Controlled nitric oxide release for tissue repair and regeneration

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Abstract: The discovery that nitric oxide (NO) plays crucial roles in mammalian systems has spurred considerable interest in its translational application. However, the major problem with its application in clinical settings is the high reactivity of NO, which makes it difficult to supply NO with spatiotemporal accuracy while maintaining its bioactivity. To deliver NO at a specified rate to a preferred location for a precise period of time is highly demanded. The significant technological advancements in controlled release make it possible to control NO release at the target site with a sustained release rate and an optimum concentration, and then provide a longer therapeutic duration of NO. Moreover, biomaterials designed for regenerative applications provide carriers with support structures for controlled NO releasing. Meanwhile, engineered matrices with controlled NO release have been used as vehicles for stem cell delivery to enhance cell engraftment and further to improve therapeutic effects of transplanted cells. In this review, we will provide an overview of controlled NO release materials, as well as the mechanisms of their treatment effects in tissue-repairing processes. Furthermore, the application of on-demand NO-releasing materials as carriers for stem cell transplantation and the NO-based therapeutic applications in translational medicine will be discussed.

Key words: Nitric oxide, controlled release, biomaterial, stem cell, regenerative medicine

1. Introduction
Nitric oxide (NO), first identified as a gaseous signaling molecule and initially called endothelium-derived relaxing factor (Ignarro et al., 1987), is generated from L-arginine by constitutive NO synthase (cNOS) or inducible NO synthase (iNOS) (Marletta, 1993). As a highly reactive radical, NO has been demonstrated as an essential molecule that regulates cellular/molecular functions in mammals and plays vital roles in numerous physiological systems, such as the cardiovascular, respiratory, nervous, and immune systems, as well as biological processes associated with tissue regeneration and cancer development (Carpenter and Schoenfisch, 2012; Gao et al., 2013). Deficiency of NO is associated with a number of pathologies, such as nonhealing chronic wounds, ischemic diseases, and carcinogenesis (Stone et al., 2012; Nguyen et al., 2013; Rybinski et al., 2014; Singh and Singh, 2015). Thus, supplying exogenous NO could be a possible and efficient method for treating these kinds of diseases. However, an exogenous NO supply is inconvenient, especially considering its extremely short half-life and the rapid loss of its bioactivity. Besides, the therapeutic effects of NO on physiology are concentration-dependent (Diers et al., 2011; Inoue et al., 2011; Wang et al., 2014), and how to sustain NO levels in an optimum range is another challenge facing NO-based therapy in clinical application.

In response to the need for controlled NO release for therapeutic application, a chemically modified NO delivery system that is capable of storing NO in a relatively inert state and releasing NO in a controlled manner could be a viable solution (Zhang et al., 2012). In this review, we will focus on the recent advances in controllable NO release systems and therapeutic application of NO for tissue repair and regeneration. Moreover, the therapeutic mechanisms and theranostic applications of tunable NO release in translational medicine will be discussed.

2. Controllable NO release systems
Glycerol trinitrate (GTN), also known as nitroglycerin, has been widely used for decades to treat heart disease (Agvald et al., 2002). However, many unwanted side effects, such as headaches, endothelial dysfunction, and enzymatic tolerance, caused by the application of GTN have been reported and uncontrolled NO release is one of the culprits for these side effects (Ghaffari et al., 2006). Thus, releasing NO on demand will be of the utmost importance for developing superior therapies for various diseases caused by inadequate endogenous NO production. The advancements...
of controlled-release technology have made it possible to harness NO release at a targeted site with an optimum concentration-versus-time profile, which provides a longer therapeutic duration of action and optimizes the therapeutic effects of NO (Quinn et al., 2015). Numerous controlled NO-releasing materials have been designed with the aim of masking undesirable drug properties, which provided us with useful tools to evaluate the pivotal role of NO in mammalian systems (Maruhashi et al., 2014).

2.1. Nonenzymatic activated NO release

The prodrug strategy provides an alternative approach to convert pharmacologically inert derivatives into active drug molecules in vivo by external stimuli (Kitamura et al., 2014; Neidrauer et al., 2014). Among these, light- or humidity-manipulated NO-releasing systems provide easily adjustable parameters (e.g., wavelength, duration, and relative intensity) to regulate NO release (Wu and McGinity, 2000; Sortino, 2012; Bajpai et al., 2015; Chiang et al., 2015).

A series of NO-releasing substances that could be photolyzed by light irradiation with wavelengths ranging from ultraviolet (UV) light (Namiki et al., 1999; Hiramoto et al., 2001) to visible light (Ostrowski et al., 2012; Hitomi et al., 2014) to near-infrared (NIR) light (Eroy-Reveles et al., 2015). These compounds can potentially enhance the therapeutic efficacy of NO in regenerative medicine (Hetrick et al., 2009).

Glycosylation modification is another commonly used approach to achieve tunable NO release. In this method, galactose is utilized as a protective moiety to block NO release from the NO-storing medium; β-galactosidase is added to remove the protective group, which would finally be able to initiate NO release in a controlled manner (Figure 1) (Zhao et al., 2013). Adding β-glycosidase to remove the sugar-capping group could lead to controllable NO release. The rate of NO release could be modulated by fine-tuning the concentration of β-glycosidase, which could provide a convenient method to modulate NO release and maintain the bioavailability of NO.

2.2. Enzymatic activated NO release

For enzyme-activated NO generation, NO donors are blocked by protecting groups to prevent uncontrollable NO release, and the inactivated donors are often loaded on a precursor compound that is used as a reservoir or matrix for both controlled release of NO and delivery of NO (Clas et al., 2014). Prodrug-activating enzymes are employed to remove the protecting groups from these parent drugs, which will lead to the release of active NO.

2.3. Multifunctional NO-releasing system

Recent advances in nanoparticle engineering are creating new opportunities for the development of multifunctional nanoparticles for NO delivery. Multifunctional systems that integrate different functionalities in a coordinated and organized manner are capable of accommodating a combination of therapeutic agents with different properties, such as imaging and specific targeting. Therefore, a targeted and traceable therapeutic strategy combined with delivery NO and imaging probes provides a versatile platform that can potentially enhance the therapeutic efficacy of NO in regenerative medicine (Hetrick et al., 2009).

2.3.1. Real-time monitoring of controlled NO release

Over the past years, nanotechnology has developed rapidly and has been employed for disease diagnosis and treatment. Nanoparticles conjugated with NO-releasing...
moiety, having both diagnostic and therapeutic functions in a single integrated system, will improve and expand a more personalized, customized treatment model based on molecular-level diagnosis (Chen et al., 2013; Yang et al., 2013). Several elaborately designed NO-releasing and imaging nanoparticle products, which could achieve the objectives of maintaining the potency of NO and further exploring the mechanism of tissue repair, early diagnosis, and simultaneous monitoring of therapeutic effect and timely adjustment of therapy regimen in the context of tracing NO delivery, have been invented as tools for theranostic nanomedicine (Rizzo et al., 2013; Ryu et al., 2014). A chitosan nanosphere, which was exploited as a vehicle of a photoresponsive NO-releasing complex, was conjugated with fluorescent agents (Tan et al., 2013). The incorporation of NO-containing materials and NIR fluorescent quantum dots gave this chitosan-based agent the capability of releasing NO in a controllable fashion and monitoring the NO release in situ. The wavelength of excitation for NO release was much shorter than that of fluorescent lights emitted from the compound, and thus no mutual interferences would appear. Researchers further developed two other compounds with the competence of effective NO release and excellent properties of optical imaging, which could be promising agents for theranostics (Figure 2) (Tan et al., 2013, 2014).

Figure 1. The pattern of enzyme-activated nitric oxide (NO) release. The on-demand release of NO is achieved by controlling the decomposition process of the NO-containing compound (chitosan-NO polymer), which is blocked by a protecting moiety (galactose). NO release only occurs after β-glycosidase removes the galactose-capping group. Reprinted with permission from Elsevier (Zhao et al., 2013).
2.3.2. Site-specific delivery of controlled NO-releasing agent

A major issue associated with NO donors is to stabilize and release NO locally and directly in different tissues. Macromolecules, such as dendrimers, hydrogels, polymers, and nanoparticles, have been used and exhibit exceptional potential in directly delivering NO in a controlled spatial and temporal manner with superior biocompatibility for pharmacological applications. The NO-donating agent [Ru(terpy)(bdqi)NO](PF6)3, with the ability of controlled release of NO upon photoirradiation, was loaded onto nanoparticles, which endowed the NO donor with an epidermal targeting effect (Marquele-Oliveira et al., 2010). Photochemical studies confirmed that light irradiation could be employed to control NO release and improve NO concentration in skin. Likewise, the controlled NO-releasing dendrimers, which were capable of selectively targeting inflamed endothelium, showed significant therapeutic potential for reducing ischemia/reperfusion injury (Johnson et al., 2010).

Improving the specificity is a major goal of gene-directed enzyme prodrug therapy. Exogenous genes that encode cell/tissue-specific enzymes, such as nitroreductase (Sharma et al., 2013), β-lactamase (Yepuri et al., 2013), glutathione-S-transferase (GST) (Weyerbrock et al., 2012), and β-galactosidase (Chen and Zhang, 2013), are employed to achieve NO release at target sites. A bacterial biofilm-targeted NO-releasing agent was designed with the capability of selectively releasing NO upon contact with enzyme β-lactamase (Yepuri et al., 2013). The location-specific NO release could minimize the interruption of NO-associated side effects in normal tissue. Likewise, an Escherichia coli nitroreductase (NTR)-activated NO prodrug also had the ability of site-directed release of NO (Sharma et al., 2013); the derivative of the commonly used NO donor, diazeniumdiolate (NONOate), could not release NO until it was metabolized by β-galactosidase-expressing cells (Chen and Zhang, 2013). Moreover, O2-(2,4-dinitrophenyl)1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K), a site-specific NO-releasing compound that could be activated in glutathione (GSH)-expressing cells, showed improvement in duration time and treatment efficacy (Weyerbrock et al., 2012).

3. Mechanism for therapeutic effects of controlled NO release

NO is involved in diverse processes, such as responding to oxidative stress and inflammation, controlling blood flow, and regulating the proliferation and differentiation of cells. Administration of exogenous NO to elevate NO levels has been recognized as a strategy for tissue regeneration. Increasing evidence suggests that NO is a key mediator in different phases of tissue reparative processes, including collagen deposition (Han et al., 2012), fibroblast migration (Han et al., 2012), and angiogenesis (Kazakov et al., 2012; Most et al., 2013).
3.1. Promoting angiogenesis
Stimulating angiogenesis is a promising therapeutic strategy for tissue defects, because nutrients, oxygen, and cytokines transported by blood vessels are of essential importance for normal tissue function (Wang et al., 2015; Zhao and Li, 2015). Resuming the blood stream to the damaged area is a prompt, straightforward, and effective method for tissue repair. NO and NO-involving signaling pathways could promote angiogenesis, which was demonstrated as a vital step in wound healing (Luo and Chen, 2005; Chin et al., 2011) and ischemic disease recovery (Alkasaki et al., 2004; Kumar et al., 2007). Supplementation of exogenous NO in a controlled fashion could modulate the related biological processes and further promote angiogenesis at the site of injury (Namkoong et al., 2008). Recently, a study was performed to compare the proangiogenic potential of six different NONOate-based NO-releasing agents in a chicken embryo partial ischemia model (Majumder et al., 2014). The results showed that different platforms had different NO-releasing patterns and performed their functions in different periods of angiogenesis. In this model, spermine NONOate (SP) exhibited the best angiogenic effect among these NONOate-based compounds, which highlighted the necessity of using the most suitable NO-releasing system during specific phases of tissue regeneration.

3.2. Enhancing efficacy of stem cell-based therapy
Stem cell-based therapy has achieved successful therapeutic effects in experimental and clinical investigations in the past few decades, which provides great hope for the treatment of a wide variety of life-threatening diseases. However, the low rates of cell engraftment, viability, and retention limit the application of stem cell therapy (Li et al., 2009a, 2009b, 2009c; He et al., 2015; Yao et al., 2015). Because of these obstacles, developing methods for alleviating cell apoptosis may be of the utmost importance for stem cell therapy. Moreover, altering stem cell fate (e.g., coaxing pluripotent stem cells into committed progenitor lineages) for therapeutic applications is a feasible and safe strategy that can minimize the risk of cellular misbehavior (Li et al., 2008, 2009a, 2009b, 2009c; Su et al., 2011).

NO could participate in regulating cell proliferation, differentiation, and migration, acting as an important gaseous signaling molecule (Fuseler and Valarmathi, 2012; Curtis et al., 2014). NO was capable of favoring tissue regeneration by enhancing self-renewal ability and inhibiting the maldifferentiation of mesenchymal progenitors (Buono et al., 2012; Cordani et al., 2014). Evidence has also been provided to demonstrate that NO could promote the differentiation of mesenchymal stem cells (MSCs) into endothelial cells, which was also a promising strategy for tissue repairing. In a recent study, bone marrow multipotent progenitor cells (MAPCs) were cultured with two kinds of NO-releasing agents, NONOate and sodium nitroprusside (SNP). Under a sustained NO-releasing environment, increased levels of von Willebrand factor (vWF) mRNA and protein were found, which indicated the endothelial differentiation of MAPCs (Chu et al., 2008).

Utilizing synthetic biomaterials as vehicles for in vivo transplantation of stem cells has been demonstrated as a promising strategy for increasing the retention and survival rate of administrated stem cells. These biomaterials could not only act as a scaffold for cell anchorage but could also provide a supportive niche for cell engraftment (Li et al., 2009a, 2009b, 2009c; He et al., 2015). A recent study developed a peptide hydrogel, which could release NO in a controllable manner catalyzed by β-galactosidase, together with MSCs for myocardial infarction treatment (Yao et al., 2015). Those results revealed that the NO released from the hydrogel could significantly enhance the engraftment and paracrine effect of MSCs. Cotransplantation of MSCs with NO-releasing hydrogel could lead to better outcomes of blood vessel reconstruction and heart performance after myocardial infarction through the NO-activated VEGF/VEGFR2 pathway in a mouse myocardial infarction model (Figure 3) (Yao et al., 2015).

3.3. Antimicrobial property of NO
Wound infection remains a serious threat to patients worldwide. A certain degree of antimicrobial property of NO has been applied against pathogen invasion (Hetrick et al., 2009). A probiotic bacteria-based system that controlled the production of NO gas (gNO) by the metabolism of lactic acid bacteria in an adhesive gas-permeable membrane was offered to treat dermal wounds (Jones et al., 2012). In that study, an infected full-thickness dermal wound model was used to evaluate the preclinical efficacy of this novel patch. Over 21 days of therapy, wound closure in the gNO-producing patch-treated groups was significantly increased based on the antimicrobial activity of NO.

4. Controlled NO release for tissue regeneration
Mounting evidence indicates that an appropriate concentration of NO at the injury site is favorable for tissue repair, while deficient endogenous NO production could hamper the recovery process (Oplander et al., 2012). Exogenous supplementation of NO could be the best way to cope with the problem of NO deficiency. Curative effects showed that localized and well-timed NO replenishing in an on-demand manner could be a superior strategy to promote tissue repair compared to spontaneous NO release.

4.1. NO therapy for cardiovascular diseases
Cardiovascular disease (CVD) remains the leading cause of death and morbidity in the world (He et al., 2015; Yao et al., 2015; Zhao and Li, 2015). The nonsurgical treatment
of cardiac therapy has been proven to be a low-risk and high-efficiency treatment option for cardiac patients by restoring oxygenated blood flow back to the heart and other organs of the body (Fang et al., 2013). Extensive experimental and clinical investigations have revealed the association between vascular NO and cardiovascular disease (Rochette et al., 2013). Reduced NO production and impaired NO bioavailability are the common causes of cardiovascular diseases.

In cardiovascular disease therapy, NO is recognized as a unique reactive modulator involved in vascular function during tissue ischemia. The NO donor SNAP has showed cardioprotective effects. However, a high dosage of SNAP was required to obtain this therapeutic effect, which was toxic to cells. To tackle this issue, SNAP was conjugated with polyamidoamine dendrimers with large drug payloads. Minimized dosage of SNAP and a better curative efficacy for reducing infarct size and ischemia/reperfusion injury were obtained by the triggering of GSH (Johnson et al., 2010). Additionally, conjugation to chemically modified micromolecules could confer the capability of tissue selectivity to NO donors, which has demonstrated good therapeutic effect in the ischemia/reperfusion injury model. A new type of S-nitrosothiol-based macromolecular NO-releasing complex, mannosylated (Man)-poly SNO- bovine serum albumin (BSA) and galactosylated (Gal)- poly SNO-BS, was designed and synthesized (Katsumi et al., 2009). These complexes made it possible to target NO delivery in the compromised tissue and prolong the retention of NO donors in the systemic circulation. NO released from these macromolecules restored proper circulation after a period of restricted blood flow.

After myocardial infarction, apoptotic and necrotic cardiomyocytes are progressively replaced by fibroblast-like cells, which lead to the impairment of cardiac function. Stem cell therapy is a new therapeutic

Figure 3. NapFF-NO hydrogel enhances the therapeutic effect of AD-MSCs for myocardial infraction. Encapsulation of AD-MSCs by NapFF-NO hydrogel could prevent transplanted cells effusing from injection positions. NO molecule released from NapFF-NO hydrogel catalyzed by β-galactosidase had the ability to facilitate angiogenic cytokine secretion of AD-MSCs, resulting in promoting angiogenesis, AD-MSC survival, and cardiac function. Reprinted with permission from Elsevier (Yao et al., 2015).
approach for repopulating damaged myocardium via cell transplantation (Li et al., 2009a, 2009b, 2009c; Yao et al., 2015). Despite this, the beneficial effects of transplanted cells are limited due to low retention of these cells during the early posttransplantation period (Du et al., 2015; He et al., 2015). Transplantation of stem cells in biomaterials to produce better retention and engraftment in the infarcted heart has been widely reported. Transplantation MSCs with controllable NO-releasing hydrogel exhibited relatively high effects on heart function improvement, which could be attributed to the hydrogel-enhanced cell engraftment and survival and the NO-promoted angiogenesis and blood vessel reconstruction in ischemic myocardium (Yao et al., 2015).

4.2. Tunable release of NO for wound healing

Patients suffering from chronic wounds and impaired healing conditions will become even more burdensome in both human health and economic terms (Eming et al., 2014). Innovative therapies for treating unhealed wounds or for speeding up the repair of acute wounds and maximal restoration of tissue function are needed. Topical application of therapeutic agents combined with controlled NO release have provided a safe, efficient, and feasible remedy for treating these lesions without interrupting normal tissues (Oplander et al., 2013).

The therapeutic effects exhibited by NO are in connection with its concentration (Burke et al., 2013), release time, and cell types (Fleissner and Thum, 2011; Krause et al., 2011). It is absolutely vital to deliver NO to the target area in a well-contained manner, for excessive amounts of NO could pose a greater risk of injury to certain tissues. Nanoparticle-based drug delivery systems have been broadly used as a promising method to improve the efficacy of existing drugs with the ability to achieve increased therapeutic duration, enhanced bioavailability, and controlled drug release (Probst et al., 2013). Conjugating NO donors to nanoparticles (NO-NPs) could significantly increase the therapeutic effect of NO. In different treatment groups, skin-injured mice were respectively treated with NO-NPs, nanoparticles without NO, and DETA NONOate (Blecher et al., 2012). In the treated groups, the wound area in NO-NP-treated mice decreased by 29.4% compared to 1.9% in nanoparticle-treated mice and 2.3% in DETA NONOate-treated mice, which showed higher therapeutic efficiency of controlled NO release compared to the group with spontaneous NO release. Likewise, the treatment effect of another kind of NO-NP during the wound repair process was evaluated (Han et al., 2012). The NO level was increased in the NO-NP-treated group compared to the nanoparticle-treated group and the untreated control group. A high wound closure rate was obtained and complete wound closure was achieved only in the NO-NP-treated group.

Ordinary bandage dressings are widely used in wound management with certain advantages. Combined with NO-releasing technology, several fabrics and bandages have been applied in medical practice and exhibited a greater potency to accelerate wound healing. Comparing topical application of NO-releasing films with an extremely short period, erythema disappeared and an increasing rate of blood flow was obtained (Marcilli and de Oliveira, 2014). Likewise, applying electrospinning technology, humidity-sensitive NO-releasing bandages and dressings were developed and assessed (Lowe et al., 2015). The wound healing rate of the NO-treated groups was significantly faster than that of the control group. Additionally, the average capillary density in the NO-treated group was about 60% higher than that of the control group.

NO-releasing sutures hold promise in treating injury located in deep tissue. PCL-coated melt-spin acrylonitrile-based sutures could release NO continually (Lowe et al., 2014). NO could be released from the sutures after they were exposed to moist environments. With PCL coating, the NO release process could be prolonged, which could prevent infection and promote wound healing.

4.3. Controlled NO release to injured tendon and muscle

Tendon and muscle injuries are common, which result in billions of dollars in health care expenses. NO is a function-preserving and repair-stimulating mediator in injury and disease, which has been demonstrated to have great therapeutic potency in both tendinopathies and muscle injuries, for it is remarkably effective for preserving function and stimulating repair of the injured tissue (Murrell et al., 2008; De Palma et al., 2014).

Addition of NO through nitric oxide-paracetamol could enhance tendon healing by improving collagen content and organization in damaged tendons (Murrell et al., 2008). In a cGMP-dependent pathway, NO was capable of stimulating the proliferation of satellite cells, which are critical to sustain muscle regeneration during numerous rounds of damage (Kuang et al., 2008). It has been demonstrated that the enhanced self-renewal ability of satellite cells induced by treatment with the NO-donating drug molsidomine was sufficient to increase the number of satellite cells during repetitive acute and chronic damages and favored muscle regeneration (Buono et al., 2012). NO-based therapy also exhibited the ability of enhancing the regenerative potential of the damaged muscle by inhibiting the maldifferentiation of mesenchymal fibroadipogenic progenitors into adipocytes (Cordani et al., 2014).

5. Summary

The current existing controllable NO-releasing systems have gradually demonstrated their superiority, such as the ease of manipulation, site specificity, longer duration of NO release, excellent biocompatibility, and reduced side
effect profiles. Based on the understanding of therapeutic mechanisms, preliminary evaluations have showed that these NO-releasing agents have great potential for regenerative medicine and tissue engineering. Controlling the extent, rate, and time of NO release can be a very practical approach to optimize its performance. Moreover, the development of high-quality NO controlled-release formulations provides optimism for NO-releasing platforms for regenerative therapy.

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