir of NONOate from protons diffusing from the surroundings and thus delay proton-catalyzed NO liberation. To form a self-assembled core−shell structure, a pro-amphiphilic diblock copolymer was designed. Reversible addition−fragmentation transfer (RAFT) polymerization, a living radical polymerization, was employed to synthesize well-defined block copolymers. As reported earlier in our group,[17] various analogues of acrylamides with cyclic secondary amine side chains have been successfully homo- and copolymerized using RAFT polymerization with excellent control and low polydispersity. We sought to form a hydrophobic poly(*N*-diazeniumdiolate) (poly(NONOate)) by reacting NO with precursors; poly(*N*-acryloylpiperazine) (PAZ) or poly(*N*-acryloylhomopiperazine) (PAZh) or poly(*N*-acryloyl-2,5-dimethylpiperazine) (PAZd) block, which are all water-soluble but yielding an insoluble poly(NONOate)s.

Homopolymeric poly(NONOate)s were synthesized to determine (in)solubility, and then an amphiphilic diblock copolymer containing poly(NONOate) was synthesized with poly(*N*-acryloylmorpholine) (PAM) as a hydrophilic block. The PAM block serves to obtain micellization rather than precipitation as the hydrophilic precursor is reacted with NO to form the hydrophobic poly(NONOate). Three poly(NONOate)s were prepared from monomers in the family of piperazine (a 6-member ring) with different number of carbons and/or substituents on the cyclic ring (see Scheme S1−2 in the Supporting Information). The average conversion of amine groups into NONOate groups was determined 71% by Griess assay. The poly(NONOate) was then derived from the precursor by reaction with NO (see Scheme S4 in the Supporting Information); *tert*-Butoxycarbonyl (Boc) chemistry was used to protect the secondary amine-containing monomer during RAFT polymerization, which was later deprotected to restore the secondary amine groups in the polymer. From solubility tests, both poly(sodium 1-(*N*-acryloylpiperazin-1-yl)diazen-1-ium-1,2-diolate) (PAZ·NONOate, actual NO payload: 6.4 μmol/mg) and poly(sodium 1-(*N*-acryloylhomopiperazin-1-yl)diazen-1-ium-1,2-diolate) (PAZh·NONOate, actual NO payload: 6.0 μmol/mg) (see Chart 1 for structures) were soluble in water, yet insoluble in any other organic solvent (see Table S1a-b in the Supporting Information). Therefore, neither of PAZ·NONOate nor PAZh·NONOate was considered as a suitable candidate for the hydrophobe of self-assembled aggregates after NONOation. In contrast, poly(sodium 1-(*N*-acryloyl-2,5-dimethylpiperazin-1-yl)diazen-1-ium-1,2-diolate) (PAZd·NONOate) was insoluble in water in distinction from its water-soluble precursor, PAZd (see Table S1c in the Supporting Information). Thus, PAZd, selected as the pro-hydrophobic block NO acceptor, was copolymerized with PAM (see Scheme S3 in the Supporting Information). The soluble poly[(*N*-acryloylmorpholine)-*block*-(*N*-acryloyl-2,5-dimethylpiperazine)] (PAM-PAZd) diblock copolymer as prepared by deprotecting the precursor, i.e. poly[(*N*-acryloylmorpholine)-*block*-(1-Boc-4-acryloyl-2,5-dimethylpiperazine)] (PAM-BocPAZd), was reacted with NO in deoxygenated water under a pressurized NO supply and in the presence of base (see Scheme S5 in the Supporting Information). As more amine groups of PAZd are gradually converted into NONOate groups, the PAZd·NONOate block segregates more from the PAM block. In this way, *in situ* formation of aggregates of poly[(*N*-acryloylmorpholine)-*block*-(sodium 1-(*N*-acryloyl-2,5-dimethylpiperazin-1-yl)diazen-1-ium-1,2-diolate)] (PAM-PAZd·NONOate) is affected in aqueous media (Figure 1a). As a result, PAM-PAZd·NONOate was self-assembled into micelles, as shown in transmission electron microscopy (TEM) images with 2% negative staining using sodium phosphotungstate (Figure 2).

The morphology of the formed micelles depends on the block length ratio between the two polymer blocks, higher hydrophobic block ratios giving bigger hydrodynamic diameters than lower ones. Longer hydrophilic blocks yielded longer worm-like micelles: PAM_{146}-PAZd·$NONOate_{57}$ being ca. 110 nm (see Figure S9a in the Supporting Information) whereas PAM_{146}-PAZd·$NONOate_{23}$ being ca. 80 nm (see Figure S9b in the Supporting Information). Interestingly, despite the copolymer comprising a smaller fraction of hydrophobe, PAZd·$NONOate_{23}$, than hydrophile, PAM_{146}, spherical micelles were not the dominant in morphology, but rather worm-like micelles. However, with an excess amount of base, ca. 10−20 times, PAM_{142}-PAZd·$NONOate_{23}$ tended to form spherical micelles (Figure 2) with approximately 50 nm average diameter, as confirmed by dynamic light scattering (DLS) and TEM. The critical micellar concentration (cmc) of the PAM_{142}-PAZd·$NONOate_{23}$ spherical micelles was determined to be 2.2×10^{-4} M by the pyrene

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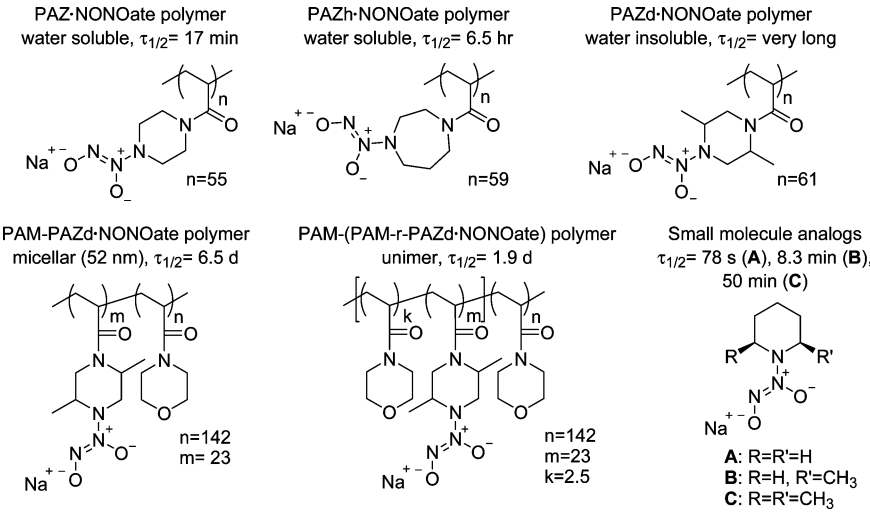
Chart 1. Structures of NO Donors Explored in This Work[a]



[a] The homopolymers PAZ·NONOate and PAZh·NONOate were both soluble in water and released NO relatively quickly, with the more hydrophobic PAZh derivative releasing more slowly than the more hydrophilic PAZ derivative. The homopolymer PAZd·NONOate was insoluble in water, and the precipitates released NO too slowly to accurately measure. The amphiphilic block copolymer PAM_{142}-$PAZd{\cdot}NONOate_{23}$ formed 52 nm spherical micelles, the hydrophobic core of which protected the NONOate domains from water and substantially delayed NO liberation. Disrupting micelle formation by copolymerization of small quantities of the hydrophile PAM in the hydrophobic PAZd block eliminated this effect and resulted in more rapid NO liberation. Small molecule analogs are shown for comparison.

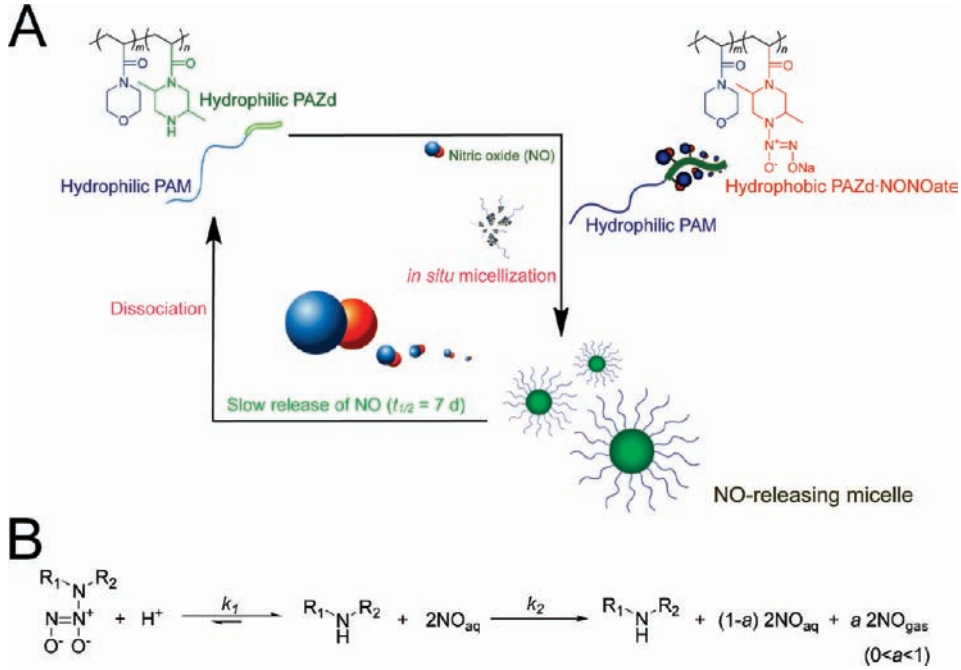


Figure 1. *In situ* formation of PAM-PAZd·NONOate micelles under pressurized nitric oxide (NO) supply. (A) PAM-PAZd·NONOate micelles formed when NO reaction on the water-soluble PAZd domain yielded the water-insoluble PAZd·NONOate. Restricted access of protons, needed for NO liberation, to the micelle core yielded delayed NO release, to around 1 wk half-life under physiological conditions. (B) Dissociation of diazen-1-ium-1,2-diolate (NONOate) and release of NO. NO analyzer detects the level of NO_{aq}.

method.[17] The average size and size distribution of these micelles did not change after rehydrating lyophilized micelles (see Figure S11 in the Supporting Information). It may be that steric hindrance as the polymer chains adjust their conformations as the degree of diazeniumdiolation increases is the reason that NONOation may not be completed to 100% conversion. Thermogravimetric analysis (TGA) demonstrated 84% conversion, leading to a total NO payload of 1.5 μmol/mg of micelle (theoretical NO loading capacity: 1.8 μmol/mg, see Figure S12 in the Supporting Information). Thus, the worm-like morphology dominant in PAM_{146}-$PAZd{\cdot}NONOate_{23}$ could be a continuum toward spherical micelles, PAM_{142}-$PAZd{\cdot}NONOate_{23}$. The size of PAM_{142}-$PAZd{\cdot}NONOate_{23}$ is almost the same as that of

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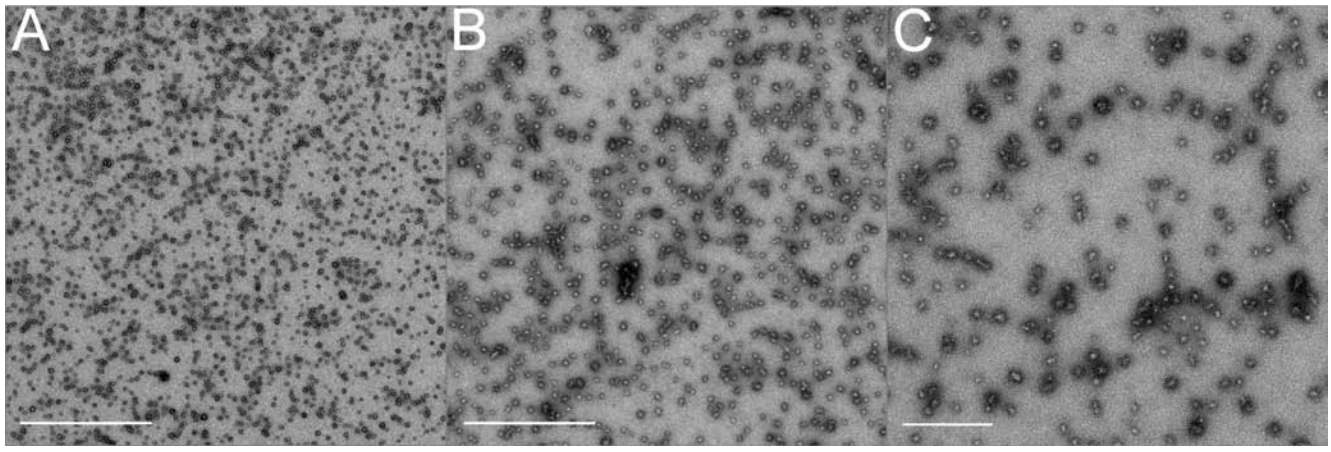

Figure 2. Spherical PAM_{142}-PAZd·$NONOate_{23}$ micelles are formed *in situ* with hydrodynamic diameter of ca. 50 nm in average: TEM images with different magnifications; (A) 10 k (Scale bar represents 1 μm), (B) 20.5 k (Scale bar represents 500 nm), and (C) 33 k (Scale bar represents 200 nm). Over time in the electron beam, NONOate degradation and evolution of bubbles were observed (not shown). Subscripts to PAM-PAZd·NONOate denote the degree of polymerization.

micelles prepared from the precursor, poly[(*N*-acryloylmorpholine)-*block*-(1-Boc-4-acryloyl-2,5-dimethylpiperazine)] (PAM_{142}-$BocPAZ_{23}$) (i.e., without deprotection of the secondary amine) (average diameter: 47 ± 1.1 nm by DLS).

According to the chemical equation in Figure 1b, the release kinetics of NO can be formulated in eqs 1−3 as reported eariler[19] with proper modification of the coefficients.

$$[\mathrm{A}] = A_0 e^{-k_1 t} \tag{1}$$

$$[\mathrm{B}] = \frac{2A_0 k_1}{k_2 - k_1}[e^{-k_1 t} - e^{-k_2 t}] \tag{2}$$

$$[\mathrm{C}] = \frac{2A_0}{k_2 - k_1}[k_1(e^{-k_2 t} - 1) - k_2(e^{-k_1 t} - 1)] \tag{3}$$

where [A] is the concentration of poly(NONOate), [B] is the concentration of NO_{aq}, [C] is the concentration of NO_{gas}, and k_1 and k_2 are the kinetic constants. A_0 is the initial concentration of poly(NONOate).

UV spectra of water-soluble PAZ·NONOate and PAZh·NONOate show distinct maxima at a wavelength 250 nm, which is typical for NONOate groups.[20] As shown in Figure 3a, the maximum at 250 nm decreases over time, which is common for water-soluble poly(NONOate)s, suggesting the NONOate groups are decomposed over time in phosphate-buffered saline (PBS; 10 mM, pH 7.4).

Interestingly, PAZh·NONOate is decomposed more slowly than PAZ·NONOate, as observed from the change of the UV spectrum. Moreover, analogous to PAZh·NONOate, another new peak at 330 nm evolves over time, which is attributed to *N*-nitroso-compound formation. To more precisely evaluate the release kinetics of NO under physiological conditions, the NO generated from lyophilized polymers was monitored in PBS (10 mM, pH 7.4) using an NO analyzer over the entire release period (Figure 3b). According to eqs 1 and 2, the half-life ($t_{1/2}$) of NO_{aq} generated from poly(NONOate) can be calculated from the kinetic constant (k_1). As summarized in Chart 1, PAZ·NONOate shows a half-life of 17 min, longer than reported for a monomeric piperazine-based NONOate ($t_{1/2}$ = 5 min).[19] The longer release pattern that we observed with the soluble poly(NONOate)s is probably due to a more structurally hindered conformation of the NONOate groups grafted in the polymer chains. Notably, there is also a remarkable difference in the half-life of NO release between PAZ·NONOate and PAZh·NONOate. At 37 °C, NO release from PAZh·NONOate is far slower than from PAZ·NONOate, i.e. 6.5 h (Chart 1), suggesting that the addition of one more carbon in the 7-membered ring affects the release rate of NO. This can also be explained by the structural difference influencing the hydrophobicity of the surrounding NONOate groups, slowing the proton transfer rate to NONOates due to the presence of the additional carbon in the 7-membered rings.

While we learned from NO release from the soluble poly(NONOate)s, our greater interest was in NO release from PAM-PAZd·NONOate micelles. Spherical micelles, namely PAM_{142}-PAZd·$NONOate_{23}$ release NO strikingly slower than PAZ·NONOate or PAZh·NONOate; releasing NO over 3 weeks (Figure 3 and Chart 1). Is this the effect of the core−shell structure according to our design, or is it due to some other reason? We examined the release pattern of PAZd·NONOate homopolymer first, which was revealed to be extremely stable under physiological conditions. This suggests that the poor water solubility of PAZd·NONOate does not allow aqueous coreactants, here protons, to penetrate through the bulky (precipitate) particles of PAZd·NONOate.

To confirm the mechanism of prolonged NO liberation from the micelles, we disrupted the micelle core by synthesizing a terpolymer with PAM as one block and a randomly copolymerized (less) hydrophobic block, composed of PAM and PAZd·NONOate: poly[(*N*-acryloylmorpholine)-*block*-((*N*-acryloylmorpholine)-*ran*-(sodium 1-(*N*-acryloyl-2,5-dimethylpiperazin-1-yl)diazen-1-ium-1,2-diolate))] (PAM_{142}-*bl*-($PAM_{2.5}$-*r*-PAZd·$NONOate_{23}$)). Because the hydrophobic core of diblock terpolymer contains more hydrophilic constituent, that is, PAM, the terpolymer became unstable in assembly as micelles so as to be in unimolecular state, with the diffusion rate of protons to the NONOate moieties in the core or in the unimers being higher than in the diblock copolymer with the (more) hydrophobic core. Consistent with our hypothesis and design, the terpolymer showed distinctively faster release than PAM_{142}-PAZd·$NONOate_{23}$ micelles, ca. 1.9 d half-life (Figure 3 and Chart 1).

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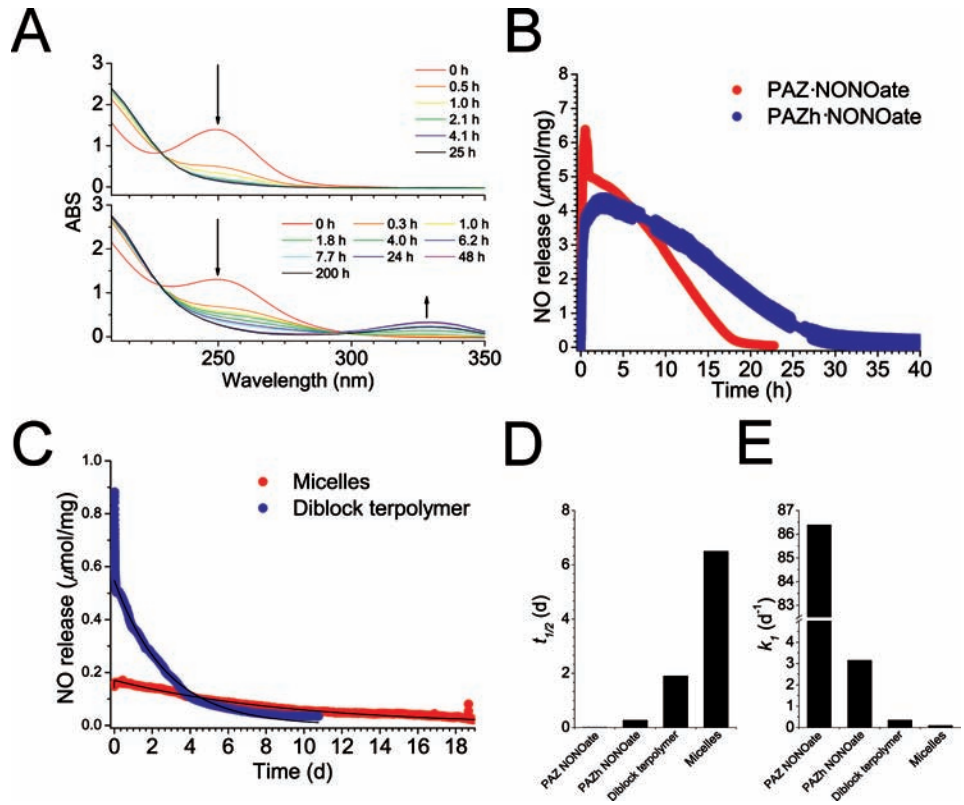

Figure 3. Micelles release nitric oxide (NO) far slower than homopolymers and/or diblock terpolymer: (A) UV absorbance profile over time for PAZ·NONOate (top) and PAZh·NONOate (bottom). Arrows indicate the evolution of the profile over time. (B) NO release profiles from PAZ·NONOate (red circle) and PAZh·NONOate (blue circle). (C) NO release profiles from PAM_{142}-PAZd·$NONOate_{23}$ micelles (red circle) and PAM_{142}-*bl*-($PAM_{2.5}$-*r*-PAZd·$NONOate_{23}$) terpolymer (blue circle), which is designed to break micellization. Black lines are fitted curves according to eq 2. (D) Half-lives and (E) kinetic constants ($k1$) of dissociation of NONOate groups of homopolymers (PAZ·NONOate and PAZh·NONOate), PAM_{142}-PAZd·$NONOate_{23}$ micelles, and PAM_{142}-*bl*-($PAM_{2.5}$-*r*-PAZd·$NONOate_{23}$) diblock terpolymer. Results from part (B) to part (D) were recorded on NO analyzer at 37 °C in 10 mM PBS (pH 7.4).

Therefore, our working hypothesis that the core−shell micellar structure protects NO-bound hydrophobic moieties in the core seems to be supported, demonstrating that micellization of a pro-micelle (i.e., a fully soluble AB block copolymer that becomes an amphiphilic AB* block copolymer, B* being the NONOate) by NONOation can strikingly slow NO release from the NO prodrug micelle.

Finally, we examined how micelles can be distributed in a dense tissue such as the arterial media by *ex vivo* infusion into a rabbit carotid artery under mild pressure. Freshly harvested rabbit carotid arteries were used without injury to the endothelium. With respect to the size effect in arterial infusion, Westedt et al. showed that particles with around 100 nm of diameter can penetrate the intima and remain within the media, while micrometer-sized beads are too large to penetrate the intima and media.[21,22] Taking worm-like PAM_{146}-PAZd·$NONOate_{23}$ micelles (ca. 80 nm of diameter by DLS) as the more challenging case, bigger than PAM_{142}-PAZd·$NONOate_{23}$ spherical micelles, we explored two conditions for *ex vivo* infusion: static infusion for 1 min and pulsed infusion for 10 s durations. The transmural pressure was maintained at 1 atm in both cases. Comparing the static infusion to the pulsed infusion, as seen in Figure 4, the polymer micelles were readily delivered into the media in both cases, with more being delivered with the pulsed protocol.[22]

So far, a number of interesting materials designs have been presented to extent the half-life of NO-generating materials, including metal organic frameworks,[23,24] zeolites,[25] poly(ethylene imine) (PEI)-based microspheres,[26] poly(methylmethacrylate) (PMMA)-based microbeads,[27] and *N*-diazeniumdiolate (NONOate)-modified silica microparticles[28] and gold nanoparticles[29] have been presented. Despite such successful examples in the aforementioned works, the size of polymeric particles is mostly in a micrometer-regime and/or the release rate of NO is rather fast. There are examples of NO-releasing materials which do so over the course of relatively long time spans, especially in hydrogel forms,[30] for example based on primary amine complexes; it is, however, well-known that primary amines form very unstable NONOates upon reaction with NO gas.[31]

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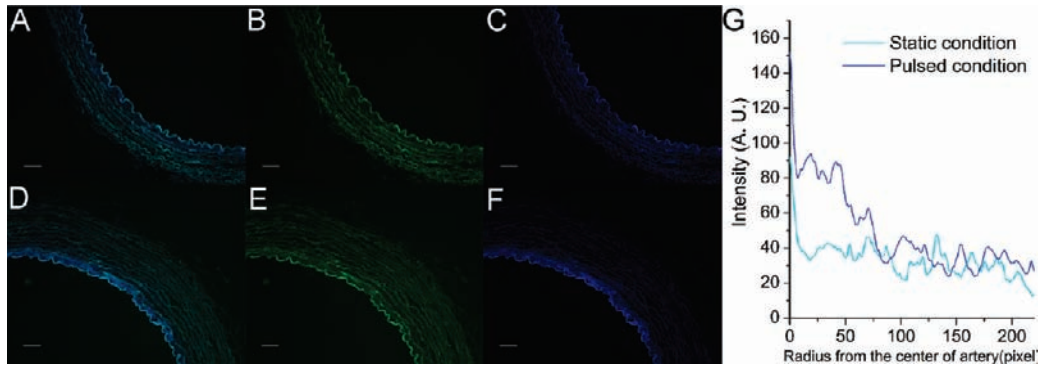

Figure 4. PAM_{146}-PAZd·$NONOate_{23}$ micelles were infused ex vivo and shown to penetrate the arterial intima and media of rabbit carotid artery. Fluorescence microscopy images from part (A) to part (C) represent static infusion and part (D) to part (F) represent pulsed infusion. Part (B) and part (D): autofluorescent elastin in the artery, part (C) and part (E): micelles, and part (A) and part (C): merged. Graph in part (G) represents the distribution of micelles across the artery. Scale bar represents 50 μm. The intensity values are an average of 4 different places from the images and calculated by MetaMorph software.

4. Conclusion

We present a design based on polymer chemistry and physicochemical response in which NONOation drives micellization of a soluble diblock copolymer comprising a domain of precursor of poly(NONOate). Careful selection of the solubility of the precursor of poly(NONOate)s allowed it to be soluble in the native state but insoluble in water as the poly(NONOate), this difference driving micellization and the resulting hydrophobic core restricting access to the protons that are required for NO generation. The slowed influx of protons provides a slowed liberation of NO accompanied by solubilization of the micelle to the native, soluble polymer. We emphasize the need for either soluble or nanoscopic NO delivery forms, since the NO after liberation from the NONOate is extremely unstable and thus only locally active; nanoparticulate structures distributed throughout a tissue structure are particularly attractive, as contrasted with an NO-containing structure atop a tissue surface. While we demonstrate herein only that distribution throughout the arterial media is possible in the context of post-PTCA restenosis prevention, other applications such as for accelerating wound-healing[30] or preventing post-operational abdominal adhesions[32] could be envisioned. It is interesting also to consider dosing of NO depending on the applications. Based on the rate of NO production from the normal healthy endothelium, an NO flux on the order of 10^{-10} to 10^{-15} μmol cm^{-2} min^{-1} would represent a reasonable target for cardiovascular applications.[16,33,34] From a reasonable concentration of the PAM_{142}-PAZd·$NONOate_{23}$ micelles (e.g., 2.2 $\times$ 10^{-3} M, 10 times higher than the cmc) and our measured NO payload (i.e., 1.5 μmol/mg), we calculate an NO flux as 7.9×10^{-13} μmol cm^{-2} min^{-1} from the PAM_{142}-PAZd·$NONOate_{23}$ micelles,[35] which is in a range of aforementioned endogenous NO flux. Thus, the PAM_{142}-PAZd·$NONOate_{23}$ micelles would appear to release physiologically relevant level of NO when administered. In cancer, NO may play an important role in apoptosis,[36] and dysregulation of NO production and elimination has been observed to correlate with malignancy and invasion.[37,38] In such applications, a higher, more sudden dose may be desirable.[39]

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Supporting Information Available: Information is available on materials used in this work, full methods, and characterization results with associated references. This material is available free of charge via the Internet at http://pubs.acs.org.

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