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Nitric Oxide Release Part II. Therapeutic Applications

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Summary

A wide range of nitric oxide (NO)-releasing materials have emerged as potential therapeutics that exploit NO's vast biological roles. Macromolecular NO-releasing scaffolds are particularly promising due to their ability to store and deliver larger NO payloads in a more controlled and effective manner compared to low molecular weight NO donors. While a variety of scaffolds (e.g., particles, dendrimers, and polymers/films) have been cleverly designed, the ultimate clinical utility of most NO-releasing macromolecules remains unrealized. Although not wholly predictive of clinical success, in vitro and in vivo investigations have enabled a preliminary evaluation of the therapeutic potential of such materials. Herein, we review the application of macromolecular NO therapies for cardiovascular disease, cancer, bacterial infections, and wound healing.

1. Introduction

Prior to 1987, nitric oxide (NO) was mainly known as an atmospheric pollutant produced from industrial processes, automobile exhausts and electrical storms. Following the seminal work by Furchgott, Ignarro and Murad, who independently identified NO as the endothelium derived relaxation factor (EDRF),¹⁻³ much research has continued to investigate the impact of this gaseous free radical in vascular homeostasis, neuronal and immunological processes.⁴⁻⁹

Although NO's bio-molecular role is still not completely understood, researchers have used current knowledge to propose and formulate NO-based therapies some of which have demonstrated success in clinical settings.¹⁰⁻¹⁶ In general, current therapies may be categorized into two groups: (1) drugs that alter the body's enzymatic production of NO and (2) materials that actively release NO or one of NO's redox analogs. While several promising therapies have been based on manipulating nitric oxide synthase (NOS) activity to alter endogenous NO concentrations,¹⁷⁻²¹ this review highlights the therapeutic potential of exogenous NO and the advantages of using macromolecular scaffolds for NO delivery.

Although gaseous NO has proven successful for select medical applications (e.g., topical for dermatological and inhaled for pulmonary treatments), NO donors have been developed to enable the chemical storage and delivery of NO to benefit a wider range of applications. While many classes of NO donors exist, including metal nitrosyls, *S*-nitrosothiols (RSNOs), organic nitrites and nitrates, and *N*-diazeniumdiolates (NONOates),²² few have been translated to the clinic. Organic nitrates, such as isosorbide mononitrate and glyceryl trinitrate, are widely used to treat angina, anal fissures, heart failure, and pulmonary hypertension, but are disadvantaged by the risk of hypotension, headaches and evolving tolerance.²³ The metal nitrosyl sodium nitroprusside is also a potent vasodilator but requires co-administration with other drugs to prevent cyanide poisoning. Thus, researchers are

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actively seeking new NO donor vehicles capable of delivering precise NO doses directly to specific tissues for improved therapeutic output and reduced side effects.^{24, 25}

As introduced in Part III of this review series (Nitric Oxide Release Part III. Measurement and Reporting), the therapeutic consequence of any NO-based drug depends strongly on the concentration and duration of NO delivered. For example, micromolar NO concentrations are required to inhibit the growth of tumor cells, while picomolar NO concentrations have an angiogenic effect leading to cell proliferation.²⁶ As a result, effective NO-based therapies must store and deliver only relevant NO doses for specific durations. In addition, NO delivery must be selective due to NO's short half-life (seconds) limiting its sphere of influence to ~100 μm from its source.²⁷ Control over these parameters (i.e., delivery site, NO concentration, and rate of NO release) remains key for developing useful therapeutics. Tolerance and toxicity issues arise when drugs cannot be applied specifically to the diseased area. Unfortunately, LMW NO donors disperse rapidly and are not easily manipulated for targeting. In contrast, macromolecular scaffolds are more easily modified to facilitate targeting and controlled NO release. As introduced in Part I of this review series (Nitric Oxide Release Part I. Macromolecular Scaffolds), examples of macromolecular scaffolds include but are not limited to proteins, dendrimers, zeolites, metal-organic frameworks, inorganic particles, and synthetic polymers/films. Macromolecular-derived NO release is characterized by many advantages such as enhanced storage and sustained release kinetics. Additionally, the scaffolds may be modified with ligands for targeting and/or tuning clearance and biocompatibility. As a result, the effectiveness of the NO-releasing vehicle may be enhanced while avoiding side effects typical of LMW NO donors. Below, we highlight key reports that have evaluated NO-releasing macromolecular materials in treating cardiovascular diseases, cancer, infection, and wound healing to aid in the designing of improved NO-based therapies.

2. Cardiovascular Disease

In the vascular endothelium, NO is generated to maintain proper blood flow and pressure.²⁸ When NO is produced from vascular endothelial cells, it influences the cellular activities of smooth muscle cells, platelets, and immune cells (Figure 1). After generation, NO diffuses into vascular smooth muscle cells and reacts with the iron of soluble guanylate cyclase. This activation of guanylate cyclase results in the production of cyclic guanosine monophosphate (cGMP), leading to relaxation of the smooth muscle cells and an overall dilation of blood vessels. Deficiencies in NO occur when the endothelium is injured or not functioning properly as is the case for several cardiovascular conditions, including atherosclerosis, heart failure, hypertension, arterial thrombotic disorders, coronary heart disease, and stroke.⁹ A decrease in NO levels may result from an overall decrease in NO production, an impaired availability of bioactive NO, or an increase in NO inactivation by reactive oxygen species. To address deficient NO levels, NO has been added exogenously.²⁹ For example, gaseous NO has been administered via inhalation to deliver NO directly to the pulmonary vasculature, thus relieving pulmonary artery pressure without causing systemic vasodilation and hypotension.²⁸ However, inhaled NO only treats well-ventilated areas of the cardiovascular system. Such therapy is also inconvenient, costly, and potentially dangerous due to the use of pressurized tanks of NO gas. Alternatively, LMW organic nitrates and nitrites have been used for centuries as cardiovascular therapeutics particularly to initiate dilation of vascular smooth muscle.²⁸ Glyceryl trinitrate (GTN) is perhaps the most popular and widely used organic nitrate for the regulation of blood pressure, although its success is hindered by the induction of tolerance. The pathophysiology of GTN tolerance is complex and dependent on several factors including GTN bioactivation, desensitization of vascular soluble guanylate cyclase, oxidative stress, and mitochondrial aldehyde dehydrogenase inactivation. Sodium nitroprusside has also been used clinically, but its success is limited by

cyanide toxicity.²⁹ Clearly the need for more effective NO donor therapies capable of delivering therapeutic NO levels to diseased cardiovascular tissue with minimal side-effects is warranted and remains an active area of NO donor research.

2.1. Circulatory Dysfunctions

The systemic delivery of exogenous NO to the vasculature for treating circulatory dysfunctions is particularly difficult due to the rapid scavenging of NO by hemoglobin, which restricts the ability to maintain sustained NO levels throughout the vasculature.²⁸ Nitric oxide release from macromolecules allows for much longer and continuous delivery of NO at therapeutic levels and thus represents a promising alternative to LMW NO donors for blood pressure regulation.

In addition to prolonging NO release, the efficacy of NO-based therapies would improve with enhanced circulation time of the macromolecules in the vasculature. One popular method for increasing the plasma retention time of therapeutics is the incorporation of polyethylene glycol (PEG), which minimizes protein and cell adhesion and increases the hydrodynamic volume to decrease the rate of clearance.³⁰ Katsumi et al. demonstrated slower clearance by modifying *S*-nitrosylated bovine serum albumin (RSNO-BSA) with PEG.^{30, 31} The pegylation augmented plasma retention in a rat model and also prolonged NO release duration by ~2–4 times that of traditional LMW RSNOs (i.e., *S*-nitroso-*N*-acetylpenicillamine (SNAP) and *S*-nitrosoglutathione (GSNO)). The increased circulation time of the RSNO-BSA resulted in a more sustained reduction in mean arterial pressure in a mouse model compared to LMW RSNOs. Furthermore, the vasodilating effects of pegylated RSNO-BSA were observed even in rats with nitroglycerin-induced tolerance, suggesting a benefit over organic nitrates. Another class of NO-releasing proteins, *S*-nitrosylated cell-free hemoglobin (SNO-Hb), have also exhibited improved circulatory effects upon pegylation.³² By pegylating SNO-Hb, the NO release vehicle avoided extravasation (i.e., leakage from the vasculature) and exhibited prolonged circulation. An increase in both fractional shortening (i.e., a measure of left ventricle performance) and the lactate extraction ratio (i.e., a measure of anaerobic metabolism due to cellular hypoxia) was noted when SNO-PEG-Hb was infused directly after the onset of coronary ischemia. Additionally, coronary blood flow and myocardial oxygen consumption increased, while coronary vascular resistance decreased. These studies revealed that infusion of the long circulating SNO-PEG-Hb improved contractile and metabolic functions in ischemic myocardial tissues.

The Friedman laboratory has developed long-circulating nanoparticles with sustained NO release without the use of PEG-modification.³³ Hydrogel-glass composite nanoparticles that contain physically entrapped NO gas release their payload upon hydration in a pH dependent manner, exhibiting an NO release half-life of 4 h at pH 7.4 and 37 °C. In a hamster model, the NO-releasing particles avoided rapid clearance and macrophage uptake, remaining in circulation up to 6 h after injection. As a result of the steady delivery of therapeutically relevant NO concentrations, decreased blood pressure was achieved with a 30-fold lower NO dose when utilizing the macromolecular scaffold compared to two commercially available LMW NO donors diethylenetriamine NONOate and dipropylenetriamine NONOate. This work indicates that sustained intraluminal NO concentrations are more favorable for maintaining proper blood flow than the bolus NO release characteristic of LMW donors. An increase in vascular relaxation and microvascular perfusion was also observed following infusion of the NO-releasing particles. Prolonged circulation and proper regulation of blood pressure with minimal negative side-effects (i.e., toxicity and tolerance development) exemplify the advantage of macromolecular scaffolds over LMW donors for treating circulatory disorders.

2.2. Ischemia/Reperfusion Injury

When proper circulation is restored following a period of restricted blood flow, oxidative stress leads to inflammation and tissue damage. This condition is referred to as ischemia/reperfusion (I/R) injury. Due to its antioxidant and anti-inflammatory properties, endogenous NO is known to be a mediator/protector of I/R injury and may therefore serve to treat this cardiovascular dysfunction. Compared to the systemic NO release required for blood pressure regulation, targeted NO delivery is necessary for reducing I/R injury as the cytoprotective activity is only relevant at the compromised tissue. To translate the use of RSNO-modified BSA (SNO-BSA) for hepatic I/R injury during liver surgery, Katsumi et al. targeted the macromolecules to the liver by further conjugating mannose or galactose to the BSA.³¹ Mannosylated macromolecules target hepatic nonparenchymal (structural) cells via mannose receptor-mediated uptake, whereas galactose-modified macromolecules target hepatic parenchymal (functional) cells via asialoglycoprotein receptors.³¹ The inclusion of the targeting ligands resulted in significantly greater NO delivery to the liver compared to the blood as detected with electron spin resonance spectroscopy. Indeed, the targeted NO delivery successfully assuaged hepatic injury as indicated by a suppression of alanine and aspartate aminotransferase levels, nearly complete prevention of an increase in neutrophil population, and inhibition NF- κ B activation.

In comparison to proteins, dendrimers are a macromolecular scaffold with greater chemical flexibility as their highly branched structure provides numerous end groups per molecule that can be functionalized with targeting and NO donor ligands. This versatility, along with their typically small size and monodispersity, make dendrimers excellent candidates for NO delivery. Johnson et al. reported the ability of generation 4 poly(amido amine) (PAMAM) dendrimers modified with SNAP (G4-SNAP, 63 SNAP molecules per dendrimer) to reduce I/R injury in an isolated perfused rat heart as a function of glutathione (GSH)-initiated NO release.³⁴ The amount of infarcted (i.e., necrotic) tissue was significantly reduced upon pretreatment (i.e., before the onset of ischemia) with the NO-releasing dendrimers (4 %Infarct) compared to controls (12 %Infarct). The large number of SNAP molecules per dendrimer scaffold allowed for the concentrated delivery of NO near the cell surface, promoting NO diffusion to intracellular organelles and leading to cardioprotective effects. The addition of GSH, a catalyst for NO release from G4-SNAP via transnitrosation, to the reperfusion media greatly decreased the dose required to successfully prevent tissue necrosis (31 nM without GSH compared to 230 pM with GSH), indicating that GSH may be a critical component of future RSNO-based I/R therapies.

2.3. Thrombosis and Restenosis

The introduction and/or removal of intravenous devices often leads to thrombosis (i.e., blood clots within a vessel) or restenosis (i.e., re-narrowing of vessels) (Figure 2). In healthy vasculature, endothelial cells generate NO to prevent thrombosis and platelet activation/adhesion. However, interventional procedures such as angioplasty and stenting often cause damage to or removal of the endothelial cells lining the artery. As a result, normal NO production from the endothelium required for vascular homeostasis is disrupted with thrombosis resulting as activated platelets aggregate and adhere along with proteins (i.e., fibrin) to the device and/or injured site. Following thrombosis at vascular injuries, smooth muscle cells (SMC) migrate to the site, and their proliferation results in neointima hyperplasia (i.e., proliferation of the cells of the inner most lining of an artery) ultimately leading to restenosis. Due to NO's innate role in the cardiovascular system, application of exogenous NO is an obvious solution to problems arising at the site of vascular device implantation and vascular injury.

To prevent thrombosis due to platelet adhesion to a surface, implanted devices may be coated with NO-releasing polymers. For example, Zhang et al. prepared NO-releasing polymers by doping medical grade polyurethanes with fumed silica particles on which amines had been tethered to the surface and subsequently *N*-diazoniumdiolated.³⁵ The NO release from these polymers mimicked the NO flux from stimulated endothelial cells (i.e., $\sim 7 \text{ pmol cm}^{-2} \text{ s}^{-1}$) and effectively decreased platelet adhesion and activation on the interior walls of extracorporeal blood circulatory tubes. As an alternative to doping polymers with NO release, polymers and films may be chemically modified with NO-release functionalities covalently bound throughout the matrix. For example, Seabra et al. designed polynitrosated polyester/poly(methyl methacrylate) blends that released up to $\sim 17 \text{ nmol}$ of NO/mg polymer and inhibited platelet adhesion.³⁶ Schoenfisch and coworkers synthesized xerogel coatings modified with either NONOates or RSNOs that exhibited a wide range of NO release totals, fluxes, and surface roughness.³⁷⁻⁴² The benefit of xerogel films include mild synthesis conditions and the ability for high NO loading. Surfaces coated with these NO-releasing xerogels exhibited decreased platelet adhesion, even at fluxes as low as $\sim 2 \text{ pmol s}^{-1} \text{ cm}^{-2}$. Although the amount of NO release required to decrease platelet adhesion in vivo is greater due to NO scavenging, recent pre-clinical animal testing indicates the ability of NO-releasing polymers to decrease thrombosis for blood-contacting medical devices.⁴³⁻⁴⁵

In response to vascular injury, thrombosis may also prove problematic by initiating excessive platelet activation and aggregation. Stasko et al. reported the ability of NO-releasing dendrimers (G4-SNAP) to inhibit platelet aggregation relative to the LMW NO donor SNAP.⁴⁶ The advantage of the macromolecular NO release was realized as $25 \text{ }\mu\text{M}$ SNAP resulted in 17% aggregation inhibition, while a dose of G4-SNAP corresponding to the same SNAP concentration caused only a 62% reduction in aggregation. The enhanced efficacy was attributed to the greater localized concentration of NO released from the dendritic structure compared to the LMW NO donor.

The recruitment and proliferation of vascular smooth muscle cells (VSMC) and endothelial cells may lead to neointima hyperplasia following thrombosis in the restenosis cascade. As NO inhibits VSMC proliferation, the prevention of restenosis with exogenous NO is achieved by decreasing platelet activation/aggregation and reducing neointima hyperplasia. Optimal treatment requires that NO is delivered directly to the vascular injury either by direct application or by some NO release trigger. Smith et al. first reported on the inhibition of VSMC proliferation and antiplatelet activity of NO-releasing polymers prepared by doping *N*-diazoniumdiolate-modified polyamines into poly(ethylene glycol).⁴⁷ More recently, Kapadia and colleagues designed NO-releasing nanofiber gels composed of two LMW NO donors, 1-[(2-carboxylato)pyrrolidin-1-yl]diazene-1-ium-1,2-diolate (PROLI/NO) and 1-[*N*-(3-aminopropyl)-*N*-(3-aminopropyl)diazene-1-ium-1,2-diolate (DPTA/NO), encased within the hydrophobic interior of the nanofibers.⁴⁸ While both NO donor-doped formulations reduced neointima formation, dopant leaching and resulting toxicity may prove problematic as the NO donor was not covalently attached to the polymer backbone.

In comparison to polymeric materials that require direct application to the vessel injury, site-specific delivery may be achieved using NO-release vehicles capable of targeted and NO release. For example, Huang et al. designed echogenic liposomes (ELIPs) loaded with gaseous NO that released their payload via Doppler ultrasound.⁴⁹ The application of the triggerable NO-releasing ELIPs was found to effectively reduce neointimal hyperplasia in injured carotid arteries of rabbits. The encapsulation and localized NO delivery resulted in less NO scavenging by hemoglobin and an overall increase in therapeutic payload, as indicated by a 7-fold greater increase in NO delivered to VSMCs compared to NO-saturated solutions. Although the use of trapped gaseous NO eliminates the concern of NO-donor

byproduct toxicity, tunability of NO release kinetics is constrained as the diffusion of NO out of the liposome is rapid.

As detailed above NO-based therapies have a long history in cardiovascular diseases.⁵⁰ Materials that release NO have a positive effect on blood flow regulation, ischemia/reperfusion injury, thrombosis and restenosis. Furthermore, the delivery of NO via macromolecules is beneficial compared to LMW donors in terms of decreased tolerance and localized delivery. Conflicting evidence remains as to whether rapid or sustained NO delivery is more beneficial for preventing neointimal hyperplasia, thus investigation into a variety of other NO donors and scaffold compositions must continue.⁵¹

3. Cancer

Nitric oxide is also intricately involved in cancer biology where both NO concentration and lifetime governs whether it acts as a tumor progressor or suppressor (Figure 3).²⁶ Large concentrations of NO (i.e., micromolar) produce reactive nitrogen species, which along with reactive oxygen species result in oxidative and nitrosative stress, leading to DNA base deamination, nitrosylation of enzymes, impaired cellular function, enhanced inflammatory reactions, inhibited mitochondrial respiration and cell apoptosis.⁵² Lower NO concentrations (i.e., picomolar) present anti-apoptotic effects and promote angiogenesis thereby increasing nutrient delivery and facilitating tumor growth. Elevated levels of all three isoforms of nitric oxide synthase have been found in human solid tumors, indicating that NO is a mediator of tumor progression.⁵³ The production of NO is part of the macrophages' antitumor immune response mechanism, yet an over-expression of NO levels in tumor sites has been found to correlate with poor clinical outcome.²⁶

The complex relationship between NO concentration and tumor development/regression has ignited much research into both pro- and anti-NO cancer therapies. Anti-NO therapies are essentially NOS inhibitors, species that decrease NOS activity to subsequently decrease tumor growth.²⁶ The use of NOS inhibitors requires long term, systemic administration that may result in hypertension and tumor regrowth if treatment is ceased prior to complete eradication.⁵⁴ Pro-NO cancer therapies aim to increase NO concentrations at the tumor site to initiate apoptosis and/or necrosis of cancer cells.⁵³ Many LMW NO donors have shown anti-tumor efficacy, including diethylenetriamine NONOate, GTN, sodium nitroprusside, furoxan-based derivatives, and NO-releasing aspirin.^{26, 53} Nitric oxide therapies have been tested against numerous healthy and tumor cell lines, revealing that susceptibility is dependent on concentration, cell type, and the oxygen environment surrounding the site of NO delivery.^{53, 55} The main advantage of NO over other antitumor therapies is a decreased toxicity toward healthy cells, particularly at concentrations toxic to tumor cells.

While extensive in vitro and in vivo testing of LMW NO donors for cancer therapy has been reported,^{26, 53, 55, 56} few drugs have proceeded to clinical trials. The sensitive concentration dependence of NO's tumoricidal properties are likely the cause for the insecurity about pro-NO cancer therapies. Indeed, both the concentration and delivery of NO must be precisely controlled for NO-based chemotherapies to be successful. For this reason, localized delivery and triggerable NO-release strategies represent the future of NO-based cancer therapies. Macromolecular NO-release scaffolds are uniquely poised to accelerate such therapies as nanoparticles, dendrimers and liposomes have proven successful vehicles for targeted delivery of other antitumor therapies.⁵⁷⁻⁵⁹

Stevens et al. evaluated the utility of NO-releasing silica nanoparticles as an antitumor therapy in which NONOates were covalently incorporated throughout the particle scaffolds, which were formed upon the co-condensation of tetraethylorthosilicate with *N*-(6-aminoethyl)aminopropyl trimethoxysilane (AHAP3/TEOS) or 3-methylaminopropyl-

trimethoxysilane (MAP3/TEOS).⁶⁰ Both NO-releasing particle compositions exhibited greater toxicity toward A2780 human ovarian cancer cells than an equivalent LMW NO donor (sodium 1-(pyrrolidin-1-yl)diazene-1,2-diolate or PYRRO/NO). Using a fluorescent marker to measure intracellular NO (4,5-diaminofluorescein diacetate, DAF-2 DA), the nanoparticle scaffolds were observed to deliver significantly greater NO concentrations to the interior of the tumor cells compared to PYRRO/NO. While the macromolecule resulted in more efficient NO delivery, some of the NO payload was inevitably released to the surrounding media prior to particle-cell interaction. In the future, therapeutic effects of these scaffolds may be enhanced by preventing/slowing NO release until the particle has reached its target (i.e., tumor cells).

One strategy for decreasing the decomposition rate of NO donors until a target is reached involves temporarily protecting the NO donor group from its decomposition initiator. For example, Stevens et al. included a hydrogen bond stabilizing group to decrease the NONOate degradation rate of silica nanoparticles.⁶⁰ Findlay et al. achieved greater protection by synthesizing the LMW NO donor *O*²-[2,4-dinitro-5-[4-(*N*-methylamino)benzoyloxy]phenyl] 1-(*N,N*-dimethylamino)diazene-1,2-diolate (PABA/NO).⁶¹ PABA/NO must first undergo nucleophilic aromatic substitution by glutathione before the NONOate decomposes under physiological conditions. While the therapeutic potential of this NONOate-based prodrug has been exhibited in animal models, the effective therapeutic lifetime is still limited. Most of the NO payload from the LMW donor is lost while the drugs are still in circulation. Kumar et al. further delayed NO release by encapsulating PABA/NO within hydrophobic polymer nanoparticles of polystyrene-*b*-poly(ethylene glycol) (PS-*b*-PEG) and poly-L-lactide-*b*-poly(ethylene glycol) (PLA-*b*-PEG).⁶² Decreased water uptake and steric hindrance limited the access of glutathione and protons to PABA/NO, resulting in an increase in the NO-release half-life from 15 min to 3 and 5 h for PLA-*b*-PEG and PS-*b*-PEG, respectively. The antiproliferative activity against human leukemia U937 and human non-small cell lung cancer H1703 cells was slightly increased by encapsulation within the macromolecular scaffold. Of note, the particle diameter (220-450 nm) and circulation lifetime (5 h) of the PABA/NO-encapsulated nanoparticles are in the ranges considered to be necessary for delivery to solid tumors by the enhanced permeation and retention effect.⁶³

Employing an external trigger such that NO is released only when intended and not while in circulation represents an alternative strategy for controlling NO donor decomposition. As numerous photo-labile NO donors exist (e.g., RSNOs and metal nitrosyls), light-initiated NO release is a potential candidate that would allow for precise spatial and temporal control of NO release.⁶⁴ Sortino and coworkers reported platinum nanoparticles decorated with nitroaniline derivatives that act as NO “photocaging” ligands and release NO only upon irradiation with visible light (i.e., 400 nm).⁶⁵ After a 3 h incubation period followed by a 30 min irradiation period with visible light, a dose-dependent decrease in cervical cancer HeLa cell viability was observed with up to 30% cell death achieved following treatment with the NO-releasing particles (1-100 μ M). While promising, the use of these particles for tumor therapy would be greatly increased if they were active in the near infrared (NIR) wavelength range to allow for greater penetration depth in tissues.⁶⁶

Based on the work today and that which is known about NO's role in cancer biology, the use of NO for cancer treatment can only be achieved by the delivery of high concentrations specifically to the tumor site without the possibility of residual low concentrations that promote tumor growth. While prodrugs and photoactive macromolecular scaffolds are more promising than LMW NO donors, the use of NO alone for cancer treatment is a daunting task because of its dual role as a tumor promoter and repressor. The future of NO-based antitumor therapy will likely involve co-administration with other therapies.^{16, 64} Indeed,

NO has been found to act as a chemosensitizer where it causes tumor cells to become more susceptible to traditional cancer therapies. Chemoresistance that is often observed in solid tumors with hypoxic conditions has also been inhibited by pretreatment with NO.¹⁶ Administering NO to the hypoxic regions of solid tumors also increases in blood flow and enhances susceptibility to radiotherapy.¹⁶ Additionally, some conventional drugs may be modified with NO-release moieties, resulting in a synergistic effect due to the combined therapy. For example, NO-releasing non-steroidal anti-inflammatory drugs (NSAIDs) are an excellent example of the advantage of combination therapy.^{56, 67} Not only were the antitumor properties enhanced by combining NO release with the NSAID treatment, but also the serious gastrointestinal and renal side effects common to NSAID usage were eliminated due to NO's ability to decrease leukocyte adhesion to the vascular endothelium. With the synthetic tunability of macromolecular scaffolds, the co-loading of multiple therapies within one delivery vehicle, either chemically or physically, may lead to a new generation of NO-based cancer therapies.

4. Antibacterial/Antibiofilms

Since 1962 only two new classes of antibiotics have reached the market, and bacterial resistance has already been reported for one (daptomycin).⁶⁸ The introduction of new antibiotics that are merely analogues of previously used drugs further compounds the problem as resistance typically develops within 2 years.⁶⁹ There is a dire need for a new generation of antibiotics that do not foster resistance or at least slow the rate that resistance is induced. Nitric oxide represents an excellent antibacterial candidate as it is at the crux of the innate immune system of higher organisms.⁷⁰ As such, NO-based antimicrobials have become a popular area of research to answer the desperate and constant need for better antibiotics.

As depicted in Figure 4, NO exhibits nitrosative and oxidative action alone and upon reaction with oxygen or reactive oxygen intermediates (e.g., superoxide and hydrogen peroxide) to form even more oxidative/nitrosative species including peroxynitrite, RSNOs, nitrogen dioxide, dinitrogen trioxide, and dinitrogen tetroxide.⁷⁰ These reactive species may then interact with microbial proteins, DNA and metabolic enzymes, ultimately disrupting vital cellular functions and structures leading to potent antimicrobial efficacy. Antibiotics that function through a number of mechanisms often decrease the probability of microbial survival, replication, and ensuing resistance.⁷¹ Nitric oxide is capable of acting on both planktonic bacteria and biofilm bacteria communities,⁷² the latter of which are more difficult to treat due to a protective exopolysaccharide matrix that resists penetration of immune cells and antibiotics.⁷³

In the development of NO release as an effective antibacterial agent, macromolecules have again proven more effective than LMW NO donors.^{74, 75} For example, Ishima et al. found that the half maximum inhibitory concentration (IC₅₀) of *S*-nitrosylated human serum albumin against *Salmonella typhimurium* was lower by a factor of 5000 compared to LMW NO donors, GSNO and propylamine NONOate.⁷⁴ A similar trend was observed by Hetrick et al. where a 3-log reduction in planktonic *Pseudomonas aeruginosa* cultures was achieved with only 0.22 mM NO using *N*-diazoniumdiolate-modified silica nanoparticles compared to 4.5 mM NO from LMW NO donor PROLI/NO.⁷⁵ Using DAF-2 DA, Hetrick et al. indicated that the enhanced efficacy of the NO-releasing macromolecule was a result of more efficient intracellular NO delivery.

When designing antimicrobial agents, it is important to note that a number of factors influence the macromolecules' killing efficacy. Hetrick et al. reported on the role of chemical composition on antibacterial/antibiofilm properties using two *N*-diazoniumdiolate-

modified silica nanoparticles (AHAP3/TEOS and MAP3/TEOS).⁷⁶ The MAP3/TEOS particles were characterized by a 2-fold larger NO payload and 3-fold faster NO release kinetics and caused nearly a 1000-fold increase in *P. aeruginosa* biofilm killing compared to AHAP3/TEOS particles. Clearly, the manner or rate by which the NO is delivered greatly affects the biocidal action with evidence suggesting more rapid release of large NO payloads are superior to slow release. The chemical composition not only governs the NO payloads and release kinetics but also greatly impact the direct interactions between the NO release scaffolds and the microbes. For example, most bacteria membranes carry a net negative charge, thus positively charged macromolecules would likely be characterized as having enhanced association and more efficient NO delivery. Physical features of the scaffold will also govern the extent and rate of scaffold-microbe interactions, ultimately affecting in the percentage of NO delivered directly to the microbe. Carpenter et al. observed an inverse relationship between nanoparticle size and antibacterial efficacy with smaller diameter particles resulting in faster particle-bacteria association and enhanced killing efficacy.⁷⁷ Moreover, characteristics of the targeted microbe (e.g., Gram stain and species) will influence NO's biocidal action. For example, NO-releasing silica nanoparticles have generally proven to be more effective against Gram-negative than Gram-positive bacteria.⁷⁶ Hetrick et al. attributes this to the thicker peptidoglycan layer of Gram-positive bacteria membranes.⁷⁶ Even among the same species, different strains may prove more or less responsive to treatment with NO-releasing macromolecules. For example, Martinez et al. found wide ranges of minimum inhibitory concentrations of NO-releasing hydrogel/glass composite nanoparticles against eleven different strains of methicillin-resistant *Staphylococcus aureus* (MRSA) (i.e., 312-2,500 $\mu\text{g mL}^{-1}$) and against nine strains of methicillin-susceptible *Staphylococcus aureus* (MSSA) (i.e., 312-1,250 $\mu\text{g mL}^{-1}$).⁷⁸ The ability of NO-releasing macromolecules to kill bacteria is clearly dependent on many chemical and physical features of both the scaffold and target microbe. These factors must be taken into consideration collectively in the design and application of NO-based antibacterials.

As might be expected, NO release has also proven effective at reducing bacterial adhesion to surfaces. Coatings that inhibit bacterial adhesion are actively being sought after as the success of many medical implants remains hindered by implant-associated infection. Similar to platelet adhesion, NO release via NO-donor modified xerogels films have been shown to reduce bacterial adhesion.^{37, 38, 40, 42, 79-83} The potency of NO to prevent bacterial adhesion was proven effective even in environments where adhesion was promoted by the presence of fibrinogen.⁸⁴ For *P. aeruginosa* under non-static (flow) conditions, a NO flux of $\sim 21 \text{ pmol cm}^{-2} \text{ s}^{-1}$ was reported as the minimum level of NO release required to reduce bacterial reduction (by 65%). Nevertheless, bacteria that did manage to adhere to these NO-releasing surfaces were not viable after 7 h as a result of NO's biocidal properties.⁸³ While this minimum level of NO may differ for other bacteria species and in vivo, the authors indicate that future NO-releasing coatings designed to prevent bacterial adhesion should at least exhibit NO fluxes above this threshold.

When designing indwelling medical devices, key properties include biostability, biocompatibility, and an antimicrobial nature. Biodegradable implants release their drug payload, then degrade into non-toxic byproducts such that additional procedures are not necessary for implant removal. Coneski et al. designed biodegradable NO-releasing polyesters composed of glutaric or adipic acid co-polymerized with glycerol and/or pentaerythritol.⁸⁵ The polymers were modified with cysteamine or penicillamine and upon subsequent nitrosation, released up to $0.81 \mu\text{mol NO cm}^{-2} \text{ g}$ over 6 d. These RSNO-modified macromolecules reduced bacterial adhesion of *P. aeruginosa* via an NO-dose dependent manner up to 80% that of controls. Additionally, complete degradation in physiological conditions (pH 7.4 and 37 °C) was achieved within nine weeks.

For sites at or near the epidermis that are prone to infection, photodynamic antimicrobials represent a promising avenue for delivering therapeutics with excellent spatial and temporal control. Nitric oxide donors that release NO upon exposure to light provide an opportunity to combine the advantages of NO's antibacterial properties with the control of photodynamic antimicrobials. For example, Riccio et al. reported on RSNO-modified xerogels that release NO upon exposure to broad-spectrum light. These materials reduced *P. aeruginosa* adhesion by 73–93% relative to controls.⁴² Seabra et al. also described the preparation of RSNO polyester coatings with light-initiated release as unique antibacterial surfaces.⁸⁶ For better spatial control, Halpenny et al. cast xerogels doped with a photoactive metal nitrosyl ($\text{Mn}(\text{PaPy}_3)(\text{NO})[\text{ClO}_4]$) onto the end of a fiber optic cable.⁸⁷ This concept was tested in vitro by inserting the NO-donor-modified fiber tips into planktonic cultures (10^5 CFU/mL) of *P. aeruginosa*, *E. coli*, MSSA, and MRSA. *E. coli* viability was greatly reduced after a 5 min illumination period, whereas a greater NO-exposure duration (10 min) was required to eradicate *P. aeruginosa* and MSSA. Greater survival of MRSA was observed under the same exposure conditions, again indicating the strain-dependence of NO's antibacterial efficacy.

In summary, the broad-spectrum and multifaceted antibacterial roles of NO are clear. The opportunity for biofunctionalization with peptides, proteins, carbohydrates, lipids, and nucleic acids is also more available with macromolecules in order to enhance efficacy by targeting cellular structures of the bacteria.⁸⁸ The ultimate goal of developing new antibacterial materials is to completely eradicate all pathogens at a location to inhibit the development of resistance.⁷¹ Since bacteria communities at infection sites protect themselves from antibiotic treatment by forming dense biofilms, the future of next generation antibacterial agents is dependent on drugs that can penetrate the protective biofilm matrix and eradicate bacteria buried within.⁸⁹ The numerous mechanisms by which NO influences bacterial cell viability and its proven ability to eradicate bacterial biofilms⁷⁶ suggests that decreased resistance emergence is feasible using NO-based antimicrobial agents.

5. Wound Healing

As shown in Figure 5, the wound healing cascade consists of blood coagulation, inflammation, cell proliferation, lesion contraction, and remodeling. This process begins immediately following injury and may continue for several months thereafter. By platelet adhesion and clot formation, coagulation may occur to limit further blood loss. The secretion of growth factors activates fibroblasts, endothelial cells, and macrophages in attempts to reestablish normal homeostasis.^{90, 91} During the inflammation phase, neutrophils, macrophages, and lymphocytes collectively defend against invading microorganisms and help to remove damaged tissue. The proliferation phase, which involves fibroblast migration, angiogenesis and re-epithelization, begins three days following injury and may last up to several weeks. During lesion contraction, the area of damage is reduced to prepare for remodeling, the last phase of wound healing. This final phase consists of changes in the extracellular matrix (ECM) composition, decreases in macrophage and fibroblast density, and reduction in growth factor migration. Finally, avascularization occurs upon decreased metabolism of neovascular tissues to form a flat surface. Rapid recovery with minimal scarring and maximal function are the ultimate goal of wound healing. In the case of severe injuries or because of preexisting conditions, the cascade leading to proper wound healing is not achievable without therapeutic intervention.⁹² Given the immense evidence for NO's antibacterial properties and the beneficial secondary effects of NO in each phase of wound healing (e.g., modulating inflammation, angiogenesis, cell proliferation, matrix deposition, and remodeling^{90, 91}) the concept that NO-based therapeutics are advantageous for promoting proper wound healing is becoming apparent.

Given the obvious complexity of the wound healing cascade, it is not surprising that proper repair of wounds and damaged tissues remains a major healthcare and biomedical challenge.⁹² Ideal wound healing therapies would augment each distinct stage of the process. Prior to the 1960s, wound treatments consisted mainly of passive wound dressings that had no active role other than protecting the wound from physical contact. To date, active wound dressings are used for both acute and chronic wounds to protect and provide optimal environments that prevent infection and promote the events of the wound healing cascade. The most ideal wound dressings maintain a moist environment, reduce infection at the wound site, remove wound fluids, exudates, and necrotic tissue, and stimulate growth factors to promote granulation tissue. Hydrogels represent an excellent wound dressing scaffold as they maintain a moist environment and allow for oxygen permeability, strength, and elasticity. Combining NO release with hydrogel technology has thus become an active area of wound dressing research. Masters et al. tested NO-releasing hydrogels on inflicted diabetic rat wounds to model diabetic foot ulcers.⁹³ Hydrogels of blended NO-donor and poly(vinyl alcohol) released up to 0.48 $\mu\text{mol NO g hydrogel}$. Although treatment with NO resulted in a slightly longer time required for wound closure, the condition of the tissue in terms of granulation and scar tissue thickness was improved for NO-treated wounds compared to controls. Alternatively, Amadeu et al. prepared hydrogels from Pluronic F-127, a well-known biologically inert polymer, doped with GSNO and applied them daily to rat excisional wounds for 4 d.⁹⁴ The extent of wound re-epithelialization, measured as the difference between the total lesion area and the area of the wound remaining open, was reported to be 77% greater for the NO-treated group. Twenty-one days after treatment with NO-releasing hydrogels, the surrounding tissue was improved as indicated by a decrease in inflammatory cells and an increase in fibroblast cells and collagen deposition. In a subsequent study, this material was applied during different phases of wound healing to specifically investigate NO's role in each step of the wound healing cascade.⁹⁵ The GSNO-doped Pluronic F-127 hydrogel was applied to rat excisional cutaneous wounds during the inflammatory phase only (days 0–2), proliferative phase only (days 3–6), or during both phases (days 0–6). No significant differences were observed between wounds treated only during the inflammatory phase or only during the proliferation phase for any of the parameters evaluated, although wound healing was improved for both compared to controls. In general, NO treatment during both phases resulted in superior wound healing compared to single-phase NO treatment. These results indicate that continuous application of NO throughout the first two phases of wound healing may be the most beneficial due to the enhancement of re-epithelialization. Future NO-releasing macromolecules should be capable of releasing NO for at least 1 week to elicit optimal wound healing.

The wound healing process is often further complicated due to infection. As such, NO-releasing hydrogel/glass nanocomposites were evaluated as topical wound healing agents for both control (non-infected) and MRSA-infected skin lesions of mice.⁷⁸ The wound size of NO-treated skin lesions was smaller for both non-infected and MRSA-infected skin lesions compared to controls (non-treated). In addition to greater collagen content, the collagen was thicker and more mature for both infected and non-infected wounds treated with the NO-release vehicles.

Much work still remains before clinical application of macromolecular NO-releasing therapies can be realized, even though the positive effects of NO on wound healing are obvious. Ultimately, exact doses of NO concentration and duration will govern the success of these materials. The ability to deliver reproducible amounts of NO in a controlled manner is necessary and may only be achieved by understanding the chemistry of the NO donors within the macromolecules. Furthermore, NO-releasing scaffolds that are selected for study as wound dressings must maintain proper wound healing environments. Superior wound dressings may be achieved by coupling NO release with other therapies. For example, chitin

and chitosan are biopolymers that may prove ideal as wound dressing compositions due to their natural adhesive nature, permeability to oxygen and antibacterial/antifungal properties.⁹⁶ Covalent modification of the amines within these polymers to provide NO storage sites in the chitosan structure may result in synergistic effects for wound dressings that are both antibacterial and mediators of wound healing.

6. Conclusions

Since the discovery of endogenously produced NO, researchers across many disciplines have attempted to harness the therapeutic potential of this small gaseous molecule known to be a potent vasodilator, tumor mediator, neurotransmitter, antimicrobial agent, platelet adhesion regulator, and angiogenesis promoter. With the knowledge that NO influences human diseases, the biomedical community now needs methods to efficiently store and controllably deliver specific NO doses necessary for desired therapies. In this tutorial review, we have provided evidence that macromolecular scaffolds are an propitious approach to meeting this goal. Macromolecules in the forms of proteins, dendrimers, particles, hydro- and xerogels, and polymers have been developed to controllably store and release NO. As such, these materials exhibit favorable advantages in treating conditions compared to LMW NO donor analogues. To enable more collaborative efforts in developing NO-releasing macromolecules, researchers across disciplines must establish standard methods for reporting concentration and durations of NO release such that the community can fully understand how specific NO release characteristics govern biological responses. The last part of this serial review (Nitric Oxide Release Part III. Measurement and Reporting) serves to develop such a standard. In the future, consistent reporting of NO release parameters may facilitate more rapid clinical translation of NO-based therapeutics to positively impact treatment of human disease.

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Figure 1.
Nitric oxide's role in the vascular endothelium and its effects on cellular activities.

Figure 2. Implant-induced (A) thrombosis and injury-induced (B) thrombosis leading to restenosis.

Figure 3.
The dual role of nitric oxide in cancer biology.

Figure 4.

The numerous antibacterial mechanisms of nitric oxide and its byproducts (A) lead to decreased bacterial viability and decreased adhesion on NO-releasing surfaces (B) compared to control surfaces (C). Images of bacteria were obtained using atomic force microscopy.⁹⁷

Figure 5.
Timeline of events that occur during the wound-healing cascade.