Organic Nitrate Functional Nanoparticles for the Glutathione-Triggered Slow-Release of Nitric Oxide

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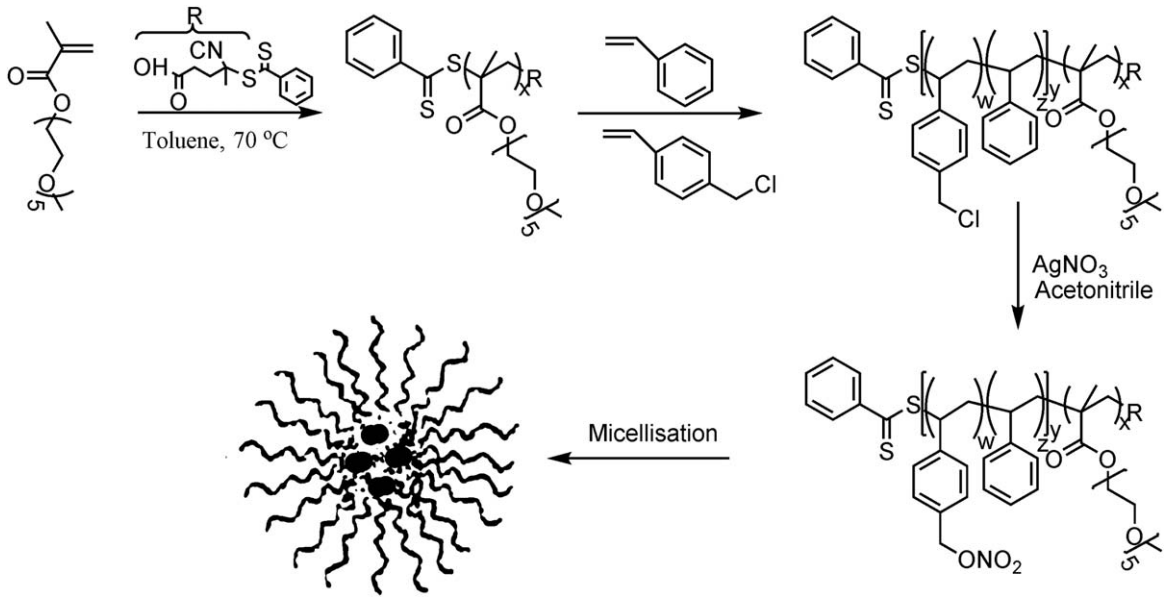
INTRODUCTION In 1992, nitric oxide (NO) was proclaimed "Molecule of the Year" by the journal Science as endogenous NO plays an important role in many bioregulatory systems, including smooth muscle relaxation, platelet inhibition, neurotransmission, and immune stimulation.[1–3] NO is an important cellular signaling molecule and deficiency has been implicated in a number of diseases, including diabetes,[4] liver fibrosis,[5] cardiovascular illness,[6] neurodegenerative diseases,[7] and several cancers.[8] The controlled exogenous delivery of NO to biological systems is challenging as NO gas has only limited solubility in water (2–3 mM) and is extremely reactive with oxygen (and other gases) resulting in a short half-life time in the body (0.1–5 s).[9] Thus, the delivery of NO using a compound that can release NO (NO donor) under defined conditions is expected to have significant application. An ideal NO donor would: (i) be stable for an extended period of time, (ii) nontoxic and noninflammatory, and (iii) release NO at a pharmacologically relevant dose over a defined timespan to exert biological efficacy.

Several small-molecules suitable for NO release have been reported. Organic nitrates, S-nitrosothiols (RSNOs), and *N*-diazeniumdiolates (NONOates) are the three most important classes of NO donors. Unfortunately, the clinical utility of small-molecular NO-donors has been limited as they are deficient in both stability and specificity. For example, NONOate compounds have a half-life of just a few minutes at 25°C.[9] In addition, a major concern is that prodrug NO-donors are nonselective leading to a high risk of systemic side effects. In recent work, NONOate was successfully conjugated to a range of polymeric systems, including a matrix of ethylene/vinylacetate,[10] star polymers,[11] and micelles.[11–14] We previously reported on micellar conjugates of S-nitrosoglutathione for controlled intracellular delivery of NO in combination with chemotherapy to treat neuroblastoma cells.[15]

In this present study, we focused on the organic nitrate class of NO donors, which do not spontaneously release NO but require enzymatic or chemical activation/degradation using enzymes, acids, alkalis, metal ions, or thiols.[9] Together with NONOates and RSNOs, organic nitrates are an important class of NO donors. Glyceryl trinitrate has been used for over a century to treat angina, and sodium nitroprusside is used intravenously in cases of acute hypertensive emergency. However, there are several limitations with the use of nitrate-based NO donors or sodium nitroprusside when administered for prolonged periods with associated cardiac and cancer risks.[9] Organic nitrates increase the efficiency of cytostatic therapy retarding the development of drug resistance.[16] Kodela et al. attached nitrate and H_2S releasing groups to aspirin forming a scaffold that inhibited the growth of several types of cancer such as colon, breast, pancreas, lung, prostate, and T-cell leukemia.[17] This article describes, for the first time, the synthesis of polymers containing nitrate groups for the controlled release of NO under specific conditions.

Additional Supporting Information may be found in the online version of this article.



SCHEME 1 Synthesis route for the preparation of P(OEGMA)-*b*-P(VBC-*co*-ST).

Synthesis of Amphiphilic Block Copolymer P(OEGMA)-b-P(VBC-co-ST) via Reversible Addition Fragmentation Chain Transfer Polymerization

Poly(oligoethylene glycol methacrylate)-*block*-copoly(styrene-*co*-vinyl benzyl chloride), P(OEGMA)-*b*-P(ST-*co*-VBC), block copolymer was synthesized using a living radical polymerization (reversible addition fragmentation chain transfer [RAFT] polymerization).[18,19] First, P(OEGMA) macro-RAFT agent was prepared in toluene at 70°C in the presence of 4-cyanopentanoic acid dithiobenzoate as a RAFT agent. The polymerization was stopped at ~70% monomer conversion ($M_{n,\ theo}$ = 12,200 g mol^{-1}, $M_{n,\ GPC}$ = 12,000 g mol^{-1}, PDI = 1.12). The monomer conversion was determined using nuclear magnetic resonance (NMR) spectroscopy by comparing the intensity of vinyl proton peaks (6.1 and 5.6 ppm) to the ester proton peaks —OCH_2 (4.1 ppm). Restricting the

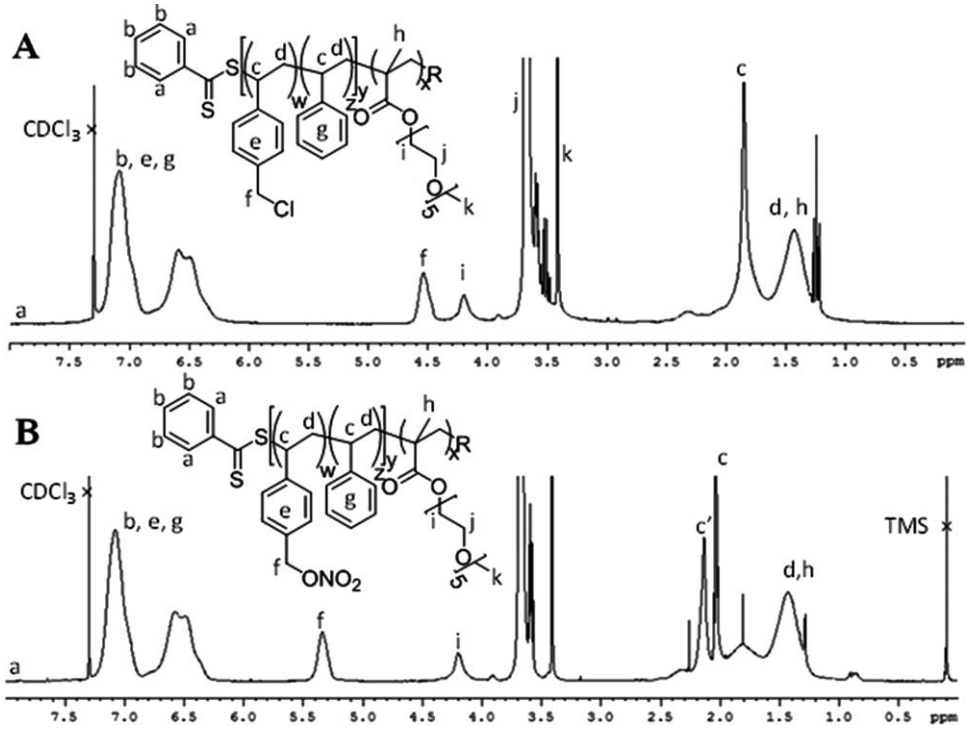


FIGURE 1 ^{1}H-NMR spectra: (A) purified P(OEGMA)$_{40}$-*b*-P(VBC$_{56}$-*co*-ST$_{158}$) in $CDCl_3$ (300 MHz). (B) Purified product obtained after modification with silver nitrate in $CDCl_3$ (300 MHz; recorded in $CDCl_3$).

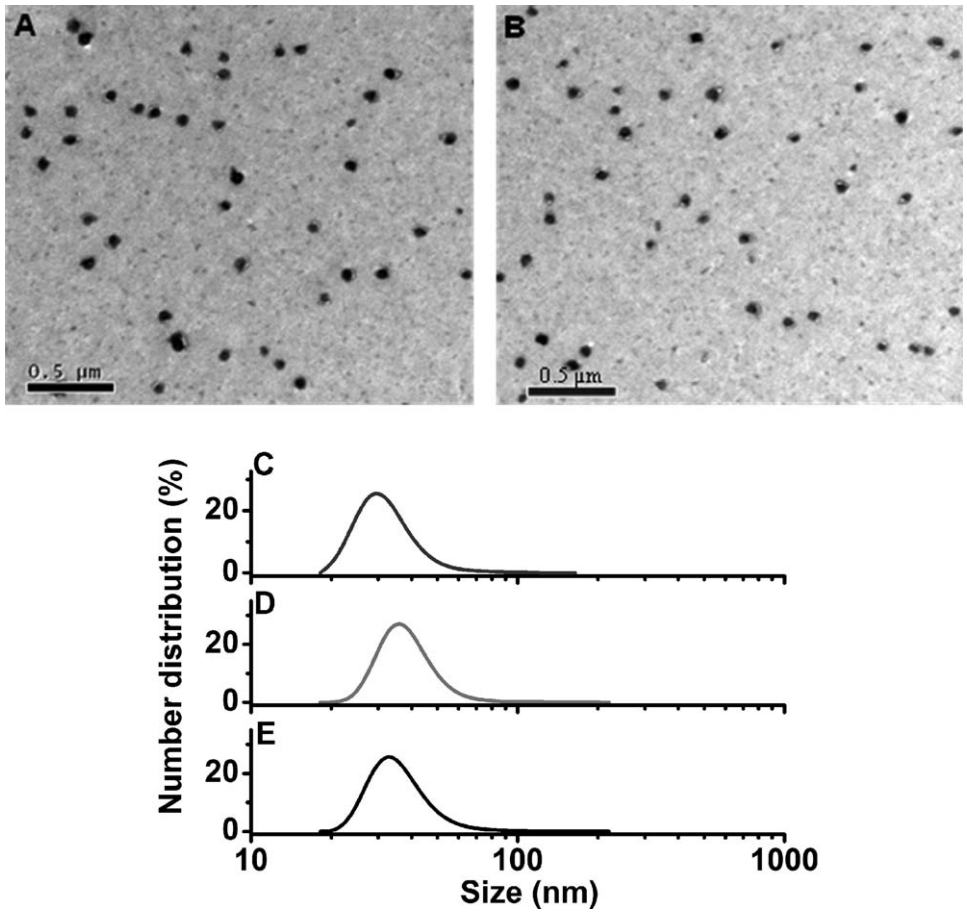


FIGURE 2 TEM micrographs of self-assembled micelles in water (concentration of micelle solution = 1 mg mL^{-1}) of (A) P(OEGMA)-*b*-P(VBC-*co*-ST) copolymers before modification. (B) P(OEGMA)-*b*-P(VB-NO_2-*co*-ST) copolymer after modification with silver nitrate. Note: OsO_4 vapour was used for staining, scale bar = 0.5 μm; Number distribution versus size (nm) assessed by DLS (concentration of micelles solution = 1 mg mL^{-1}) of block copolymer P(OEGMA)-*b*-P(VBC-*co*-ST) after modification with nitrate (C) at 25°C, (D) at 37°C, and (E) at 60°C in water.

polymerization to medium-range monomer conversion helped avoid formation of significant amounts of dead polymer that occurs typically at high monomer conversions (more than 90%). Subsequently, the P(OEGMA) macro-RAFT agent was chain extended in bulk at 100°C in the presence of VBC and ST monomers generating amphiphilic block copolymers. The number of chloro-pendant groups was manipulated by adjusting the feed ratio of the two monomers (Supporting Information [SI], Table S1). GPC traces (SI,

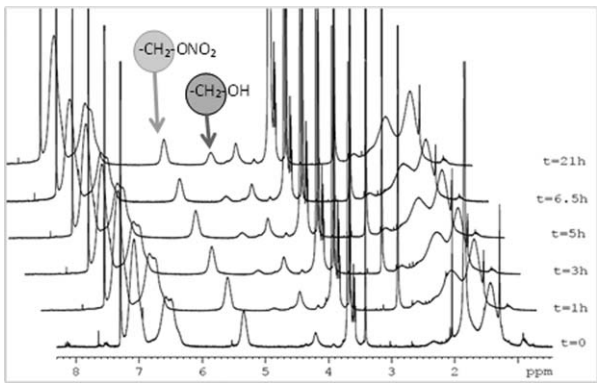


FIGURE 3 Stack plot of ^1H NMR spectra of nitrated modified polymer after treatment with glutathione at 37°C (recorded in $CDCl_3$).

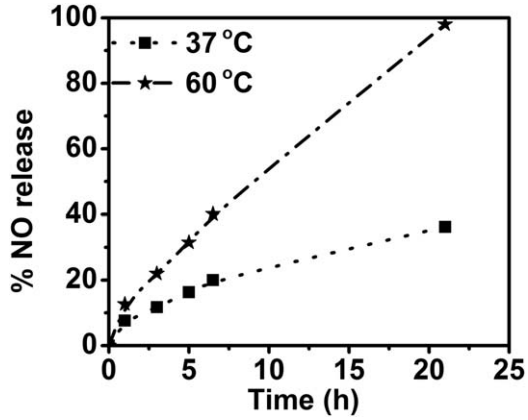


FIGURE 4 Nitric release from nitrate-containing micelles in the presence of glutathione (GSH) in water ([GSH] = 5 mM) at 37 and 60°C.

Fig. S1) confirmed chain growth consistent with block copolymer formation. The polydispersities (PDI) before and after chain extension remained low (<1.20), indicating living polymerization characteristics. After purification by precipitation in diethyl ether, the number of repeating units of OEGMA, VBC, and ST was determined using ^{1}H NMR analysis exploiting the characteristic signals at δ 4.2, 4.5, and 6.3–7.3 ppm attributed to P(OEGMA), VBC, and ST, respectively. P(OEGMA)-*b*-P(VBC-*co*-ST) ($M_{n,\ theo}$ = 37,000 g mol^{-1}, N_{OEGMA} = 40, N_{VBC} = 56, N_{ST} = 158, $M_{n,\ GPC}$ = 36,000 g mol^{-1}, PDI = 1.18) was used as a polymer scaffold for further experiments.

Nitrate Modification of P(OEGMA)-b-P(VBC-co-ST)

The chloro-pendant groups in the P(OEGMA)$_{40}$-*b*-P(VBC$_{56}$-*co*-ST$_{158}$) copolymer were substituted with nitrate groups using silver nitrate ($AgNO_3$) (Scheme 1). After purification by several precipitations in methanol/diethyl ether, NMR analysis was invoked to confirm the successful postmodification reaction (Fig. 1). After reaction with silver nitrate, we observed the presence of a signal at 5.5 ppm attributed to —C*H*$_2$—ONO$_2$ nitrate groups and the absence of the —C*H*$_2$—Cl signal at 4.6 ppm, confirming complete postmodification after 14 h. GPC data showed the molecular weight increased slightly after modification (SI, Fig. S1). Fourier transform infrared spectroscopy (FTIR) was also used to confirm the successful conversion of chloro-moieties to nitrate, as evidenced by the disappearance of the characteristic absorption of 650 cm^{-1} (—C*H*$_2$—Cl groups) and the presence of strong bands consistent with —C*H*$_2$—ONO$_2$ at 1625 and 1250 cm^{-1} [SI, Figs. S3(A) and 3(B)].

Self-Assembly of Block Copolymer Before and After Modification

The self-assembly of amphiphilic diblock copolymers in aqueous media to form different morphologies for drug delivery has been intensively investigated.[20–26] Eisenberg and Soo have investigated the factors that control the morphology of self-assembled copolymers, such as block length, the water content, the nature and the presence of additives (ions, homopolymers, and surfactants), and the polydispersity (PDI) of the block copolymers.[27] It is known that the micellization is not only governed by molecular design factors but it is also influenced by the process used (preparation protocol).[28] In this article, the copolymer was dissolved in N,N-dimethylformamide (DMF), a common solvent for both hydrophilic and hydrophobic blocks, followed by the dropwise addition of water. The final solution was dialyzed against distilled water for 2 days using a tubular membrane (molecular weight cut-off of 3500 Da) to remove DMF. The sizes of the aggregates were determined by dynamic light scattering (DLS) [Fig. 2(C)] and transmission electron microscopy (TEM) [Fig 2(A,B)]. TEM reveals that the aggregates formed were spherical micelles with particle sizes of about 30 nm in accord with the DLS data.

NO Release from Nitrate-Containing Micelles

The generally accepted mechanism for the conversion of nitrate to NO by a nonenzymatic pathway is a direct chemical reaction of organic nitrate with thiol compounds. We used glutathione a tripeptide present in the cytoplasm in mammalian cell (with a concentration ranging from 0.5 to 10 mM)[29] to stimulate the release of NO from the nitrate-functional micelles.

$$R{-}ONO_2 + 2R'SH + 2H^+ \rightarrow R{-}OH + NO + R'S{-}SR' + H_2O \quad (1)$$

The direct chemical reaction of nitrate polymer and glutathione results in the release of NO and the production of a hydroxyl group. Equation (1) gives the overall reaction.[30] The production of hydroxyl group was monitored by ^{1}H NMR analysis. The copolymer was analyzed by FTIR after reaction with glutathione (5 mM) at 37°C (21 h) [SI, Fig. S2(C)]. The NO absorption bands at 1625 and 1250 cm^{-1} decreased after 21 h, while the appearance of a —OH absorption band at around 3300 cm^{-1} confirmed the formation of hydroxyl group as proposed in eq (1).

The NO release kinetic from nitrate micelles was examined by comparing the —CH$_2$—ONO$_2$ proton peak at 5.5 ppm and the —CH$_2$OH proton peaks at 4.6 ppm using ^{1}H NMR analysis at 37 and 60°C over a period of 21 h (Fig. 3; SI, Fig. 3). The intensity of the signal at 5.5 ppm attributed to —C*H*$_2$—ONO$_2$ decreased gradually over time. Interestingly, the release rate of NO at 37°C is slower than that observed at 60°C. NO of 36% was released over a period of 21 h at 37°C, while 99% released was measured at 60°C for the same period of time (Fig. 4).

CONCLUSIONS

In summary, we report on the synthesis of micelles with typical sizes of 30 nm as delivery vectors for NO. NO was released by the specific degradation of nitrate groups by exposure to glutathione (a natural peptide compound containing a thiol group). Kinetic studies showed that nitrate-functional nanoparticles could slowly release NO at 37°C. Such nanoparticles may present significant therapeutic opportunities, where long biocirculation and stealth properties are combined with a sustained slow NO release for benefits in cardiovascular therapy.

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