Nitric oxide-releasing vehicles for biomedical applications

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Nitric oxide (NO) is involved in several physiological processes, such as the control of vascular tone, the inhibition of platelet aggregation, smooth muscle cell replication, the immune response, and wound healing processes. Several pathologies have been associated with dysfunctions in the endogenous production of NO. Thus, there is great interest in developing NO-releasing drugs and in matrices which are able to stabilize and release NO locally and directly in different tissues. Over the past few years, a very promising strategy for biocompatible NO-delivery systems has emerged based on the use of nanobiotechnology for targeted NO-release for biomedical applications. In this work, the current state-of-the-art approaches to NO-release from nanomeric materials, their preparation, and promising biomedical applications are reviewed. Such materials, comprised of dendrimers, liposomes, metallic and silica nanoparticles, polymeric micro and nanoparticles, semiconductor quantum dots, carbon nanotubes, and nanoporous solid materials exhibit exceptional potential in directly delivering NO in a controlled spatial and temporal manner with superior biocompatibility for pharmacological applications.

Introduction

Nitric oxide (nitrogen monoxide, NO) is a gas and free radical with an unshared electron. It plays a key role in the regulation of several physiological and pathophysiological processes, such as host defense, neuronal communication, regulation of vascular tone, neurotransmission, glaucoma and neural degeneration, vasodilation, pulmonary hypertension, penile erection, angiogenesis, inflammation, immune response, septic shock, platelet aggregation, gastrointestinal mobility, hormone secretion, gene regulation, hemoglobin delivery of oxygen, stem cell proliferation and differentiation, bronchodilation, and wound healing.[1–14]

NO mediates its biological effects mainly by activating guanylyl cyclase and increasing guanosine 3′,5′-cyclic monophosphate (cGMP) synthesis from guanosine triphosphate (GTP).[15] However, many NO–cGMP-independent pathways have been discovered recently.[16] The endogenous synthesis of NO is mediated through nitric oxide synthase (NOS) which

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catalyzes the conversion of L-arginine to NO and citrulline.[17] NOS is a homodimer with each monomer containing reductase and oxygenase domains. The prosthetic groups are flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, and the cofactors BH4 and Ca-calmodulin tetrahydrobiopterin.[18] At low concentrations, NO is quite stable and minimally reactive, however, at higher concentrations, it can interact with transition metals, heme-containing proteins, and thiol groups leading to the oxidation of functional groups on RNA, DNA and proteins.[19–23] In inflammatory processes, overproduced NO can rapidly react with reactive oxygen species, such as superoxide ($O_2^{\bullet -}$), to form the very reactive peroxynitrite ($ONOO^-$), which can in turn nitrate proteins.[16] The biological effects of NO have been shown to depend on the site and source of its production, as well as on its concentration.

Many different classes of NO donors have been prepared and applied in biological systems, including disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO),[24] 1-[*N*-(3-ammoniopropyl)-*N*-(n-propyl)amino]-diazen-1-ium-1,2-diolate (PAPA/NO), 1-[*N*-(3-aminopropyl)-*N*-(3-ammoniopropyl)diazen-1-ium-1,2-diolate (DPTA/NO),[24] 1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate (DETA/NO),[25] *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetylcysteine (SNAC),[20,26–28,14] ruthenium derivatives,[29] and *N*-nitrosomelatonin (NOMela).[30] These NO donors can be incorporated into, or chemically linked to, biopolymers in order to mimic the endogenous NO production directly at the target site.[26,31] Over the past few years, due to the wide range of biological effects of NO, there has been a great effort to develop agents that release NO in a controlled manner in living systems, where it might have therapeutic effects.[31] These NO-releasing vehicles can be used in target tissues and sites of application to modulate the *in situ* kinetics of NO release.[26,28,31,32] Many vehicles, such as NO donor complexes, have already been developed to chemically store and release NO. Some of these compounds include the thiol nitroso derivatives, the nitrosohydroxylamines, the nitrogen-bound diazeniumdiolates[33,34] and the nitrosyl ruthenium complexes that induce vasorelaxation.[29]

In order to increase the utility of NO release systems, current research is aimed at coupling NO donors to nanoparticles. This new and very promising area of research is named nanobiotechnology. Pharmaceutical nanoparticle properties and drug delivery from these materials have been widely studied in order to increase their solubility, stability, and their specificity targets.[35–37] The combination of NO donors with nanoparticles represents a very promising strategy for the pharmaceutical applications of NO. The aim of this feature is to review the latest strategies in nanobiotechnology related to the biochemistry and clinical medicine of NO donors in order to improve the biological activities of NO for biomedical applications. Another area that is leading to major improvement, but will not be discussed in this mini-review, is the use of nanobiosensors for NO detection.[38]

The following are important nanostructures in the field of nanobiotechnology related to NO release, which will be discussed in this review: dendrimers, liposomes, metallic and silica nanoparticles, polymeric micro and nanoparticles, semiconductor quantum dots, carbon nanotubes, micellar nanoassembly, and nanoporous solid materials.

(a) Dendrimers

Dendritic architecture is found at the meter scale in tree branching and roots, and at the centimeter and micrometer scales in circulatory topologies of the human anatomy (*e.g.* lungs, kidneys, liver, spleen, and cerebral neurons).[39,40] Since nanostructures are dendritic structures that include glycogen, amylopectin, and proteoglycans,[39] the resulting dendrimer products are monodisperse compared to bulk polymers, and their size (2–20 nm) varies with dendrimer type and exterior functionality. The "divergent method", in which growth of a dendron originates from a core site, was the first synthetic strategy used to create dendrimers.[41] This approach involved

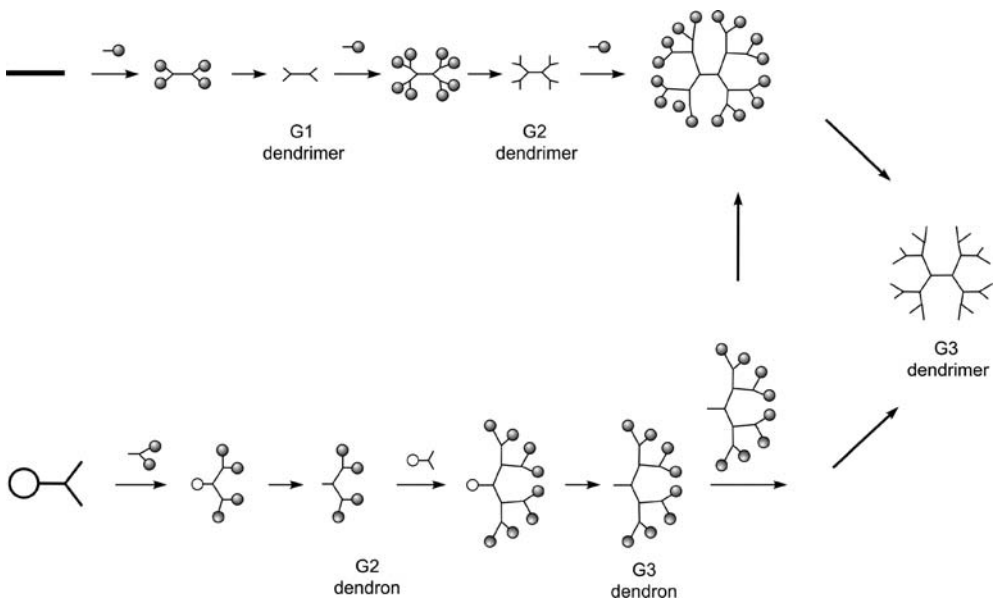

Fig. 1 Dendrimer synthesis (schematically depicted). Top: divergent strategy. Bottom: convergent strategy (modified from ref. 40 by permission of The Royal Society of Chemistry).

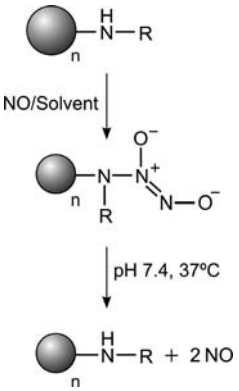

Fig. 2 The formation of sodium stabilized diazeniumdiolates (NONOates) followed by decomposition under physiological conditions to yield 2 mol of NO and initial dendrimer conjugate (*n*) 16, 64 (modified from ref. 42 by permission of the American Chemical Society).

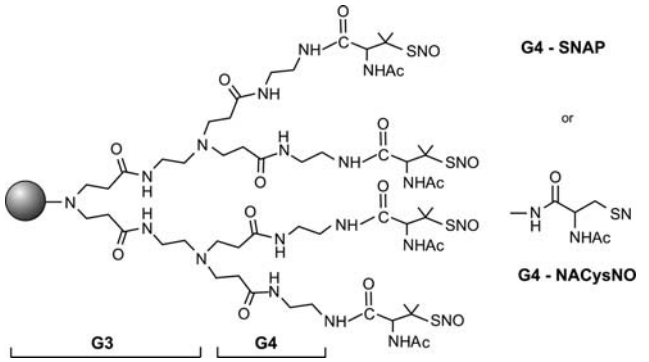

Fig. 3 Generation 4 polyamidoamine (PAMAM) dendrimers containing a completely modified exterior (64 thiols) of *S*-nitroso-*N*-acetyl-D,L-penicillamine (G4-SNAP) or *S*-nitroso-*N*-acetylcysteine (G4-NACysNO) (modified from ref. 45 by permission of the American Chemical Society).

assembling monomeric modules in a radial, branch-upon-branch motif according to certain dendritic rules and principles. The second method followed a "convergent growth process" and proceeded from what would become the dendrimer surface inward to a reactive focal point, leading to the formation of a single reactive dendron (Fig. 1).[40]

In accordance with this, the synthesis and characterization of nanoparticle-NO-donors capable of storing and releasing large quantities of NO from dendritic scaffolds have been reported (Fig. 2).[42]

The primary advantage of NO-releasing dendrimers over other vehicles is their high NO loading capacity per molecule of dendrimer.[42] In addition, it is possible to change the external characteristic of the dendrimer, *e.g.* its solubility, or engineer it for a specific function.[40] For example, a dendrimer with a lipophilic external surface may be incorporated into hydrophobic polymers to impart thrombo-resistivity to them, on the other hand, hydrophilic dendrimers may allow for NO delivery *in vivo*.

Schoenfisch's group[42] has reported a new class of NO-releasing macromolecules prepared from dendrimers functionalized with primary and secondary amines. Secondary amine diazeniumdiolates play an important role in the amine's stability since the total NO released from secondary amine conjugate dendrimers is significantly greater than from the primary amine dendrimers.[42] Kinetic studies have shown that NO release from dendrimer conjugates is proton initiated, which is consistent with the mechanism of diazeniumdiolate dissociation. In this case, the dendridic effect is demonstrated by the increased storage capacity of this architecture and the half-lives of the NO donors exceed, by several fold, those of the corresponding small molecules.[42] However, careful control of NO release is not possible with diazeniumdiolate-modified dendrimers. Finally, the authors suggest that the next step would be to introduce multiple functionalities on the dendritic exterior to equip it with targeting ligands (*e.g.*, folic acid) to selectively deliver NO to cancer cells.[42]

S-nitrosothiols (RSNOs), such as *S*-nitrosoglutathione (GSNO) and *S*-nitrosoalbumin, are the main carriers of NO *in vivo* since they regulate several biological processes including vasodilation, platelet activation, neurotransmission, and tissue inflammation.[43] RSNOs have been the subject of several studies and pharmacological strategies addressing the importance of NO in living systems. Several publications have shown the potential therapeutic effects of RSNO adducts, including their toxicity toward cancerous cells,[44] their antimicrobial activity,[20] their cardioprotective effects during ischemia/reperfusion injury, and their inhibition of platelet aggregation.[45,46] One of the most documented clinical applications of RSNOs is as antiplatelet agents and inhibitors of platelet aggregation which do not affect vascular tone.[47] The drawback of these RSNOs is that they are unable to deliver NO to a specific site of action. Additional problems include thermal instability and their rapid systemic clearance which have both seriously hindered the clinical development of RSNO therapeutics.[48] In order to partly avoid these problems, Stasko *et al.*[45] have reported the synthesis of two thiol-derivatized generation 4 polyamidoamine (PAMAM) dendrimers capable of storing up to 2 μmol NO mg^{-1} of dendrimer when converted to the corresponding RSNO-donors (Fig. 3). The authors compared the antiplatelet aggregation effects of the *S*-nitroso-*N*-acetyl-D,L-penicillamine dendrimer (G4-SNAP) with those of free *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) molecules in order to confirm the biological activity of the dendrimer bound to RSNO. The G4-SNAP and the *S*-nitroso-*N*-acetyl-L-cysteine (G4NACysNO) dendrimers stored and released 1.7 ± 0.2 and 2.1 ± 0.2 μmol NO mg^{-1}, respectively, in the presence of initiators of RS-NO decomposition such as Cu^{2+} or light.[45]

Since thiol groups present on the surface of platelets may undergo *trans*-*S*-nitrosation reactions with RSNOs, the ability of RSNO-dendrimers to cause the nitrosation of platelet surface thiols leads to a careful control of NO release in biological systems.[45] Through the selective *trans*-*S*-nitrosation reaction between the dendrimer-RSNO and thiol groups on the surface of the platelets, the antiplatelet activity of G4-SNAP dendrimer was 45% more effective at preventing platelet aggregation than the corresponding free SNAP (25 μM).[45] Stasko and co-authors state that one of the most important advantages of the dendritic scaffold over other potential RSNOs drug delivery vehicles is the easy and versatile functionalization of its exterior surfaces.[45]

Benini *et al.*[49] have pursued the hypothesis that a similar platform, previously reported by Stasko and Schoenfisch,[42] could be used for local and controlled delivery of NO using PAMAM dendrimers functionalized with nitrosyl ruthenium complexes. Benini and collaborators[49] synthesized a $K[Ru^{III}(Hedta)Cl]$

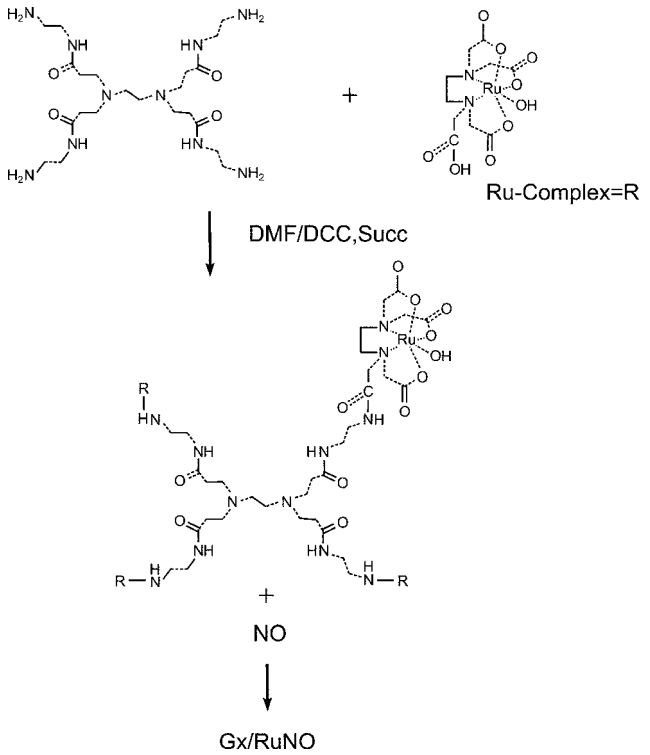

Fig. 4 The preparation of a K[Ru^{III}(Hedta)Cl] complex and its functionalization to release NO (Modified from ref. 49).

complex in which the EDTA ligand was pentadentate, with one of the carboxylate arms unbound, and the sixth Ru^{III} ligand was a chloride ion. The presence of an uncoordinated carboxylate allowed anchoring of the complex to the terminal amine groups of PAMAM dendrimers through amide bonds (Fig. 4).

The PAMAM functionalized dendrimers with [Ru^{II}(edta)H_2O] are designated, for simplicity, as Gx/Ru, where x = 0 (4 NH_2 terminal groups), x = 2 (16 NH_2 terminal groups), and x = 3 (32 NH_2 terminal groups).[49] It is relatively easy to load NO to the dendrimer Gx/Ru by purging NO gas with argon. As PAMAM functionalized dendrimers with [Ru^{II}(edta)NO], designed as Gx/RuNO, liberate NO when reduced *in vivo* and *in vitro* several biological assays were performed to demonstrate the effects of NO-releasing dendrimers. These experiments were carried out using denuded normotensive rat aortic rings precontracted with noradrenaline. The induction of slow, delayed relaxation was initiated after 15 min and maximum relaxation was only achieved after 37 min with Gx/RuNO (x = 0 and 3.3 M).[49] This behavior was similar to that observed for [Ru^{II}(edta)H_2O] (62 min) which induced a slow, delayed relaxation with a maximum relaxation effect in the second hour.[50] The antitrypanosomal activity showed that the G0/RuNO dendrimer was more effective against *T. cruzi* (100%) than free [Ru^{II}(edta)NO]$^-$ (89%).[49]

(b) Liposomes

Liposomes have been used for carrying and delivering NO in various biomedical applications.[51] Phosphatidylcholine and cholesterol, as well as positively and negatively charged lipids, are used to prepare liposomes by a method that involves hydration of the lipid film, sonication, followed by freezing and thawing.[52] NO incorporation into liposomes can be performed by pressurizing the lipid dispersion with pure NO gas or NO mixed with another gas, followed by a sonication step. Huang *et al.*[53] have reported that cationic liposomes provide the highest NO delivery and incorporation (6 μL NO/mg lipids) into cells, with rapid passive NO release in the first half hour, followed by slow NO release for the next several hours. In addition, incubating NO-releasing liposomes with cultured cells leads to an increase in the production of intercellular guanosine 3′,5′-cyclic monophosphate (cGMP) and the effective NO scavenger, hemoglobin, does not affect NO activity. The authors demonstrated that by incubating encapsulated NO with vascular smooth muscle cells, the NO-bioactivity was preserved, even in the presence of an NO scavenger.[53]

A recent work described the characterization of a novel NO-releasing nanocomposite, wherein the NO release can be photostimulated and dynamically modulated at biological levels.[54] The NO-releasing material was encapsulated within lipid vesicles with a diameter of 150 nm and consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), cholesterol, and dihexadecyl hydrogen phosphate dissolved in chloroform. A thin lipid film was formed on the walls of a glass vial and *S*-nitroso-*N*-acetylpenicillamine (SNAP) solution at pH 6.0 with EDTA was added and then extruded. Stimulation by a light source produces an increased NO release from the nanocomposite material in a very short period of time (Fig. 5).[54] The NO release profile decreases with distance perpendicular to the surface, as expected for diffusion from a surface through an aqueous environment. The authors found a linear relationship between NO surface concentration and photon flux, and they suggest that this relationship can be used to tune the material's response.[54]

Nanotechnology allied with NO-releasing materials can be a useful tool in the treatment of coronary restenosis.[55] When

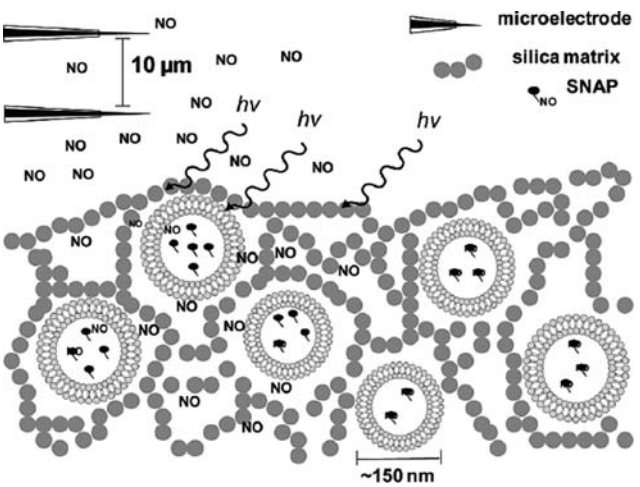

Fig. 5 A compartmentalized nanocomposite material for light controlled NO release. In the dark, NO is bound in the donor molecule, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), which cannot diffuse through the liposome compartment membrane. The liposome is trapped within pores of the silica cell culture substrate. Upon exposure to light the SNAP controllably releases NO, which diffuses to the surface of the material. (Reproduced from ref. 54 by permission of the American Chemical Society.)

cationic liposomes carrying plasmid DNA encoding inducible and endothelial nitric oxide synthases (NOSi and NOSe, respectively) are administered to human umbilical vein endothelial cells (HUVEC) cultures with various inhibitors and inducers of eNOS, the peripheral eNOS side effects must be considered during clinical administration.[56] Others researchers have examined local iNOS plasmid application in injured femoral and coronary artery models,[57] and in the foxhound dog model after an arterial graft.[58] *In vivo* studies of eNOS plasmid DNA formulated in cationic lipids and cholesterol have also been carried out.[59,60]

Since a primary goal is to direct NO releasing compounds (such as NONOates, *i.e.*, compounds containing the N_2O_2-group) to the infected cell, and thereby concentrate high levels of NO near infectious agents, it has been hypothesized that liposomes may facilitate this process since they are readily taken up by macrophages.[61] To determine whether liposomes would enhance the uptake of these NONOates by macrophages, the amount of NO released by macrophages after incubating these cells with either liposome-encapsulated NONOates or non-encapsulated NONOates was compared. The results demonstrated that macrophages released 71% and 78% more NO after exposure to liposomes (dimyristoylphosphatidylcholine (DMPC) : dimyristoylphosphatidy-glycerol (DMPG) : cholesterol, in mole ratios of 9 : 1 : 7.5) containing spermine/NO (SPER/NO) and diethylenetriamine/NO (DETA/NO), respectively, than macrophages treated with non-encapsulated SPER/NO and DETA/NO.[61]

(c) Nanoparticles

(c.1) Metallic nanoparticles. Thiol-stabilized metal nanoparticles, monolayer-protected clusters, are hybrid systems for bio-applications, and it is known that these small-sized surfaces are easy to chemically modify by the introduction of functional units. This has generated a large interest in their biomedicinal applications.[62–66]

The design, fabrication, and properties of hydrosoluble platinum nanoparticles decorated with the NO caging compound monolayer-protected clusters (MPC-3) have been reported (Fig. 6).[63]

Carboxy-terminated monolayer-protected clusters (MPC-1) has been synthesized as a suitable scaffold for NO photoreleasing nanoparticles (designed thiol **3**, see Fig. 6).[63] In this case, NO photorelease takes place through a nitro-to-nitrite photorearrangement followed by rupture of the O–NO bond with the formation of NO and a phenoxy radical (see Fig. 6). The results unambiguously showed that MPC-3 is stable in the dark and can release NO upon irradiation with visible light. The biocompatibility of these nanohybrid systems and their potential in photoactivated anticancer therapy have been explored *in vitro* using tumor cell lines.[63] HeLa cells exhibited negligible mortality in the dark, accounting for the excellent biocompatibility between both types of modified Pt nanoparticles. Results showed that cells treated with MPC-3 were sensitive to light-induced death and the extent of photoinduced cell mortality was strictly dependent on the nanoparticle concentration.[63]

A recent report claims that gold nanoparticles catalyze NO production from endogenous RSNOs in blood serum through

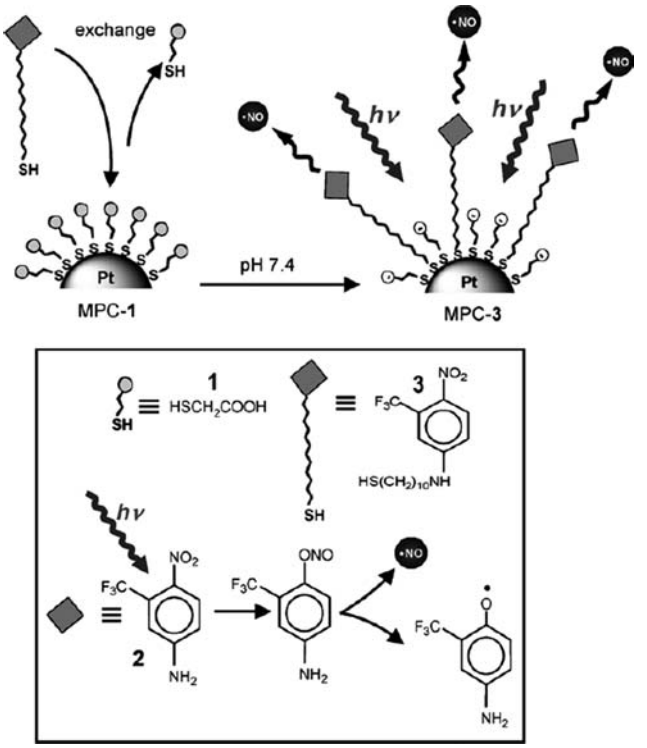

Fig. 6 A schematic view of the construction of the NO photocaging monolayer-protected clusters (MPC-3) and mechanism for NO photorelease from the photoactive unit 2. (Reproduced from ref. 63 by permission of The Royal Society of Chemistry.)

the formation of Au-thiolate on the surface of the gold nanoparticles.[67] Furthermore, whenever the gold nanoparticles are used in conjunction with a drug and its excipient as a probe in the living body, caution must be taken to avoid causing oxidative stress, since gold nanoparticles probably exert toxic effects on the body *via* a dose-dependent increase in reactive nitrogen species levels after treatment with the nanoparticles.[67] This occurs

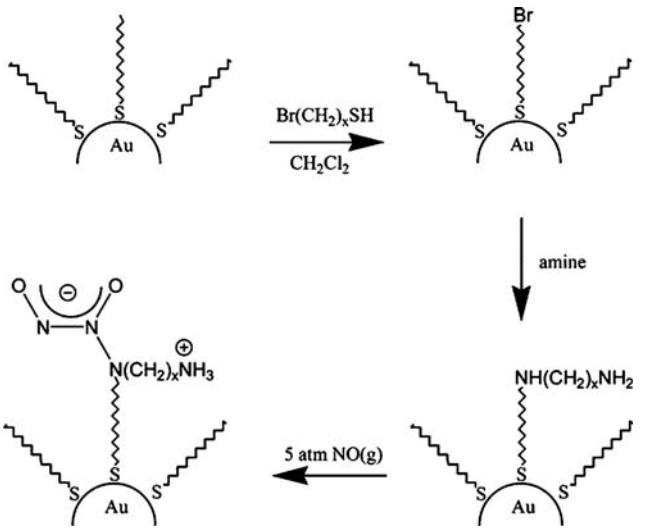

Fig. 7 A synthesis scheme for the preparation of NO-releasing gold nanoparticles (reproduced from ref. 69 by permission of the American Chemistry Society).

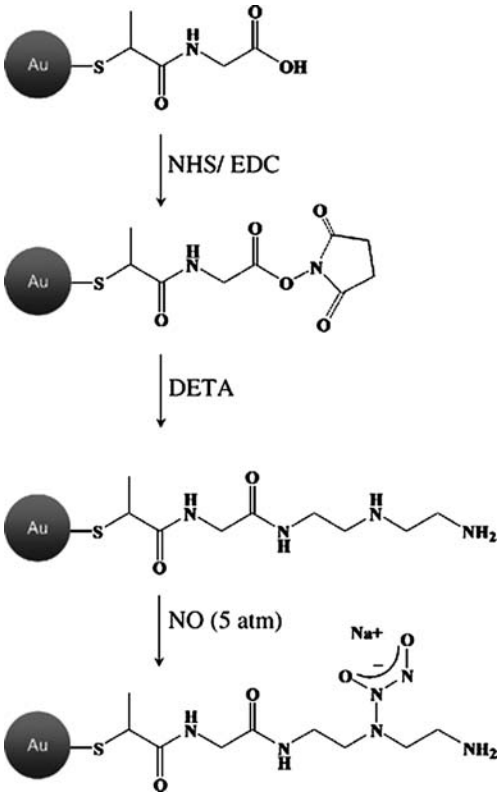

Fig. 8 Strategy for diethylenetriamine (DETA) functionalization of tio-monolayer-protected clusters (MPCs) by amide coupling, followed by *N*-diazeniumdiolate formation (reproduced from ref. 33 by permission of the American Chemical Society).

because NO is known to react rapidly with superoxide to produce the harmful peroxynitrite ($ONOO^-$) species, which easily interacts with lipids, DNA, and proteins *via* direct oxidative reactions or indirectly causing radical-mediated damage.[67,68]

The monolayer-protected gold cluster nanoparticles are very small in size (1–5 nm), have high stability, and are easily functionalized.[69] The metallic nanoparticle surfaces are easily altered by thiols containing the desired functional groups. The characteristics of these gold nanoparticles, as described above, could prove useful for a wide range of biomedical and pharmaceutical applications (*e.g.*, *in vivo* sensor design, topical creams for wound healing, and/or the dilation of blood vessels) (Fig. 7).[69]

Water-soluble NO-releasing monolayer-protected gold clusters with tiopronin (~3 nm) were functionalized with amine ligands and subsequently exposed to 5 atm of NO gas to form diazeniumdiolate NO donors covalently bound to the gold cluster.[33] Unfortunately, these particles did not efficiently store NO (<0.023 μmol mg^{-1}), therefore to increase the NO storage capacity of these gold nanoparticles, polyamine-stabilized gold clusters (~5 nm) (pentaethylenehexamine-PEHA) were synthesized and exhibited significantly enhanced NO-release (0.386 μmol mg^{-1}) (Fig. 8).[33]

(c.2) Silica nanoparticles. The synthesis of a new NO-releasing scaffold prepared from amine-functionalized silica nanoparticles was initiated through inorganic–organic hybrid silica and prepared *via* co-condensation of tetraethoxy- or tetramethoxysilane and aminoalkoxysilane with ethanol (or methanol), water, and ammonia.[70] Afterwards, the amine functional groups in the silica were converted to the NO donor *N*-diazeniumdiolate *via* exposure to high NO pressures under basic conditions (20–500 nm) with NO payloads of 50–1780 nmol mg^{-1} (Fig. 9).[70]

Shin *et al.*[70] have pointed out that the diversity of NO release kinetics, scaffold size, and favorable toxicity represent the distinct advantages of silica over previously reported nanoparticle systems. Based on the capacity to tune NO storage and release and the small size of the silica nanoparticles, this material could be important in new NO-based therapies.[70] Silica nanoparticles prepared using the pre-formation approach exhibited an up to 6–7 fold greater NO payload and NO release duration than silica synthesized *via* a post-formation method.[71]

The same group has reported the synthesis of diazeniumdiolate-modified aminoalkoxysilanes prior to nanoparticle formation, in which particle aggregation was reduced due to decreased hydrogen bonding interactions between amines during particle formation.[72] The synthesis resulted in 99% amine-to-diazeniumdiolate conversion efficiency and greater yields of NO per mole of aminoalkoxysilane precursor compared to previous synthetic procedures used to generate NO-releasing silica nanoparticles (Fig. 10).[72]

The bactericidal efficacy of the 45 mol% *N*-(6-aminohexyl) amino-propyltrimethoxysilane (AHAP3) NO-releasing silica nanoparticles has been evaluated against *P. aeruginosa*, and has been shown to be significantly more effective than NO released from the small molecule NO donor.[72] *In vitro* cytotoxicity experiments with fibroblasts have confirmed that NO-releasing silica nanoparticles are largely nontoxic to these cells at concentrations capable of killing *P. aeruginosa*.[72]

NO-releasing fumed silica particles (0.2–0.3 μm) have been synthesized by tethering alkylamines onto the silica surface using

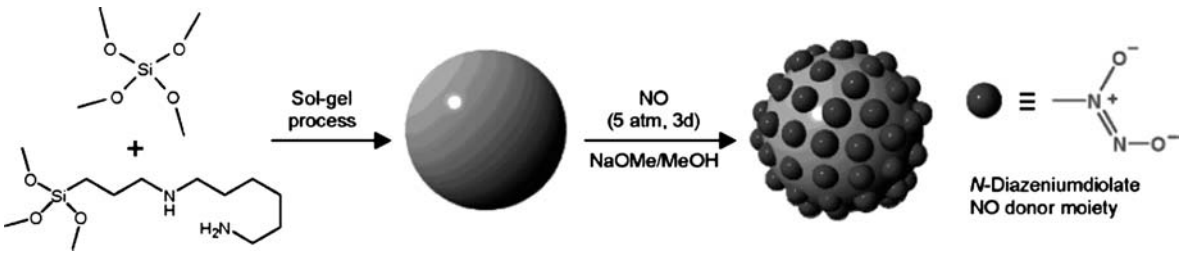

Fig. 9 The synthesis of *N*-diazeniumdiolate-modified silica nanoparticles using tetramethoxysilane (TMOS) and *N*-(6-aminohexyl)aminopropyltrimethoxysilane (AHAP3) as tetraalkoxy- and aminoalkoxysilane precursors (reproduced from ref. 70 by permission of the American Chemical Society).

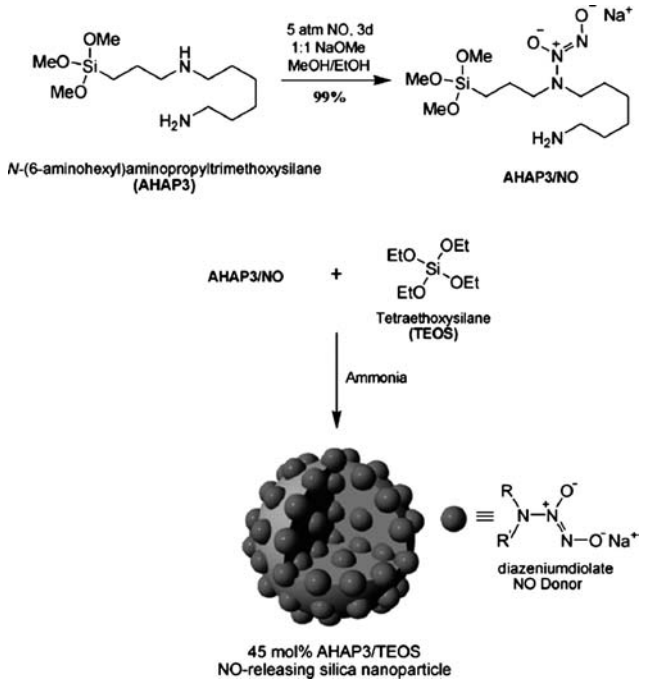

Fig. 10 The synthesis of *N*-(6-aminohexyl)aminopropyltrimethoxysilane (AHAP3) NO donors and co-condensation with tetraethoxysilane (TEOS) to form NO-releasing silica nanoparticles: R = $-(CH_2)_3Si\equiv$ and R′ = $-H_2N(CH_2)_6-$. (Reproduced from ref. 72 by permission of the American Chemical Society.)

amine-containing silylation reagents, which are converted to the corresponding *N*-diazeniumdiolate groups upon reaction with NO gas at high pressure in the presence of methoxide bases (Fig. 11).[73] It has also been shown that the resulting NO-releasing fumed silica particles can be embedded in polymer films, creating coatings that are thrombo-resistant, due to NO release at fluxes that represent normal endothelial cells. A polyurethane coating containing 20 wt% of NO-releasing particles prepared with a pendant hexane diamine structure (*i.e.*, Sil-$_2$N[6]-N_2O_2Na)

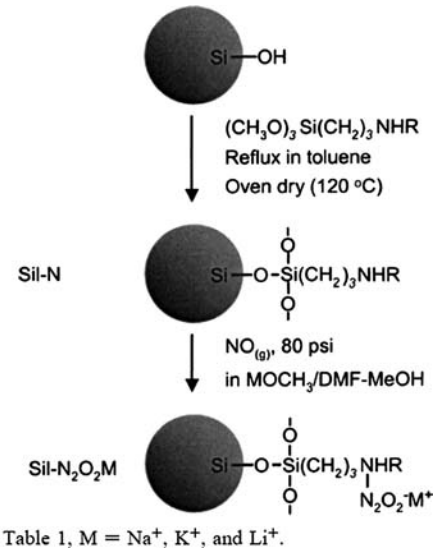

Fig. 11 The synthesis route to Sil-N_2O_2M particles (reproduced from ref. 73 by permission of the American Chemical Society).

exhibited improved surface thrombo-resistivity in a rabbit model.[73]

(c.3) Polymeric micro and nanoparticles. Due to its biocompatibility and biodegradability, poly-lactic-co-glycolic acid (PLGA) is approved for therapeutic use by the Food and Drug Administration (FDA).[74] PLGA is one of the most widely used polymers for preparing micro and nanoparticles[35,75] and a PLGA microparticle encapsulated with the NO pro-drug diethylenetriamine/NONOate (DETA/NONOate) has been produced. PLGA microparticles can protect the pro-drug from dissociation under acidic conditions and promote controlled release of NO, which is useful for medical applications, *e.g.* vaginal-specific delivery of NO.[76] The microparticles were fabricated using various solvent evaporation/extraction techniques, such as water in oil in oil (W/O/O)], followed by freeze-drying (~20 μm). The effects of microparticle fabrication conditions as well as particle size distribution, morphology and drug entrapment efficiency were also investigated. *In vitro* NO release studies performed at vaginal pH (pH 4.0) revealed that PLGA 50/50 microparticles produced by W/O/O (36% loading) released NO over 14 h following an initial burst of 50% NO released within 30 min.[76]

In addition to PLGA encapsulated with NONOates, Tfouni's group[77] reported the synthesis and characterization of the ruthenium nitrosyl complex (*trans*-$[Ru(NO)(NH_3)_4(py)](BF_4)_3^-$ H_2O), loaded into a polymeric PLGA microparticle (0.9–6.0 μm) using a double emulsification method. The choice of loading conditions was dictated by the characteristics of both the ruthenium nitrosyl complex and the microparticle matrix. At concentrations lower than 1×10^{-4} M, the nitrosyl complex did not show toxicity in B16–F10 murine cells, whereas, free in solution it was toxic at higher concentrations ($>1 \times 10^{-3}$ M), with cell death attributed to NO release following the reduction of the complex.[77] The nitrosyl complex was not cytotoxic in the dark, however it was upon light irradiation. This phototoxicity suggests that cell death is due to NO release from the complex. This model proposes an interesting system for carrying and locally delivering NO, under irradiation, to tumor cells.[77]

Similar to Tfoni's group[77,78] Mascharak's group[79–81] has also been investigating metallic complexes in biocompatible polymeric materials that photorelease NO for specific clinical applications. For example, Eroy-Revels *et al.*[80] reported the NO release with high quantum efficiency upon irradiation with visible light of a sol–gel hybrid material containing the photoactive manganese nitrosyl, $[Mn(PaPy_3)(NO)]ClO_4$. The NO-releasing sol–gel material was coated with the biocompatible polyurethane to enhance the material's stability in biological fluids. An advantage of this material is the rapid generation of NO upon irradiation with visible light at specific sites of action where NO might have biological effects, such as the induction of tumor cell death in photodynamic therapy.[82] As reported in these works, photochemical NO-release materials play an important role in the photodynamic therapy.[80,82]

Chitosan is another broadly biodegradable and biocompatible polymer that can enhance the transport of polar drugs across epithelial surfaces for several biomedical applications.[83] Diethylenetriamine (DETA) and propionic aldehyde (PA) were employed individually to modify the primary amine groups on chitosan. Two diazeniumdiolates (chitosan/NO adducts) were

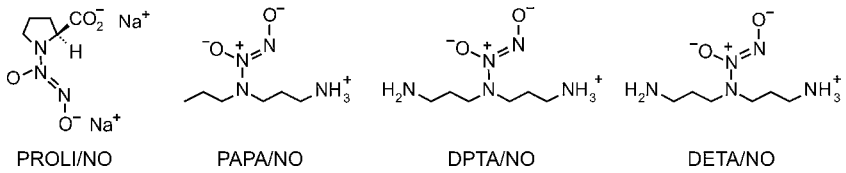

Fig. 12 The diazeniumdiolate nitric oxide donors, PROLI/NO, PAPA/NO, DPTA/NO, and DETA/NO. (Modified from ref. 24.)

prepared by suspending the chitosan derivatives in a sodium methoxide–methanol solution under 6 atm of NO for seven days.[84] The NO release profiles of the chitosan derivatives in PBS (pH 7.4, 37 °C) were investigated by the Griess assay. The results show that increased nucleophilic sites on DETA-modified chitosan results in high NO loadings, and the maximal NO release from chitosan/DETA/NO and chitosan/PA/NO was 1945 nmol mg^{-1} and 1156 nmol mg^{-1}, respectively.[84]

(c.4) Other nanoparticles. Nanotechnologies applied to NO action have led to the creation of a novel approach that successfully inhibited neointimal hyperplasia in a rat carotid artery injury model.[24] The diazeniumdiolates evaluated in this study include disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO), 1-[*N*-(3-ammoniopropyl)-*N*-(n-propyl)amino]diazen-1-ium-1,2-diolate (PAPA/NO), 1-[*N*-(3-aminopropyl)-*N*-(3-ammoniopropyl)diazen-1-ium-1,2-diolate (DPTA/NO), and 1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)-amino] diazen-1-ium-1,2-diolate (DETA/NO) (Fig. 12).[24] Among the studied gels, disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO) nanofiber gel was the most effective neointimal hyperplasia inhibitor. The authors are aware that before this new therapy can be used in clinical procedures, the toxicity as well as the efficacy of NO-releasing micro and nanopaticles must be evaluated in long-term studies using a large-animal model. All the facts indicate that this novel NO-based therapy has promising clinical potential, and its use in vascular and cardiovascular surgical procedures to prevent restenosis and the associated patient morbidity appears possible in the near future.[24]

Recently, a report revealed that a hydrogel/trehalose composite with NO is composed of nanosized building blocks that show varying degrees of aggregation.[85] This structure allowed the generation of NO through the thermal reduction of added nitrite by glucose after exposure of these robust biocompatible nanoparticles to moisture, leading to the initiation of sustained release of the trapped NO over an extended period of time. Nitrite-containing glassy matrices were prepared in a phosphate-buffered (pH 7.5) sodium nitrite solution and the sugars were then added. It was estimated that each nanoparticle was about 10 nm in diameter comprising aggregates.[85] Compared with the control particles, fibroblasts treated with NO-releasing hydrogel/glass composite particles exhibit minimal toxicity. These results support the use of these nanoparticles *in vivo* and demonstrated their potential as biocompatible therapeutics. The authors state that these new NO-releasing nanoparticles can be easily designed for different therapeutic circumstances.[85]

Photochemical NO release from biomaterials is an important tool for controlled release of NO in biomedical applications.[54,63,86] A recent review[86] covers the preparation and photochemistry of a series of transition metal complexes designed to release NO upon visible or near infrared excitation, including the works of Tfouni's,[77,87] Mascharak's[88] and Neuman's groups (the latter with quantum dots).[89,90] In particular, semiconductor quantum dots (QDs) nanocrystals (~ 2–100 nm) represent an attractive class of materials, since they are currently used as fluorophores in biomedical applications.[91] QDs are capable of enhancing photochemical NO production from electrostatic assemblies due to their high extinction coefficients.[89] An advantage of QDs is their functionalization can lead to attachment of key molecules at the nanocrystal surface to improve their water solubility and/or stability and biological specificity.[92] Such functionalization can also be used to incorporate NO precursors or carriers. Neuman *et al.*[89,90] describe the energy-transfer processes between water-soluble core/shell CdSe/ZnS QDs and the cationic complex, *trans*-Cr(cyclam)$(ONO)_2^+$. They observed an increase in the photoproduction of NO from the transition metal centers in the presence of CdSe/ZnS core-shell QDs. This is evidence for electron and/or energy transfer from QDs to nearby molecules capable of generating important radical species such as NO.

However, metals such as cadmium and selenium, two of the most widely used metals in QD core metalloid complexes, are known to be acutely and chronically toxic in vertebrates and are of considerable human health and environmental concern.[93,94] QDs may pose risks to human health and to the environment, and the toxicity of these materials needs to be carefully evaluated for biomedical applications.[93]

(d) Carbon nanotubes

Carbon nanotubes can be used for the release of NO at target sites. In this context, silver-gold bimetallic nanoparticles were prepared, as reported by Alvarez-Puebla *et al.*,[95] by the citrate reduction method and silver ions, followed by titration with $HAuCl_4$ solution (Fig. 13).

Next, multiwalled carbon nanotubes (5 nm diameter, 5–20 μm in length, and 95% purity) were sonicated at pH 10 in a solution containing NaCl and poly(allylamine hydrochloride)(PAH) forming CNTs@PAH.[96] The intermediate sample CNTs@PAH was reacted with a bimetallic nanoparticle suspension under sonication forming the CNT@AuAg hybrid colloid. The influence of gold on NO formation at physiological pH was studied by mixing a nitrosothiol solution with the CNT@AuAg hybrid colloid, where a RSNO bond was immediately cleaved due to the high affinity of gold for thiols, liberating NO (Fig. 14).[96] It has also been demonstrated that metal nanoparticles adsorbed onto carbon nanotubes can be used as a trigger for the generation of NO from an external RSNO.[96]

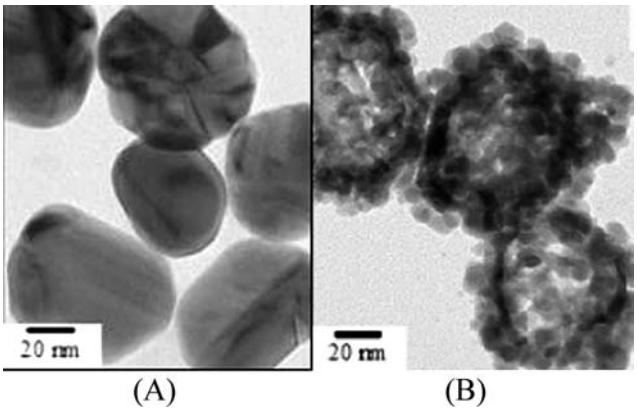

Fig. 13 Transmission electron microscopy images of (A) silver citrate colloids, and (B) silver citrate colloids treated with 15 mL of 10^{-3} M gold solution. (Reproduced from ref. 95 by permission of the American Chemical Society.)

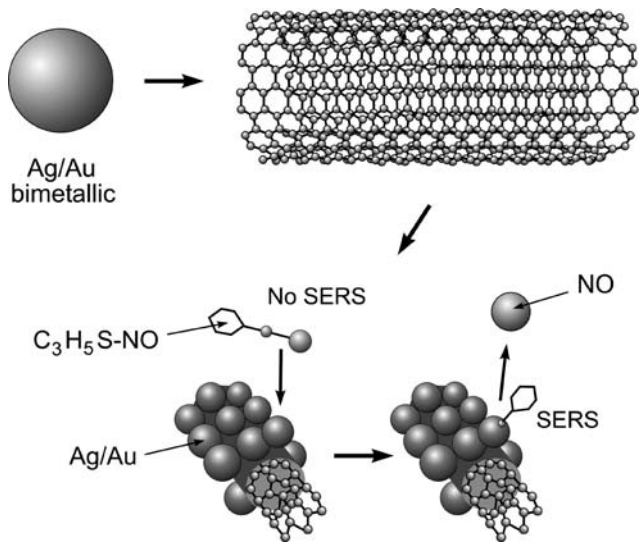

Fig. 14 A carbon nanotube containing RSNO as NO donor. (Modified from ref. 96 by permission of the American Chemical Society.)

It is known that irradiation of a metal-nitrosyl complex causes NO release when the appropriate wavelength of light is used.[87,88] This is one of the reasons why metal-nitrosyl complexes are used in photodynamic therapy to kill cancer cells.[86] However, it is difficult to find a combination that has a safe wavelength for the body as well as a fast NO release. One interesting approach is to functionalize the surface of a multiwalled carbon nanotube with a Ru–NO complex. This is possible since the nanotube can absorb light and transfer it to the ruthenium moiety, causing the release of NO. This approach aims to quickly release NO using light in the visible range rather than the ultraviolet one.[97]

(e) Micellar nanoassembly

Caruso *et al.*,[98] have reported the preparation of a photoactive micellar nanoassembly system, for cancer therapy, that controls the simultaneous release of NO and singlet oxygen upon stimulation with visible light. Fig. 15 shows the multifunctional micellar nanoassembly, which is composed of mesogen-bearing cationic amphiphilic *N*,*N*,*N*-trimethyl-10-{[4-nitro-3-(trifluoromethyl)phenyl]amino}decan-1-aminium bromide (compound **1**, Fig. 15) and the anionic 5,10,15,20-tetrakis(4-sulfonatophenyl)-21*H*,23*H*-porphyrin (TPPS) that releases NO and singlet oxygen under excitation at 450 nm and 532 nm, respectively. The photorelease of NO involves a nitro-to-nitrite rearrangement followed by the rupture of the O–N bond, generating NO and phenoxyl radical. The authors believe that the extension of this approach using a variety of *ad-hoc*-chosen NO photodonors and porphyrin derivatives may facilitate the route for the development of novel classes of nanoscaled, photoactive systems in nanomedicine.[98]

(f) Nanoporous solid materials

Nanoporous solid materials such as zeolites and metal–organic frameworks with a variety of architectures and chemical composition have been recently considered as one of the most promising vehicles in nanotechnology for releasing NO in medical applications.[99] This is based on the fact that these nanoporous solid materials are able to absorb, store, and release free NO in a controlled manner. Zeolites are highly crystalline aluminosilicate microporous materials with a rigid three-dimensional network of channels, cages and rings that share corners to produce an open framework, which is ideal for the adsorption of small gaseous molecules, such as NO.[100]

Exposure of zeolites to NO gas results in both reversible and irreversible NO adsorption. Reversibly adsorbed NO is considered to be physisorbed and NO is readily released while irreversibly adsorbed NO is not spontaneously released, since it interacts strongly with extraframework cations in these porous materials.[99,101] Due to the high affinity between NO and zeolites, zeolites absorb and store NO in high capacity (up to *ca.* 3.5 mmol of NO per g of zeolite),[102] and it has been reported that NO-loaded zeolites are stable in the anhydrous state for several months and even more than one year.[99] NO can be released in biologically relevant amounts to target sites upon contact with water, making this an attractive approach for long-term NO storage. A major advantage of using zeolites as NO-delivery vehicles is that the rate of NO release can be modulated and tailored to the desired range for medical applications by altering the metal-ion content and structure of the zeolites, as well as the vehicle containing the zeolite. It has been reported that the total amount of NO released is approximately 1 mmol of NO per g of zeolite,[100] and an initial short-lived burst of NO release followed by slower release was observed.[102]

Biological assays with NO-containing zeolites showed that they inhibit platelet aggregation and the adhesion of human platelets *in vitro* making zeolite-containing materials good candidates for the prevention of thrombus formation.[100] Fig. 16 shows scanning electron micrographs of the surface of pressed disks of Co-zeolite in 75 : 25 wt% mixtures with powdered poly(tetrafluoroethylene) (PTFE) (a) untreated and (b) NO-loaded Co-zeolite/PTFE disks. Fig. 16 (a) shows the presence of platelet aggregates (PA) on the surface of zeolite/PTFE (Z/PTFE) while only a reduced number of platelets (P) can be observed on the surface of NO-loaded zeolites/PTFE (NO-Z/PTFE) (Fig. 16 (b)).[100] Recently, Mowbray *et al.*[103] reported that the topical

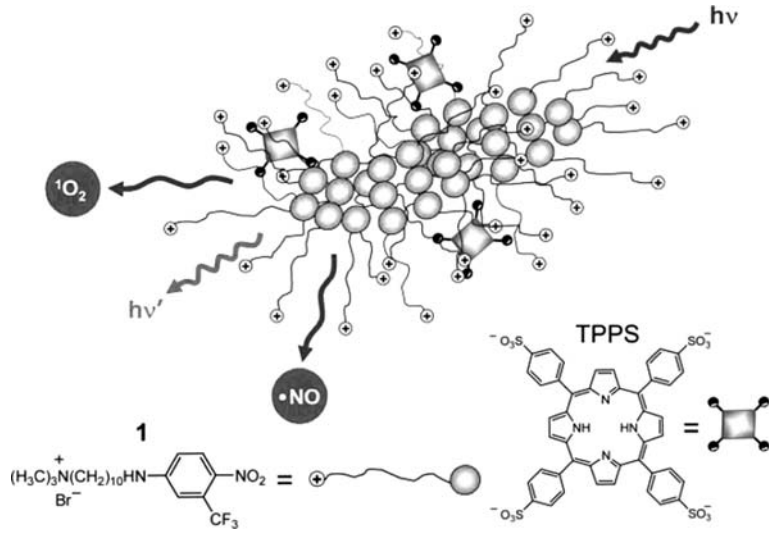

Fig. 15 Micellar nanoassembly for the photorelease of NO and singlet oxygen upon stimulation with visible light. (Reproduced from ref. 98 by permission of The Royal Society of Chemistry.)

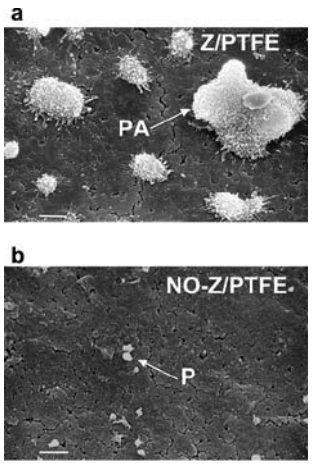

Fig. 16 Scanning electron micrographs of the surface of pressed disks of Co-zeolite in 75 : 25 wt% mixtures with powdered poly(tetrafluoroethylene) (PTFE) (a) untreated and (b) NO-loaded Co-zeolite/PTFE disks. The presence of large platelet aggregants (PA) on the surface of zeolites/PTFE disks can be observed in (a), while in (b) there are a few platelets on the surface of NO-loaded zeolites/PTFE disks. (Reproduced from ref. 100 by permission of American Chemistry Society.)

application of NO-containing zeolites increased dermal blood flow in humans. The enhanced flow was correlated with transdermal NO diffusion through the skin as assayed by cutaneous microdialysis.[103] NO released from the zeolites was able to diffuse across the epidermis and dermis to promote the local vasodilation with minimal pro-inflammatory effects, making NO-releasing zeolites good candidates for dermatological applications (such as the promotion of wound healing and treatment of cutaneous fungal and viral diseases).

In addition to zeolites, metal organic frameworks (MOFs), or porous coordination polymers, are crystalline nanoporous materials comprised of a metal or metal clusters connected three-dimensionally by organic linkers. NO can be strongly absorbed at the metal sites, and the substitution of NO by water promotes NO release from the MOFs, similar to NO release from zeolites.[99,101] Moreover, the preparation of MOFs and zeolites that contain copper(I) species (Cu^I) has been reported.[104] These materials offer an additional advantage as NO donors, since Cu^I catalyzes the biotransformation of nitrite to NO; hence the amount of NO produced is enhanced by catalytic gas production.[104]

Again, by changing the composition and structure of the MOF it is possible to control the rate of NO release and adjust it for the desired biological application. For example, a low flux of NO is required for anti-thrombogenic applications (typically 1×10^{-10} mol cm^{-2} min^{-1})[105] while higher doses are necessary for anti-bacterial effects.[106] Upon exposure to moisture, MOFs can release *ca.* 7 mmol NO per g of material,[101] and the NO released from MOFs was found to inhibit platelet aggregation[107] and promote relaxation of porcine arteries.[101]

Since zeolites and MOFs contain transition metal ions, the potential toxicity of the metals to biological systems is of concern.[107] For example, NO-releasing zeolites or MOFs when in contact with biological fluids such as blood, will lose their metal ions. Therefore, further studies are needed before these materials can be considered as NO donors, in practice.

(g) Patents and innovations

Many patents describe inventions using nanotechnology in combination with NO donors. The storage and release of NO from these donors has been considered a great challenge in medically-relevant situations, in part due to the gaseous nature of NO and its instability in the presence of oxygen. A patent by Toone and Stamler[108] provided a method of preparing some NO-releasing nanomaterials, particularly those comprising carbon nanotubes, containing NO or gases with NO-like biological activity, where the gas is non-covalently bound to the compound. These compounds allow for the storage of NO gas, followed by its controlled release. Compounds disclosed in the invention include polymers, articles, pills, capsules, and medical devices.[108]

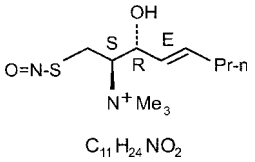

Fig. 17 The chemical structure of *S*-nitroso-*N*,*N*,*N*-trimethylsphingosine. (Modified from ref. 109.)

Another interesting patent reported liposome formation with lipids containing (a) an *S*-nitroso moiety –S–N═O, (b) an *O*-nitroso moiety –O–N═O, and/or (c) an *N*-nitroso moiety, and the preferred *N*-nitroso groups included the NONOates.[109] As a specific example, *S*-nitroso-*N*,*N*,*N*-trimethylsphingosine was synthesized by first adding a thiol group to one or more of the hydroxyl groups of *N*,*N*,*N*-trimethylsphingosine, and subsequently *S*-nitrosating the thiol. Upon delivery to the body (*e.g.*, to the vasculature) the *S*-nitroso-*N*,*N*,*N*-trimethylsphingosine of the present invention released NO, which has numerous beneficial therapeutic effects. Since it contains *N*,*N*,*N*-trimethylsphingosine (Fig. 17), this compound also inhibits platelet formation and prevents leukocyte-endothelial interactions, even after the release of NO from the compound.[109] One advantage of the described *S*-nitrosated, *N*-nitrosated and *O*-nitrosated lipids over other organic and inorganic NO donors is that they can be readily incorporated into cell membranes, promoting tissue uptake and enhancing tissue retention time. Similarly, these *S*-nitrosated, *N*-nitrosated and *O*-nitrosated lipids have an affinity for lipid deposits in the body, such as those within atherosclerotic plaques. This affinity allows for targeted NO release within such regions.[109]

Recently, another patent has described the synthesis of biodegradable and non-biodegradable nanoparticles for coating medical devices, such as intracoronary stents.[110] The nanoparticles are composed of a matrix and a bioactive agent, wherein the matrix or shell is formed of a material selected from ceramic materials, bioglass, metals, polymers, plastic, and combinations thereof. The bioactive agent is selected from many active drugs and in particular, NO donors. One method for treating a disorder in a patient consists of implanting the nanoparticle-releasing medical device, wherein the disorder is selected from, for example, atherosclerosis, thrombosis, restenosis, hemorrhage, vascular dissection or perforation, vascular aneurysm, vulnerable plaque, *etc.*[110]

In another patent, Trescony *et al.*[111] have described the preparation of a biodegradable polymer matrix impregnated with an NO donor for continuous release of NO upon hydration. In this context, poly(L-lactic acid)-CCl_4 containing a solution of $NaNO_2$ in MeOH is used to coat stents or polyurethane tubing for slow release of NO as the polymer degrades in test systems. The polymer matrix provides an acidic microenvironment that facilitates the conversion of organic or inorganic nitrite to NO. Polylactic acid slowly biodegrades to lactic acid, which facilitates the conversion of nitrite to NO. The invention also provides a material capable of releasing NO for use in the lining of blood-contacting surfaces of implantable or extracorporeal devices, so as to reduce or eliminate the undesired effects of platelet aggregation or thrombogenesis. Additionally, *in situ* production and release of NO avoids the problems associated with the short half-life of NO under physiological conditions.[111]

Schoenfisch *et al.*[112] published an invention that included at least one NO donor in combination with a second therapeutically active agent (*e.g.*, silver nanoparticles) with antimicrobial and wound healing capability. As a result, a patient may benefit from reduced dosage requirements and therefore a reduced likelihood of developing antimicrobial resistance. The compound can be formulated for local or systemic administration, for topical applications, as well as for use in coatings for medical supplies and devices.[112] In another patent, Russell *et al.*[113] proposed the use of NO-releasing zeolites for medical applications such as the inhibition of platelet aggregation to prevent thrombus formation.

(h) Toxicological evaluation of NO nanodonors

A crucial issue with regards to the clinical use of NO-releasing materials is the lack of a complete evaluation of their toxicity *in vivo*, and this applies to most of the vehicle types previously mentioned in this review. Clearance of the vehicle after NO release in biological systems must also be characterized since the majority of NO-releasing materials (dendrimers, metallic nanoparticles, QDs, carbon nanotubes, and nanoporous solid materials) contain metals in their structure, which can lead to toxicity *in vivo*. As an example, the two most widely used metals in QDs, cadmium and selenium, are known to cause acute and chronic toxicity in vertebrates.[93,94] Therefore, before these materials can be proposed for use in clinical applications, the crucial issue of their short- and long-term toxicity must first be extensively characterized and evaluated.

Summary and conclusions

Due to the crucial effects of NO as an endogenous mediator of numerous physiological processes in the cardiovascular, immune and nervous systems, and also in skin physiology, great effort has been devoted to the development of NO delivery systems for therapeutic purposes in the last few years, as reported by Miller and Megson.[34] Biomaterials that act as NO carriers and release NO in a controlled manner are highly desirable for biomedical applications. Recently, considerable improvement in the development of NO-releasing vehicles was made by combining nanotechnology and NO-delivery systems. The success of this new strategy is based on the fact that nanotechnology allied to NO delivery can overcome many of the main challenges in NO-donor therapy: generation of the desired amount of NO, for the right length of time, at the target location.

The advantage of NO-releasing dendrimers is not only that these nanoparticle-NO-donors are able to store and release large amounts of NO,[42] but also the exterior surface of the dendritic scaffold can be functionalized to improve the material's solubility or engineer it for a specific function.[45] This latter advantage is important since chemical modification of the material's surface should allow delivery of NO selectively to target cells or tissues. However, as stated by Schoenfish's group,[42] a disadvantage of this vehicle is the difficulty in controlling the NO release from diazeniumdiolates-modified dendrimers. NO control is extremely important for the biological applications of these materials, especially in the cardiovascular arena. In addition, the synthesis of dendrimers is not simple, and it involves the addition of organic solvents. Similar to dendrimers, liposomes can

incorporate and deliver high amounts of NO.[61] An advantage of these vehicles is that liposomes have been used in dermatological applications due to their biocompatibility.[51]

A major advantage of metallic nanoparticles, such as the monolayer-protected gold cluster, are their small size (1–5 nm), high stability and ease of surface functionalization.[62–66] Moreover, these materials can release NO upon irradiation, allowing their use as photoactive materials in medical applications, such as in anticancer therapy.[63] As pointed out by Shin *et al.*,[70] silica nanoparticles offer some advantages in terms of NO release kinetics, scaffold size, and lower toxicity over previously designed nanoparticle materials for NO release.

A positive aspect of polymeric micro- and nano-particles is that these materials are often comprised of the biologically friendly, FDA approved polymer poly-lactic-co-glycolic acid (PLGA).[74,75] Furthermore, PLGA micro particles protect the pro-drugs from dissociation and are able to control NO release. However, the preparation of these materials is expensive and for pharmaceutical applications the cost of micro- and nano- particle production should be lowered. PLGA can encapsulate different classes of NO donors, such as NONOates[76] and ruthenium nitrosyl complexes,[77] and release NO in a controlled manner upon irradiation. NO-releasing nano-particles are very promising materials for biomedical applications.

Transition metal complexes have been successfully used for controlled NO release under irradiation with visible light.[54,63,86] Among these materials, semiconductor quantum dot nanocrytals (QDs) offer the advantage of enhanced photochemical NO production from electrostatic assemblies.[89] Similar to dendrimers, QDs have multiple coordination sites on their surface to impart properties such as solubility, biological specificity, and to attach NO precursors.[89,92]

Carbon nanotubes can be decorated to improve NO release in biological applications. For example, metal nanoparticles[96] or Ru–NO complexes[97] adsorbed onto the nanotube surface can release NO upon irradiation with visible light.

Finally, nanoporous solid materials such as zeolites and metal–organic frameworks are very promising vehicles for NO release in pharmacological therapies due to their very rich chemistry and architecture.[99–104] These materials can absorb large amounts of NO, store the gas for over a year and release free NO without other by-products. In addition, these materials exhibit NO bioactivity and recently the dermatological application of NO-releasing zeolites in humans increased dermal blood flow due to NO diffusion across the skin with minimal inflammatory response, making these materials highly desirable candidates for dermatological applications of NO-therapy.

In summary, it is clear from the recent literature that nanotechnology will, in the very near future, enhance the application of NO in several medical disorders in which this biologically important molecule is needed. The combination of nanotechnology with NO chemistry and biochemistry to create efficient NO-releasing therapies that can be used clinically offers great promise in medicine.

The design of NO-delivery vehicles can be considered advanced in terms of the preparation of new, versatile and different classes of materials. Generally, these materials are able to load high amounts of NO, are quite stable, some are photoactive, their surfaces can be chemically modified and optimized for specific medical applications, and all of them possess demonstrable biological activity. Most materials release NO over an hour, which is useful for short-term NO therapy. However, for some medical applications, long-term NO release is necessary. For example, there is a great interest in NO-releasing blood-compatible polymeric materials for coating medical devices such as intravascular catheters, vascular grafts, coronary artery and vascular stents, and long-term vascular access devices. In these cardiovascular applications, continuous NO release over days and even months is desired.

Of course, each technology presented in this review offers advantages and disadvantages. However, the vehicle that proves to be highly efficient in NO delivery, releases NO for longer periods of time, and be fully biocompatible will, undoubtedly, find success in practical medical applications. Additionally, the therapy should be simple to perform, economically feasible, safe, and without side-effects. To this end, we need specific regulations and control of these new nanomaterials for their use in humans.

Acknowledgements

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