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Ultraviolet Light Responsive N-Nitroso Polymers for Antibacterial Nitric Oxide Delivery

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Abstract: This study investigates the incorporation of active secondary amine moieties into the polymer backbone by co-polymerizing 2,4,6-tris(chloromethyl)-mesitylene (TCM) with three diamines, namely 1,4-diaminobutane (DAB), m-phenylenediamine (MPD), and p-phenylenediamine (PPD). This process results in the stabilisation of the amine moieties and the subsequently introduced nitroso groups. Charging bioactive nitric oxide (NO) into the polymers is accomplished by converting the amine moieties into N-nitroso groups. The ability of the polymers to store and release NO depends on their structures, particularly the amounts of incorporated active secondary amines. With grafting photosensitive N-nitroso groups into the polymers, the derived NO@polymers exhibit photoresponsivity. NO release is completely regulated by adjusting UV light irradiation. These resulting polymeric NO donors demonstrate remarkable bactericidal and bacteriostatic activity, effectively eradicating *E. coli* bacteria and inhibiting their growth. The findings from this study hold promising implications for combining NO delivery with phototherapy in various medical applications.

Nitric oxide (NO) plays a crucial role in various physiological and pathological processes in the body. It serves as a regulator of vasodilation, inflammation, and immune responses during infection, inhibits platelet adhesion and aggregation, and facilitates diabetic wound healing.[1] NO acts as either a pro-inflammatory or an anti-inflammatory agent in the body, relying on its localized concentration and sources. Notably, its antiviral properties have been observed against different virus families including the coronavirus SARS-CoV-2.[2] Endogenous NO is delivered through amino acid metabolism by NO synthase (NOS) within the body. In contrast, delivering exogenous NO is administered externally, which becomes more challenging, given NO radical short biological half-life (< ~5 seconds) and limited diffusion range (20 – 160 μm),[3] hence proper delivery approaches are essentially required.

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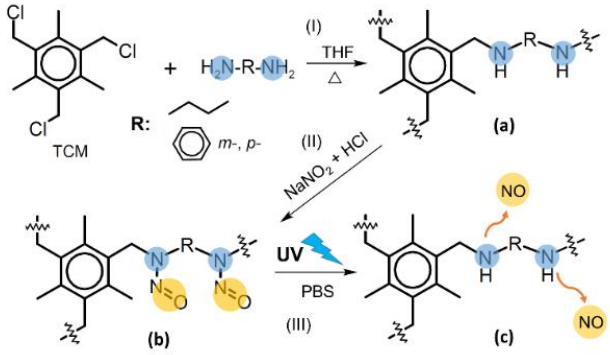
Directly inhaling NO gas and indirectly delivering NO by its carriers have been extensively investigated. The second is practically more suitable for the cost-effective and safe treatment of mild-condition patients in homes. To this end, several materials-based NO delivery systems have been developed, such as using porous materials (zeolites and metal-organic frameworks (MOFs)), polymers, molecular donors, and nanoparticles to serve as vectors for carrying NO in terms of metal-NO complexes, NO gas adsorbed in pores, N- (or S-) nitroso, and diazeniumdiolates (N-NONOates).[4] The NO can be conveniently released through water substitution reactions. The NO storage or release is mainly influenced by the number of active sites available for binding NO, the bond stability of NO to the active sites, and the porosity of the materials, which together with material properties determine the spatiotemporal delivery of NO.

Unlike inorganic materials, porous organic polymers can offer a broader range of structurally diverse polymeric NO donors to align with clinical requirements, ascribed to a large number of monomers available, and can also be decorated with various functional groups to regulate the storage and release of NO. Typically, the polymeric vectors employ embedded amine moieties to bind NO molecules. The availability of secondary amine moieties for NO to bind and the type of NO species formed determine NO storage and its release. For instance, the N-nitroso group (N-NO) can store one NO molecule per >NH group, while diazeniumdiolates (N-NONO) can store two NO molecules. These NO species can be stored within the polymer matrix through encapsulation of small NO molecular donors and grafting the amines to the polymer backbone, or by copolymerizing the monomers with secondary amines.[4f, 5] NO release is driven by the protons present in the triggering medium e.g. water or phosphate-buffered saline (PBS) solution. By modifying the chemical structures of the polymer, it is possible to manipulate the stability of the N-NO (or N-NONO) bond and adjust the surface hydrophilicity/hydrophobicity to control triggering NO release. High chemical stability is preferred to prevent the decomposition of polymers into undesirable small molecular amines. In contrast to the diazeniumdiolate derivatives, which readily release NO upon exposure to water and thus pose challenges in controlling NO delivery, the N-nitroso groups exhibit relative stability in an aqueous solution. They can be produced through a cost-effective and straightforward nitrosation reaction. Moreover, their light-responsive nature enables accurate control of NO release by manipulating light irradiation within the range of 300 – 600 nm to cleave the N-NO bonds.[6] However, the concerned issue is about the possible leaching of nitrosamine compounds as probable human carcinogens.[7] To mitigate the risk, the best solution is to copolymerise the amines into the polymer backbone. This ensures the resulting bonds possess sufficient strength to withstand the NO-releasing conditions, thereby eliminating the nitrosamine leaching and enhancing the safety and effectiveness of the NO delivery system.

Herein, this paper reports a new route for antibacterial NO delivery using copolymers incorporated with light-responsive nitroso groups. Scheme 1 shows the typical processes: (I) copolymerisation of 2,4,6-tris(chloromethyl)-mesitylene (TCM) and diamines (*1,4*-diaminobutane (DAB); *m*- phenylenediamine (MPD); *p*-phenylenediamine (PPD)) to yield copolymers **(a)** coded as TCM-*co*-A_x (*A*: DAB, PPD, and MPD; *x*, the initial molar ratio of *A* to TCM; *x* = 1.0, 1.5, 2.0);[8] (II) storing NO in the copolymers through nitrosation reaction to convert **(a)** into NO donors **(b)** (NO@TCM-*co*-A_x); and (III) releasing NO in PBS solution under UV light irradiation.

Scheme 1. (I) Synthesis of copolymers from TCM and diamines; (II) incorporation of NO in polymers *via* nitrosation reaction; (III) releasing NO under UVA irradiation.



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The monomers TCM and diamines (DAB, PPD, and MPD) have been successfully copolymerized, as verified by the IR spectra in Fig. 1 and S1. The diamine monomers are characterized by the doublet spike peaks at 3420 - 3220 cm^{-1} attributed to the -NH_2 asymmetric and symmetric stretching vibrations. These peaks disappear in the resulting copolymers and are replaced by the single bands centered at 3388 (TCM-*co*-DAB_x), 3343 (TCM-*co*-PPD_x), and 3350 cm^{-1} (TCM-*co*-MPD_x), ascribed to the -NH- stretching vibrations typically for the secondary amines. The multiple peaks at 3010 – 2800 cm^{-1} present in these copolymers are assigned to the stretching vibrations of -CH_3 and -CH_2- groups inherited from the monomer TCM. The peaks near 1600 and 1500 cm^{-1} are attributed to the aromatic carbon-carbon stretching vibrations.[9] These results indicate that the desirable secondary amine groups have been incorporated into the copolymer backbone chains. Further investigations found that the copolymerization of diamine and TCM is influenced by their initial molar ratios. If all three -CH_2Cl groups in TCM participate in the SN2 reaction with the amine monomers, the N/C molar ratios are estimated to be 0.167 for TCM-*co*-DAB_x, 0.143 for both TCM-*co*-PPD_x and TCM-*co*-MPD_x, respectively. In this study, when the initial amine/TCM molar ratio is lower than 2.0, the N/C molar ratios of the copolymers vary in a range of 0.08 – 0.12, lower than the calculated. A lower N/C molar ratio implies that not all the -CH_2Cl groups in TCM take part in the reaction with amines. Increasing the initial ratio of amine/TCM to 2.0, the N/C molar ratios of copolymers are increased to 0.154 for TCM-*co*-$DAB_{2.0}$, 0.125 for TCM-*co*-$PPD_{2.0}$, and 0.148 for TCM-*co*-$MPD_{2.0}$, respectively. These are very close to the calculation results, indicating almost all the -CH_2Cl groups in TCM are converted into the secondary amine (-CH_2NH-) groups. It predicts that the effect of the monomer initial molar ratio on copolymerization will consequently impact the NO storage in the polymers.

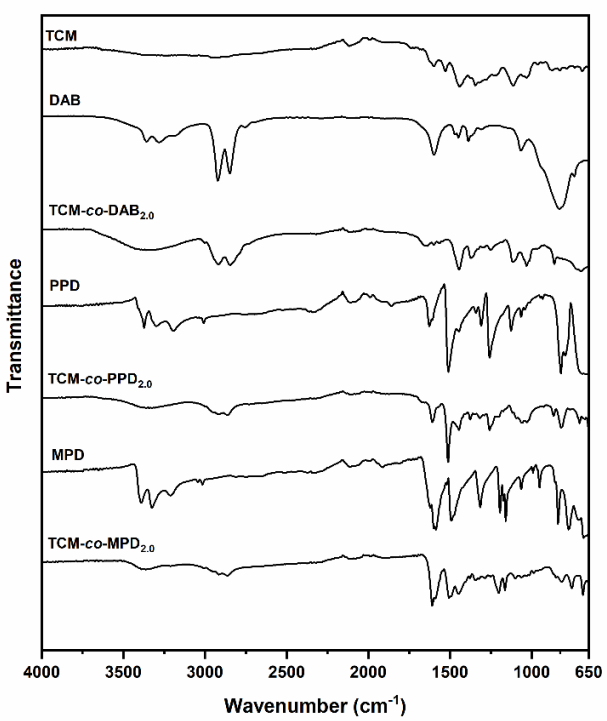

Figure 1. FTIR spectra of monomers and copolymers: TCM-*co*-$DAB_{2.0}$; TCM-*co*-$PPD_{2.0}$ and TCM-*co*-$MPD_{2.0}$.

The stability tests demonstrated that the resulting copolymers are chemically stable. Immersing them in various solutions such as PBS (pH 7.4), acidic (pH 4.0) or alkaline (pH 12), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), hexane, ethanol, methanol, and dichloromethane for seven days, the copolymer structures remained unchanged, as indicated by the consistent FTIR spectra before and after the tests (Fig. S2). The ^{1}H NMR spectra did not detect the amine species in the solutions caused by polymer decomposition. This possibly removes the concerns regarding carcinogenic nitrosamines leaching under NO-releasing conditions. Apart from the chemical stability, the copolymers

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are also thermally stable up to ~ 300 °C (Fig. S3). This makes further functionalization of these copolymers possible at an elevated temperature required.

To store NO, the copolymers were then nitrosated through a facile reaction with acidic sodium nitrate, which transforms the secondary amine groups on the backbone chains into the N-nitroso groups thus charging NO in polymers. The formation of N-nitroso groups (>N-N=O) is verified by the IR spectra in Fig. 2, in which the typical copolymers derived from amine/TCM = 2.0 are presented. The peak appearing at ~ 1435 cm^{-1} is assigned to N=O stretching vibration, and the peak at ~ 1110 cm^{-1} is ascribed to the N-N stretching vibration in the nitroso groups.[9] A pair of peaks in 1630 - 1530 and 1315 - 1260 cm^{-1} are not observed regarding the nitro group (>N-NO_2) asymmetric and symmetric stretching vibrations,[10] indicating the incorporated nitroso groups were not further oxidised.

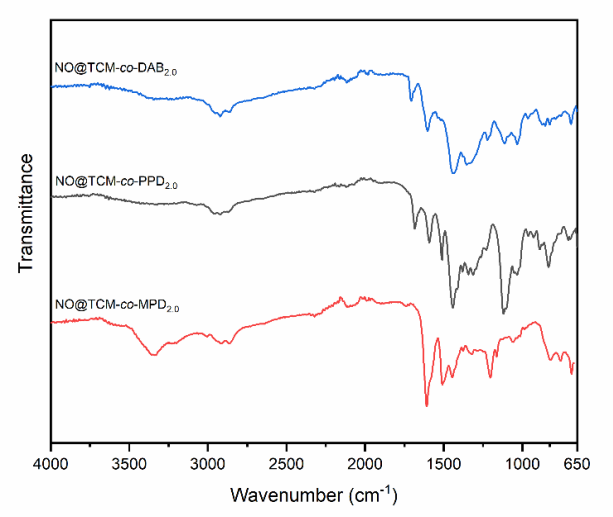

Figure 2. IR spectra of the typical copolymers incorporated with NO.

The average numbers of NO stored in the copolymer matrix have been estimated by comparing the nitrogen contents before and after the nitrosation reaction. It shows on average TCM-*co*-DAB_x, TCM-*co*-PPD_x, and TCM-*co*-MPD_x can approximately store NO of 2.90, 5.48, and 6.73 mmol g^{-1}, respectively, with corresponding conversions of 43.4, 71.0, and 72.1% (based on nitrogen). Polymer TCM-*co*-DAB_x has lower NO storage and lower conversion. That may be due to its backbone chains composed of the secondary aliphatic amines, which may cause more steric effects than the others to hinder the NO_2^- from attacking the secondary amines. It has a lower porosity characterized by the N_2 adsorption (Fig. S4),[11] to make TCM-*co*-DAB_x behave like a non-porous or macroporous polymer with a low BET surface area of ~ 5.0 m^2 g^{-1}. Comparatively, polymers TCM-*co*-PPD_x and TCM-*co*-MPD_x have relatively higher surface areas. It might be possible that fewer amine groups presenting on TCM-co-DABx external surface are converted into N-nitroso groups, resulting in a lower conversion.

Under ultraviolet light irradiation, the light-responsive N-nitroso groups release NO. The UV wavelength triggering NO release depends on the chemical environment of NO moieties in the polymers. A wide range of wavelengths from 200 to 700 nm has been used to cleave the nitroso groups in different NO donors to release NO. For example, N-nitrosated naphthalimides release NO at 365 nm;[12] S-nitroso-N-acetyl-D-penicillamine PVC polymers release NO at 460 nm.[13] This study clarified the effect of wavelength on the NO release. The nitrosated polymers respond differently to the LED UV light irradiation at 365, 385, and 405 nm. These wavelengths are in the range of UVA spectra responsible for about 95% of the UV light that reaches our skin. At 365 nm, the polymers released more NO per time than at higher wavelengths (385 and 405 nm). This is different from the simple nitroso compound molecules in which the N-NO bonds are cleaved at a higher wavelength of more than ~460 nm[14] at which less bond dissociation energy is required. This implies the complexity of the N-NO chemical environments in the polymers and the state of NO donors dispersed in the medium for NO release. More effective photon energy is therefore required to dissociate the N-NO bonds at lower wavelengths. This photodissociation mechanism does not like the photoinduced nitro-to-nitrite rearrangement and subsequent bond cleavage to yield NO radicals.[15] Irradiation wavelength as a variable can adjust NO release, and so

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does irradiation intensity. Generally, the higher the light intensity, the higher the NO release flux. These parameters make light-controlling NO delivery more flexible. In this study, these two parameters were simply set at 365 nm and 25 mW cm^{-2} respectively without further optimisation. Another critical parameter of controlling NO release is the ON-time of the light irradiation pulse. Together with the frequency, their combination provides fine modulation over the dose and duration of NO release.

The NO release profiles in Fig.3 are explicitly correlated to the polymer composition. A low molar ratio of diamine to TCM results in a low concentration of effective -NH- moieties incorporated to form >N-NO groups, accordingly, less amount of NO is stored and released. In addition to the effect of polymerisation, the amine structures affect NO release. To further understand this effect, an apparent first-order kinetics analysis was applied to NO release. It shows the NO delivery abilities of polymers follow TCM-co-PPDx > TCM-co-MPDx > TCM-co-DABx with high deliverable NO of 0.41-1.89 mmol g^{-1}. The NO release rate constant, *k*, varies within a range of approximately 5.0 - 7.0 x 10^{-4} s^{-1}. The adjacent substituent groups slightly affect the activation and rupture of the N-NO bonds. Stability tests demonstrate that at room temperature the incorporated N-nitroso groups are chemically stable in the same solutions used for the copolymer stability tests before, and do not release NO in PBS solutions if the polymers are kept in a dark environment as shown in Fig. 3(d), unlike the diazeniumdiolate groups or the NO stored in MOFs which spontaneously release NO upon contact with water. When exposed to natural light, the polymers released NO very slowly, which can be avoided if keeping polymers from light. Compared with UV irradiation, natural light can trigger very small amounts of NO release. The incorporated nitroso groups are thermally stable up to ~120 ^{o}C (Fig. S3). Increasing the temperature to ~150 ^{o}C will completely decompose them. For practical applications, the high stability of these nitrosated copolymers allows the long-term storage of NO at ambient temperature achievable.

Antibacterial tests validate the activity of the NO released from the polymers to kill *E. coli* bacteria, as shown in Fig. 4, where the mixture of polymer and *E.coli* suspension in a petri dish was irradiated with UV light at 365 nm for 20 minutes. The percent difference of colony-forming units (CFU) per mL of bacteria suspension after irradiation compared to the control CFU counts gives the antimicrobial rate. With the same dose of polymeric NO donors, the higher the rate, the stronger the bactericidal effect. Consequently, the bactericidal activity follows an order of NO@TCM-co-PPD_x> NO@TCM-co-MPD_x > NO@TCM-co-DAB_x, which is closely related to the ability of these polymers to deliver NO. Increasing polymer dosage from 0.4 to 10 mg ml^{-1} will significantly enhance their antibacterial activity. This is apparently related to the delivered NO concentration. For example, at a typical polymer dose of 1.0 mg ml^{-1}, the maximum concentrations will be 1.89, 0.92 and 0.77 µmol NO mL^{-1} for NO@TCM-co-$PPD_{1.5}$, NO@TCM-co-$MPD_{1.5}$, and NO@TCM-co-$DAB_{1.5}$, respectively. Extensive observations have been made regarding the impact of different macromolecular scaffolds and NO delivery modes on the antibacterial efficacy of NO.[16] To examine if polymers inhibit bacteria growth, after UV irradiation, the bacteria/polymers suspensions were incubated at 30, 60, and 90 minutes, respectively, in a dark environment at 37 ^{o}C in the air. It was observed, all polymeric NO donors inhibited bacterial growth within 90 min at a higher dose of 10 mg ml^{-1} (Table S4). By reducing the dose to 0.4 mg ml^{-1}, NO@TCM-co-PPDx still plays

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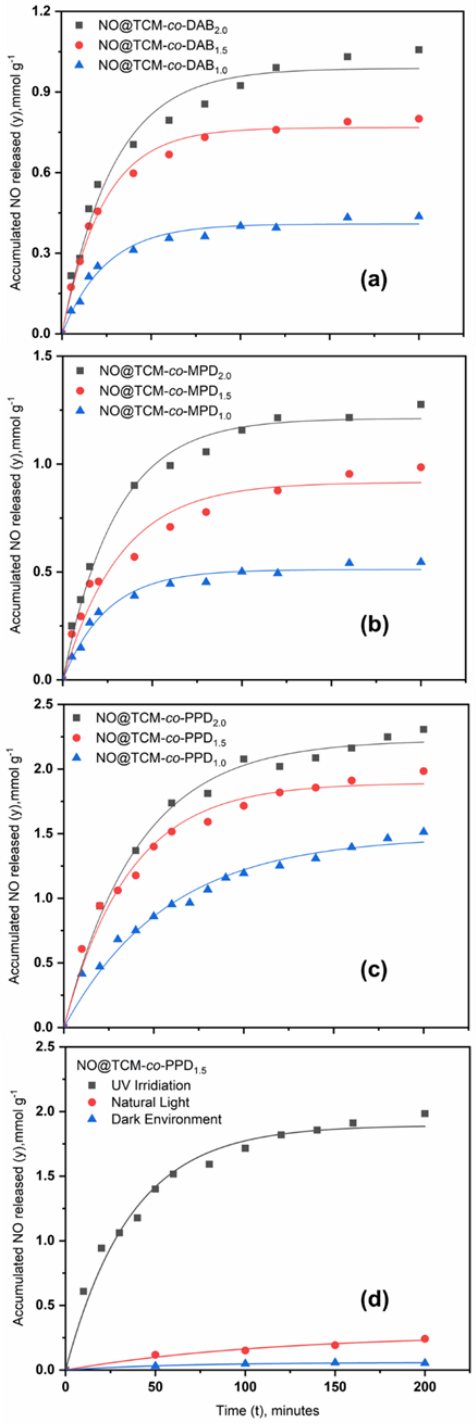

Figure 3. The accumulated amount of NO released from the copolymers with time in PBS solution under UV irritation: (a) NO@TCM-*co*-DAB$_x$; (b) NO@TCM-*co*-PPD$_x$ and (c) NO@TCM-*co*-MPD$_x$; (d) neither natural light nor dark environments trigger NO release significantly.

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the role of inhibiting bacteria growth within 90 minutes of incubation. However, polymers NO@TCM-co-MPDx and NO@TCM-co-DABx achieved comparable inhibition with shortened times of 60 and 30 minutes, respectively. The bacteriostatic effect of NO relies on its capacity to covalently bind to DNA, proteins, and lipids, effectively inhibiting or killing target bacteria.[17] The residence time and concentration of NO species in the solution influence polymers' ability to exert bacteriostatic effects.

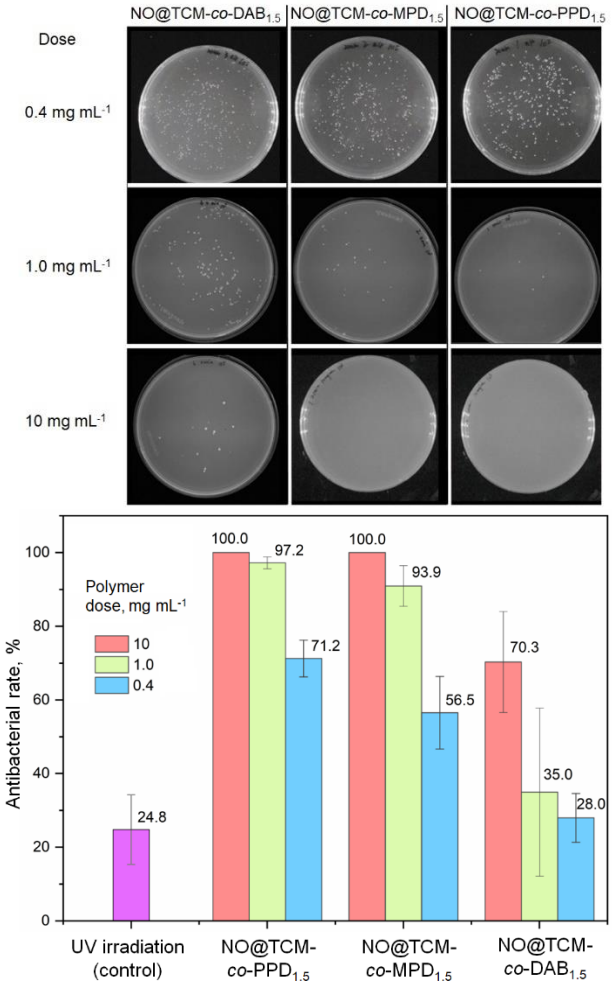

Figure 4. Antibacterial effects of the NO released from the polymers on Gram-negative *E.coli* vary with the polymer doses. (UV irradiation: 365 nm, 25 mw cm^{-2}, and 20 mins).

In conclusion, the new synthesis strategy of co-polymerisation of TCM and diamines have been investigated to incorporate the secondary amine moieties into the backbone chains, thereby stabilising the N-nitroso groups. The novel polymeric NO donors exhibit light responsiveness, enabling the precise control of NO release by adjusting the wavelength, intensity, and duration of the irradiation to achieve the spatiotemporal delivery of NO. These nitroso polymers demonstrate potent antibacterial activity. With a proper dose, they can effectively kill *E. coli* bacteria and inhibit their growth. This study showcases the promising potential of integrating antibacterial NO delivery and light therapy for practical applications.[18]

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Keywords: copolymers • photoresponsivity • nitric oxide • light irradiation • antibacterial

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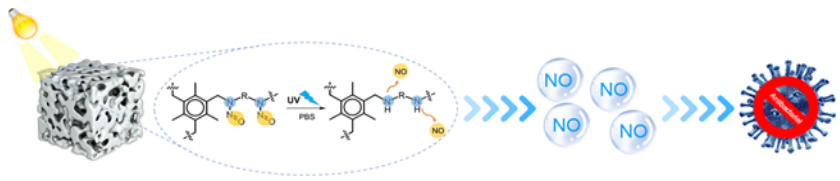

Nitric oxide (NO) exhibits potent broad-spectrum antibacterial activity, but effectively controlling the spatiotemporal delivery of NO presents a considerable challenge. This innovative solution involves integrating light-responsive N-NO nitroso groups into polymers, allowing for precise control of NO release by toggling the light ON/OFF, which holds promise for integrating NO delivery with light therapy in medical applications such as wound healing

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