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Article

Nitric Oxide Releasing-dendrimers: an Overview

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Platforms able to storage, release or scavenge NO in a controlled and specific manner is interesting for biological applications. Among the possible matrices for these purposes, dendrimers are excellent candidates for that. These molecules have been used as drug delivery systems and exhibit interesting properties, like the possibility to perform chemical modifications on dendrimers surface, the capacity of storage high concentrations of compounds of interest in the same molecule and the ability to improve the solubility and the biocompatibility of the compounds bonded to it. This review emphasizes the recent progress in the development and in the biological applications of different NO-releasing dendrimers and the nitric oxide release pathways in these compounds.

Uniterms: Dendrimer. Nitric oxide. Nitrosyl complexes. Diazeniumdiolate. Nitrosothiol.

Plataformas capazes de armazenar, liberar ou capturar NO de forma controlada e específica são de grande interesse tendo-se em vista aplicações biológicas. Dentre as possíveis matrizes que podem ser utilizadas para esse fim, os dendrímeros são excelentes candidatos. Essas moléculas têm sido empregadas em sistemas para o transporte de fármacos e apresentam propriedades interessantes tais como a possibilidade de modificações químicas em sua superfície, a capacidade de estocar altas concentrações de compostos de interesse em uma só molécula e a possibilidade de aumentar a solubilidade e a biocompatibilidade dos compostos a eles ligados. Esta revisão enfatiza os recentes avanços no desenvolvimento e nas aplicações biológicas de diferentes dendrímeros liberadores de NO e a forma em que o óxido nítrico é liberado nesses compostos.

Unitermos: Dendrímero. Óxido nítrico. Nitrosilo complexos. Diazeniodiolato. Nitrosotiol.

INTRODUCTION

Chemical, biological (Fukuto *et al.*, 2012; Toledo, Augusto, 2012) and medical aspects of nitric oxide (NO) have been subject of great interest since the discovery that mammalian cells are able to synthesize it (Palmer, Ashton, Moncada, 1988; Pagliaro, 2003; Pacher, Beckman, Liaudet, 2007). This uncharged diatomic molecule is generated endogenously by three nitric oxide synthases (NOS) enzymes – endothelial NOS, neuronal NOS and inducible NOS – which catalyses the conversion of L-arginine to L-citrulline, yielding NO (Palmer, Ashton, Moncada, 1988; Ignarro, 1990; Zweier *et al.*, 1995; Zhou, Zhu, 2009). Nitric oxide, a small molecule signaling agent (Fukuto *et al.*, 2012), is associated with many biological processes (Toledo, Augusto, 2012) as vasodilatation (Furchgott, Zawadzki, 1980; Hu *et al.*, 2003; Yetik-Anacak, Catravas, 2006; Pacher, Beckman, Liaudet, 2007), immune response (Bogdan, 2001; Bogdan, Röllinghoff, Diefenbach, 2000;

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Karpuzoglu, Ahmed, 2006), as regulator of the central and peripheral nervous system (Bredt, Hwang, Snyder, 1990; Garthwaite, 1991, 1995; Moncada, Palmer, Higgs 1991; Steinert, Chernova, Forsythe, 2010), angiogenesis and muscle contractility (Reid, 2001; Stamler, Meissner, 2001).

Due to the properties outlined above, an exogenous NO deliver is a potential therapeutic agent, inasmuch deficiencies in NO biosynthesis are related to some diseases (Baylis, 2008; Luiking, Engelen, Deutz, 2010). NO can be administered in gaseous form (respiratory route) in specific cases (Serafim *et al.*, 2012), but this compound is known to react with a wide number of molecules (proteins, thiols, heme proteins, metals and others) and also can be oxidized in solution to NO_2 by O_2 (Fukuto *et al.,* 2012; Toledo, Augusto, 2012). In addition, the NO lifetime in biological media varies from 10 ms to 1 sec (Thomas *et al.*, 2001; Flores-Santana *et al.*, 2009). Furthermore local lack or excess of NO can be deleterious for biological systems. Therefore, strategies to produce stable NO carriers systems have been developed, being much of the work focused on the synthesis of nitric oxide donors (NO-donors) (Wang *et al.*, 2002; Serafim *et al.*, 2012) or scavengers (Fricker *et al.*, 1997; Davies *et al.*, 1997).

There are some representative chemical classes of NO-donors (Figure 1), as *S*-nitrosothiols (RSNOs), diazeniumdiolates (NONOates), organic nitrates and nitrites, and metal nitrosyl complexes, which were recently reviewed (Miller, Megson, 2007; Keefer, 2011; Tfouni *et al.*, 2012; Serafim *et al*., 2012).

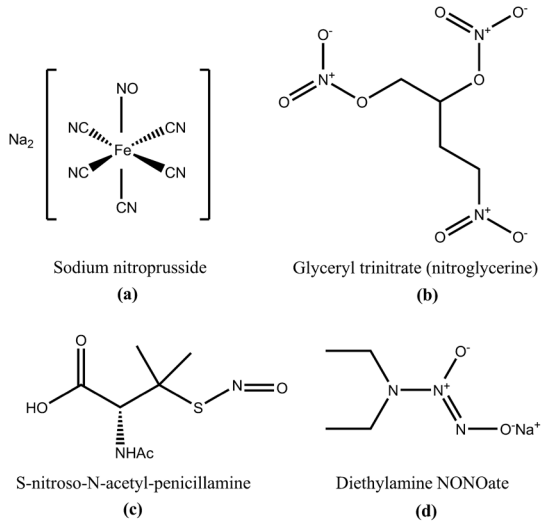

FIGURE 1 - Representative NO-donors: (a) metal nitrosyl, (b) organic nitrates, (c) S-nitrosothiols and (d) diazeniumdiolates.

Most of synthesized NO-releasing compounds have been used to help in understanding the physiologic aspects related to NO and to describe its therapeutic properties. This last topic includes as examples the protection of myocardium against ischemia/reperfusion injuries (Chan, 2002; Webb *et al*., 2004; Schulz, Kelm, Heusch, 2004), toxicity toward cancer cells (Osti *et al.*, 2012; Serafim *et al.*, 2012), trypanocidal and leishmanicidal activities (Silva *et al.*, 2007, 2009; Pereira *et al.,* 2010; Guedes *et al.*, 2010; Tfouni *et al.*, 2012).

A reasonable volume of the current research have been focused on attaching NO releasing compounds to different platforms (Eroy-Reveles, Mascharak, 2010; Tfouni *et al.*, 2010; Seabra, Durán, 2010; Riccio, Schoenfisch, 2012; Jen *et al.*, 2012), as nanoparticles (Friedman *et al*., 2008; Seabra, Durán, 2010), silica gel (Zanichelli, Sernaglia, Franco, 2006; Doro, Rodrigues-Filho, Tfouni, 2007), xerogels and dendrimers (Stasko, Schoenfisch, 2006; Stasko, Fischer, Schoenfisch, 2008; Benini, McGarvey, Franco, 2008; Lu *et al.*, 2011). These strategies aimed to improve NO-donors stability (Lu *et al.,* 2011), promote a high payload of nitric oxide on a single platform (Stasko, Schoenfisch, 2006; Stasko, Fischer, Schoenfisch, 2008) and achieve specific targets (Taite, West, 2006).

In this context, next topics deal specifically with functionalized dendrimers, tailored for NO delivery and their possible applications.

Dendrimers as a platform for NO transport

Dendrimers are molecules of the dendritic materials group, which is also composed by dendronized and hyperbranched polymers, dendrons and dandrigrafts (Carlmark *et al.*, 2009; Astruc, Boisselier, Ornelas, 2010). The main difference between dendrimers and dendrons regarding to the other dendritic polymers is related to their shape: dendrimers and dendrons are perfectly branched (Figure 2).

The concept of dendrimer-like compound was first described by Vögtle and coworkers (Buhleier, Wehner,

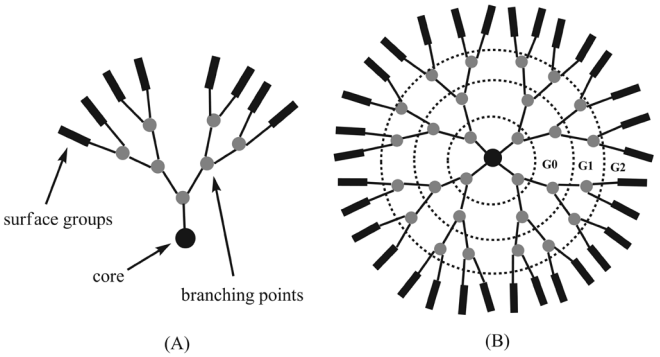

FIGURE 2 - Schematic picture of dendrons (A) and dendrimer (B). Gn (n=0–2) represent dendrimer generations: generation 0 to 2.

Vögtle, 1978), who synthesized polypropyleneimine (PPI), employing branching trough repetitive growth approach. The first dendrimer synthesis was carried out independently by two groups of researchers: Tomalia and coworkers (1985) and Newkome and coworkers (1985), whose "baptize" their molecules as "starburst polymer" and "arborol", respectively. Dendrimers are generally described as hyperbranched molecules, with a well defined structure, possessing three main architectural components (Figure 2B): core, interior layers (radially branched) which define dendrimers generations and end-groups (surface or exterior) (Tomalia *et al*., 1985).

One of the key features related to dendrimers is the possibility of tailoring its surface through chemical modifications, and their conjugation with molecules or ions of interest (Astruc, Boisselier, Ornelas, 2010; Archut *et al.*, 1998; Vögtle *et al.*, 1999). Major applications are related to the drug delivery systems development (Gillies, Fréchet, 2005; Medina, El-Sayed, 2009; Nanjwade *et al*., 2009; Wijagkanalan, Kawakami, Hashida, 2011; Sun *et al.*, 2012), solubility (Devarakonda, Hill, Villiers, 2004) and biocompatibility improvements (reduce toxicity) (Duncan, Izzo, 2005; Sadekar, Ghandehari, 2010; Ciolkowski *et al.*, 2012), magnetic resonance image (MRI) contrast (Wiener *et al.,* 1994; Margerum *et al.*, 1997; Kobayashi *et al.*, 2003; Kojima *et al*., 2011; Tang *et al.*, 2012), targeted drug delivery (Yang *et al.*, 2009; Menjoge *et al.*, 2011) and gene delivery (Dufès, Uchegbu, Schätzlein, 2005; Kim *et al.*, 2007; Mintzer, Simanek, 2009; Shcharbin, Klajnert, Bryszewska, 2009; Nam *et al.*, 2012).

The most common dendrimers (Newkome, Shreiner, 2008; Mintzer, Grinstaff, 2011) are polyamidoamine (PAMAM), polyamide, poly(L-lysine) (PLL), polypropylenimine (PPI) and PEG-polyester types, being most of them commercially available.

Together with other types of matrices (Tfouni *et al.*, 2010; Seabra, Durán, 2010; Saraiva *et al.*, 2011; Riccio, Schoenfisch, 2012), dendrimers have been used as NO-releasing carriers platforms (Taite, West, 2006; Stasko, Schoenfisch, 2006; Stasko, Fischer, Schoenfisch, 2008; Benini, McGarvey, Franco, 2008; Lu *et al.*, 2011). One of the advantages of the NO-donors dendrimers over others NO carriers systems is their ability of storage high concentrations of nitric oxide per unit of drug carrier. For example Stasko (Stasko, Schoenfisch, 2006) synthesized polypropylenimine (PPI) dendrimers of generations 3 and 5 conjugated with diazeniumdiolates (Figure 3 and Table I, compounds **1**-**7**). The highest NO storage capacity (5.6 μmol NO mg^{-1}) was achieved with the secondary amine dendrimer **1** (DAB-PO-64/NO), followed by **6** (3.4 μmol NO mg^{-1}) and **7** (3.2 μmol NO mg^{-1}), which were more stable than the ones containing primary amines (**4**, **5**) and amides (**2**, **3**) (Stasko, Schoenfisch, 2006). Syntheses of diazeniumdiolate-dendrimers (**1**-**7**) were performed using high pressure of NO gas (5 atm) in basic solution to convert primary (**4, 5**), secondary amines (**1**, **6** and **7**) and amides (**2**, **3**) to diazeniumdiolates (Stasko, Schoenfisch, 2006). Secondary amines (**1, 6, 7**) provided better conversion to diazeniumdiolates than primary amines (**4, 5**) and amides (**2, 3**), as shown in Table I.

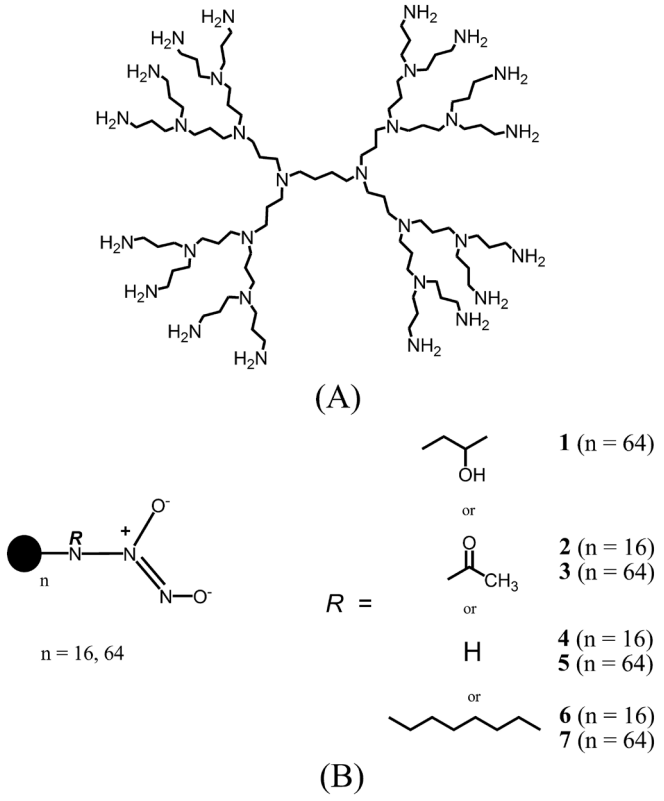

FIGURE 3 - Structure of (A) PPI dendrimer generation 3 and (B) PPI diazeniumdiolates (adapted from Stasko, Schoenfisch, 2006).

Also, PAMAM dendrimers were used as NO-releasing carriers. Stasko (Stasko, Fischer, Schoenfisch, 2008) synthesized two types of PAMAM dendrimer generation 4, with *S*-nitroso-*N*-acetyl-L-cysteine (NACysNO) or S-nitrosothiol-*N*-acetyl-D,L-penicillamine (SNAP) groups respectively attached to its surface (Figure 4 and Table I, compounds **8** and **9,** respectively). The capacity of nitric oxide storage of the two above *S*-nitrosothiol dendrimers was 2.1 ± 0.2 μmol NO mg^{-1} for **8** and 1.7 ± 0.2 μmol NO mg^{-1} for **9** (Stasko, Fischer, Schoenfisch, 2008).

Taite and West (2006) have synthesized a poly(ethyleneglycol)-lysine (PEG-lys) dendrimer in which multiple diazeniumdiolate NO-donors were generated by

TABLE I - General properties of NO-releasing dendrimers

NO-releasing dendrimer	Class of NO donor	NO-release mechanism	$t_{1/2}$ (min) (half-life of NO release)	Number of dendrimer superficial groups	% of NO conjugated on the dendrimer surface	References
DAB-PO-64/NO **(1)**		Spontaneous in physiologic condition	28	64	47 [a]	(Stasko, Schoenfisch, 2006)
DAB-Ac-16/NO **(2)**			1.4	16	<0.2 [a]	
DAB-Ac-64/NO **(3)**			2.5	64	<0.2 [a]	
DAB-Am-16/NO **(4)**	Diazeniumdiolate		12	16	2.3 [a]	
DAB-Am-64/NO **(5)**			29	64	3.9 [a]	
DAB-C7-16/NO **(6)**			77	16	38 [a]	
DAB-C7-64/NO **(7)**			86	64	36 [a]	
G4-NACysNO **(8)** G4-SNAP **(9)**	*S*-nitrosothiol	Triggered by light and/or copper	7–106 [b] 1.5–200 [b]	63 [c] / 62 ± 2 [d] 58 [c] / 49 ± 4 [d]	87 [e] 79 [e]	(Stasko, Fischer, Schoenfisch, 2008)
PEG-lys NO **(10)**	Diazeniumdiolate	Spontaneous in physiologic condition	ND	ND	83 ± 9 [a]	(Taite, West, 2006)
G0/RuNO **(11)**	Metal-nitrosyl complex (ruthenium)	Triggered by chemical reduction or light irradiation	4.12–6.08 [f]	4	100	(Benini, McGarvey, Franco, 2008)
G2/RuNO **(12)**			3.98–8.25 [f]	16	75	
G3/RuNO **(13)**			ND	32	90.6	
a-8-NO G2-PPI-ACN-NO **(14)**	Diazeniumdiolate	Spontaneous in physiologic condition	288.6	8	26.1 [a]	(Lu *et al.*, 2011)
a-16-NO G3-PPI-ACN-NO **(15)**			290.4	16	26.1 [a]	
a-32-NO G4-PPI-ACN-NO **(16)**			289.2	32	19.9 [a]	
a-64-NO G5-PPI-ACN-NO **(17)**			292.8	64	13.8 [a]	
b-8-NO G2-PPI-PEG-NO **(18)**		Spontaneous in physiologic condition	40.2	8	31.9 [a]	(Lu *et al.*, 2011)
b-16-NO G3-PPI-PEG-NO **(19)**			66.6	16	31.6 [a]	
b-32-NO G4-PPI-PEG-NO **(20)**			50.4	32	40.3 [a]	
b-64-NO G5-PPI-PEG-NO **(21)**			73.2	64	34.6 [a]	
c-8-NO G2-PPI-PO-NO **(22)**			18	8	22.7 [a]	
c-16-NO G3-PPI-PO-NO **(23)**			37.2	16	26.2 [a]	
c-32-NO G4-PPI-PO-NO **(24)**			46.8	32	27.3 [a]	
c-64-NO G5-PPI-PO-NO **(25)***	Diazeniumdiolate		63.6	64	32.1 [a]	
d-8-NO G2-PPI-SO-NO **(26)**			88.2	8	12.2 [a]	
d-16-NO G3-PPI-SO-NO **(27)**			58.2	16	10.3 [a]	
d-32-NO G4-PPI-SO-NO **(28)**			85.8	32	13.6 [a]	
d-64-NO G5-PPI-SO-NO **(29)**			97.2	64	14.5 [a]	
e-8-NO G2-PPI-ED-NO **(30)**			48.6	8	24.2 [a]	
e-16-NO G3-PPI-ED-NO **(31)**			102.6	16	21.1 [a]	
e-32-NO G4-PPI-ED-NO **(32)**			80.4	32	19.9 [a]	
e-64-NO G5-PPI-ED-NO **(33)**			112.8	64	24.7 [a]	

[a] values based on the conversion efficiency of amine or amide precursor to diazeniumdiolates. [b] depending on the trigger type (Stasko, Fischer, Schoenfisch, 2008). [c] number of thiol (NAP)/dendrimer estimated by ^{1}H NMR (the original PAMAM dendrimer has 64 end groups) (Stasko, Fischer, Schoenfisch, 2008). [d] number of thiol (NAP)/dendrimer estimated by Ellman´s assay (Stasko, Fischer, Schoenfisch, 2008). [e] estimated by the yield of NO storage efficiency (Stasko, Fischer, Schoenfisch, 2008). [f] estimated from the k_{obs} values in different pH. * similar structure of compound **1.**

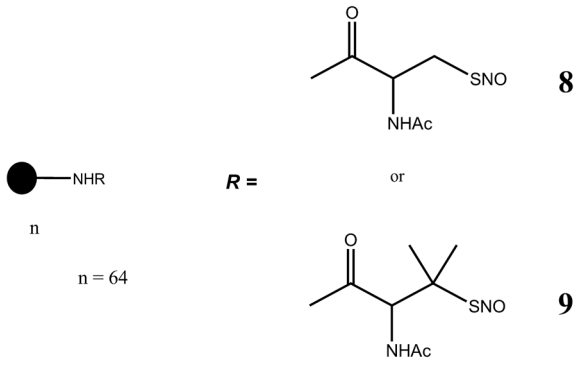

FIGURE 4 - Representation of PAMAM dendrimer with *S*-nitrosothiols (adapted from Stasko, Fischer, Schoenfisch, 2008).

the reaction of PEG-lys with NO gas (Table I, compound **10**). This reaction converted ~83 ± 9% of amines in diazeniumdiolate NO-donors.

Benini, McGarvey and Franco (2008) have functionalized PAMAM dendrimers of generation 0, 2 and 3 with the complex K[Ru(edta)Cl]. The functionalization was performed through a peptide bond between one of the carboxylate arms of the edta ligand and the superficial amines of dendrimers. This anchored chloro complex in aqueous solution yields the respective aquo species $[Ru(edta)H_2O]^-$ attached to the dendrimer surface. The reaction of this system with NO gas (6 h) yielded the NO analogous species $[Ru(edta)NO]^-$, which remain anchored on PAMAM (Figure 5 and Table I, compounds **11-13**). The percentage of superficial groups of PAMAM functionalized with ruthenium complexes were 100% for G0, 75% for G2 and 90.6% for G3 (Benini, McGarvey, Franco, 2008). According to the reported data (Benini, McGarvey, Franco, 2008), compound **13** was able to storage ~1.4 μmol NO mg^{-1}.

In addition, PAMAM dendrimer functionalized with $[Ru(edta)H_2O]^-$ can be used as nitric oxide scavenger, once $[Ru(edta)H_2O]^-$ reacts with NO to produce $[Ru(edta)NO]^-$ at the second order rate constant of ~2 x 10^7 M^{-1} s^{-1} in phosphate buffer at pH = 7.4 and 7.3 ºC (Davies *et al.*, 1997; Wanat *et al.*, 2002). NO scavengers were proposed as therapeutic agents for septic shock treatment (Davies *et al.*, 1997, Fricker *et al.*, 1997; Cameron *et al.*, 2003), and dendrimers functionalized with $[Ru(edta)H_2O]^-$ could also be used for this purpose (Benini, McGarvey, Franco, 2008).

Lu *et al.* (2011) synthesized a series of amine PPI dendrimers (generation 2–5) functionalized with different surface groups (acrylonitrile = ACN, propylene oxide = PO, styrene oxide = SO or poly(ethylene glyclol) methyl ether acrylate = PEG and 1,2-epoxy-9-decene = ED). The

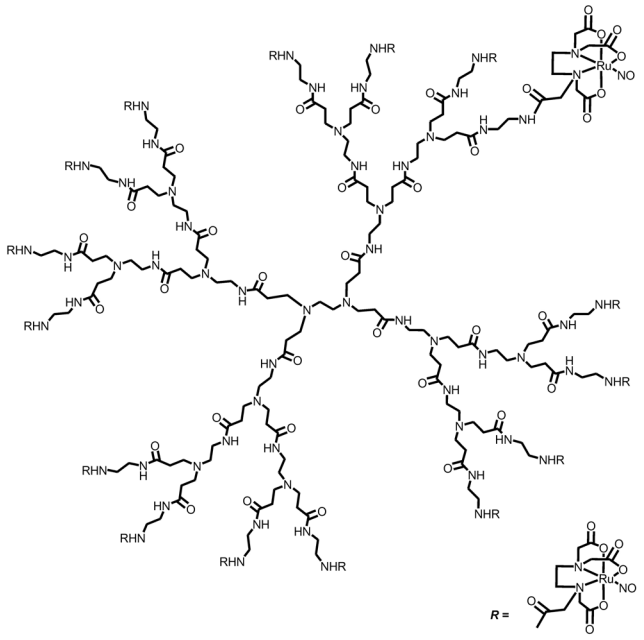

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FIGURE 5 - PAMAM dendrimer generation 2 (G2/RuNO) functionalized with $[Ru(edta)NO]^-$. (Benini, McGarvey, Franco, 2008).

authors reacted these compounds with NO gas (10 atm of NO in a methanolic sodium methoxide solution) to produce the respective diazeniumdiolates on the resulting secondary amine G2–G5 PPI (Figure 6, Table I, compounds **14-33**). The NO storage capacity of compounds **14-33** was in the range of 0.9–3.8 μmol NO mg^{-1}(Lu *et al.*, 2011), showing how dendrimer surface modifications influence the diazeniumdiolate properties (stability/release of NO).

Following a similar approach, Sun *et al.* (2012) functionalized PPI dendrimers of generation 2 and 5 with PEG, PO or SO, and then reacted the products with NO gas (10 atm, in sodium methoxide solution in methanol) to obtain compounds of similar structure of **18** and **21** for PEG, **22** and **25** for PO and **26** and **29** for the SO PPI functionalized dendrimer (Figure 6). The NO storage capacity of these compounds was in the range of 1.8-3.8 μmol NO mg^{-1} and the conversion efficiency of amines into diazeniumdiolates, between 19–35% (Sun *et al.*, 2012). Antibacterial activity of these compounds was tested against Gram-positive and Gram-negative pathogenic bacteria (Sun *et al.*, 2012), and it will be discussed in next sections.

NO-donors dendrimers: mechanism of nitric oxide release

One of the main differences observed among the NO-releasing dendrimers is their nitric oxide release

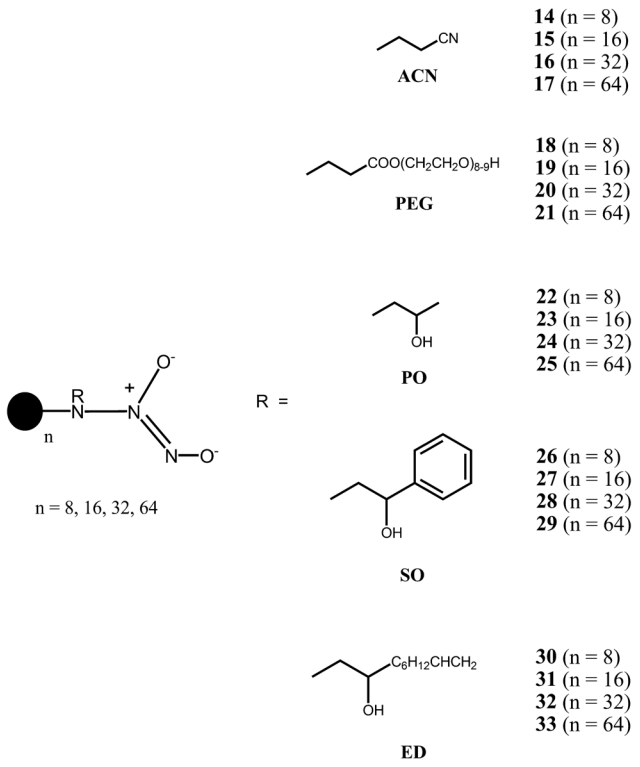

FIGURE 6 - Schematic representation of PPI dendrimer functionalized with different ligands and diazeniumdiolates (adapted from Lu *et al.*, 2011; Sun, *et al.*, 2012).

mechanism (see Table I). All diazeniumdiolate derivative dendrimers (**1-7, 10, 14-33** on Table I) release NO spontaneously under physiological conditions (pH 7.4, 37 ºC), yielding two moles of NO per mole of diazeniumdiolate (Figure 7) (Davies *et al.*, 2001; Hrabie, Keefer, 2002; Keefer, 2011).

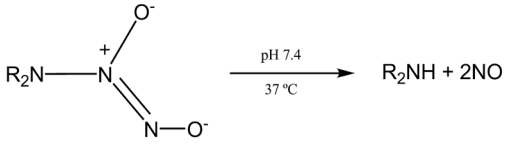

FIGURE 7 - NO release from diazeniumdiolate under physiological conditions.

Nitric oxide release in these dendrimer-diazeniumdiolates derivatives occurs through a hydrolytic degradation mechanism, initiated by protonation at the amine nitrogen (Davies *et al.*, 2001; Hrabie, Keefer, 2002; Keefer, 2011). Compound **1** and **7** (Figure 3) were able to maintain the sustained-release of NO for more than 16 h, followed by **6** (~14h), being these values the longest observed among analogous compounds (dendrimers **2-5**) and small alkyl diazeniumdiolates (Stasko, Schoenfisch, 2006). The great increase on the NO release duration time, in comparison with small alkyl diazeniumdiolates, was attributed to the dendritic effect (Stasko, Schoenfisch, 2006), which resulted in an enhancement on the diazeniumdiolate stability. This effect was also correlated with the increase in the NO release half-life ($t_{1/2}$) of NO-releasing dendrimers in comparison to the structural equivalent small molecules (Stasko, Schoenfisch, 2006).

In the other series of diazeniumdiolate PPI dendrimers (**14-33**, Figure 6), compounds **14-17** released NO for ~25 h, **18-21** for ~21 h, **22-25** for ~22 h, **26-29** for ~17 h and **30-33** for ~22 h (Lu *et al.*, 2011). For compounds **14-33**, the NO release kinetics presented a wide range of $t_{1/2}$ values, which varied from 18 to 293 min (Table 1). This broad range of values for $t_{1/2}$ was explained based on the dendrimers exterior modifications. Faster NO release (in general) observed for compounds **18-25** in comparison to others of this class (**14-17** and **26-33**) was attributed to the presence of PEG (**18-21**) or PO (**22-25**) groups on the dendrimers surface (Figure 6), which are hydrophilic and consequently facilitate the diazeniumdiolate degradation and the NO release (Lu *et al.*, 2011). This explanation was consistent with the measured $t_{1/2}$ values for NO release from compounds **26-33**, which have more hydrophobic surface groups at the exterior of dendrimer (SO or ED, Figure 6), thus resulting in higher $t_{1/2}$ values than the ones observed for compounds **18-25**.

It is interesting to note that despite the foregoing, the longest $t_{1/2}$ values for NO release in the series of diazeniumdiolate PPI dendrimers were observed for the compounds **14-17**, which have a hydrophilic cyano group on the dendrimer surface. According to the authors (Lu *et al.*, 2011), cationic groups on the neighboring of diazeniumdiolates are responsible for the additional stabilization of this class of NO-donors, thus increasing the $t_{1/2}$ values for NO release.

Also, Taite and West (2006) have synthesized another diazeniumdiolate dendrimer (**10**) that release NO for over 60 days period under physiological conditions, with the majority of the release occurring within the first 10 days.

According to Stasko, Fischer, Schoenfisch (2008) *S*-nitrosothiol (RSNO) derivative dendrimers (**8**, **9**) are able to release nitric oxide by two main pathways (Figure 8): (A) transition metal-mediated catalytic decomposition, based on Cu^{+}/Cu^{2+} redox couple and (B) photo-initiated decomposition. In the last pathway, irradiation of compounds **8** and **9** using a broad-spectrum white light resulted in homolytic cleavage of the *S*–*N* bond, generating a thiyl radical ($RS^{\bullet}$) and also liberating NO (Stasko, Fischer, Schoenfisch, 2008).

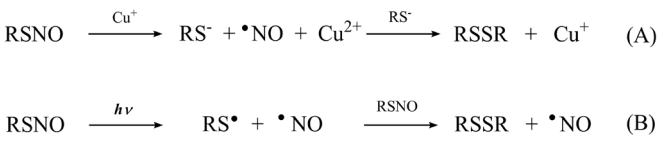

FIGURE 8 - General mechanism of nitrosothiol decomposition and NO release, (A) copper-mediated and (B) photo-initiated (adapted from Stasko, Fischer, Schoenfisch, 2008).

The rate constant for NO release was dependent on the structure of the nitrosothiol (SNAP or NACysNO) and on the NO release triggers (copper or light). This resulted in $t_{1/2}$ values for NO dissociation in the range of 7–106 min for **8** and 1.5-2.3 min for **9** when triggered by copper (0.2, 0.6 or 1 mM of Cu^{2+}), and 49–97 min for **8** and 34-200 min for **9** when triggered by light (60, 100 or 200 W) (Stasko, Fischer, Schoenfisch, 2008).

PAMAM dendrimers functionalized with ruthenium NO-donors (**11-13**) release nitric oxide after one-electron reduction, following a pathway similar to the other ruthenium nitrosyl complexes (Borges *et al.*, 1998; Gomes *et al.*, 1998; Tfouni *et al.*, 2003; Carlos *et al.*, 2004; Toledo *et al.*, 2002, 2004; Metzker, Cardoso, Franco, 2013). According to Benini, McGarvey and Franco (2008) the reaction of compounds **11-13** with Eu(II) liberates NO (Figure 9), similarly to the analogous free ruthenium nitrosyl complexes (not attached on PAMAM): the nitrosyl ligand (NO^+) is first reduced to NO^0 and then dissociates from the ruthenium coordination sphere (Benini, McGarvey, Franco, 2008). Dissociation is the reaction rate determining step.

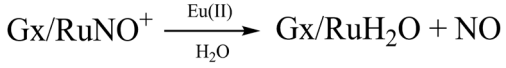

FIGURE 9 - Nitric oxide release from ruthenium nitrosyl dendrimer (Benini, McGarvey, Franco, 2008).

It is interesting to highlight that the chemical properties of ruthenium nitrosyl complexes were not altered after their attachment to the PAMAM dendrimers (Benini, McGarvey, Franco, 2008). The potential for the nitrosyl reduction (NO^+/NO^0) in the compounds **11-13** was around -0.32 V vs SCE (saturated calomel electrode), values which are very close to the ones measured for the complex $[Ru(edta)NO]^-$ (-0.34 V vs SCE). These redox potentials are accessible for the reducing agents as NADH, NADPH and cysteine present in biological media, thus enabling the *in vivo* delivery of NO from these metal nitrosyls. Also, the rate constants for NO release (k_{-NO}) in compounds **11** and **12** after one-electron reduction were in the range of 1.9–2.9 x 10^{-3} s^{-1}, which is in agreement with the previously reported (Zanichelli *et al.*, 2004) k_{-NO} value of 2.1 ± 0.4 x 10^{-3} s^{-1} for [Ru(Hedta)NO] (pH 1.0, 25 ºC). Compounds **11** and **12** showed $t_{1/2}$ values for NO release (after one electron reduction) in the range of 4.0-8.2 min (Table I) (Benini, McGarvey, Franco, 2008), which is in agreement with $t_{1/2}$ data for NO release for [Ru(Hedta)NO], that is ~5.5 min (Zanichelli *et al.*, 2004).

All together, the general features of the nitric oxide release mechanisms for all NO-donors presented above (diazeniumdiolates, *S*-nitrosothiol and metal nitrosyl complexes) were maintained when these compounds were attached to dendrimers.

Applications of NO-releasing dendrimers

Most of NO-releasing dendrimers discussed above were tested in different biological assays (Taite, West, 2006; Benini, McGarvey, Franco, 2008; Stasko, Fischer, Schoenfisch, 2008; Johnson *et al.*, 2010; Sun *et al.*, 2012). Stasko, Fischer and Schoenfisch (2008) described the ability of *S*-nitrosothiol derivative dendrimer **9** in the inhibition of thrombin-mediated platelet aggregation. According to the authors, the mechanism occurs via transnitrosation (Hogg, 2000; Walsh *et al.*, 2007; Bell, Shah, Gordge, 2007; Stasko, Fischer, Schoenfisch, 2008), a process in which a nitrosothiol transfers the nitroso group to a free thiol (present in proteins on the platelet surface). Compound **9** inhibited platelet aggregation regardless of nitrosothiol (SNAP) concentration (100, 50 or 25 mM), and at 25 mM led to a reduction of 62% in the aggregation compared to 17% for 25 mM of the analogous small molecule (similar *S*-nitrosothiol – SNAP – not attached on dendrimer). Best results for **9** in comparison to the "free" SNAP were attributed by the authors to the increase on the *S*-nitrosothiols local concentration during the transnitrosation process, due to the anchoring of the dendrimer on the cell surface (Stasko, Fischer, Schoenfisch, 2008).

Taite and West (2006) tested the capacity of compound **10** on the inhibition of platelet adhesion to thrombogenic surfaces. PEG-lys-NO dendrimers (**10**) reduced platelet adhesion in approximately 81% regarding to the analogous dendrimer that did not carry NO (PEG-Lys) (Taite, West, 2006). Another assay was performed to verify the ability of compound **10** on the regulation of vascular cell proliferation (Taite, West, 2006). First, cell viability was assessed using culture of bovine aortic endothelial cells (BAECs) and rat aortic smooth cells (SMSc), which were exposed to NO-releasing dendrimers (**10**, PEG-Lys-NO) and to ones that do not release NO (PEG-Lys). According to the authors, the NO-releasing dendrimer **10** is non toxic

for BAECs (100 ± 1% of cell viability) and SMCs (99 ± 3% of cell viability). The same test performed with PEG-Lys, resulted in cell viability of 89 ± 3% for BAECs and 87 ± 6% for SMCs. As reported (Taite, West, 2006) this result showed that even the PEG-Lys low toxicity is reduced when NO is linked to the dendrimer (PEG-Lys-NO, **10**). They also demonstrated the ability of compound **10** to selectively target an inflamed endothelium (compound **10** modified with a targeting ligand specific for inflamed endothelium – Sialyl Lewis X), as an example of the significant therapeutic potential of such NO-releasing dendrimer.

Johnson *et al.* (2010) reported the activity of *S*-nitrosothiol dendrimer **9** (G4-SNAP) in reducing ischemia/reperfusion injury. Using the same concentration of *S*-nitrosothiol (2 mM of SNAP) in G4-SNAP and in free SNAP (not attached on dendrimer), the infarct percentage (% infarct) was respectively of 3.48 ± 0.46% and 4.41 ± 1.45% (not statistically different). These values were lower than the exhibited by control (similar molecules that do not delivery NO: G4-NAP and NAP), in which the % infarct was around 11% (Johnson *et al.*, 2010).

Acute toxicity of **9** was evaluated on human umbilical vein endothelial cells (HUVEC) and pulmonary artery endothelial cells (CPA-47), using two different assays: propidium iodide uptake (PI) and the release of lactate dehydrogenase (LDH). Results demonstrated the low cytotoxicity of compound **9** in two concentrations (4 and 40 mM) after two hours of exposure: the HUVEC viability was in the range of 89.1–92.7% and the CPA-47 in the range of 87.7-92.3% (in both PI and LDH tests) (Johnson *et al.*, 2010).

The same authors (Johnson *et al.*, 2010) also evaluated the kinetics aspects of nitric oxide release from compound **9** (300 nM) initiated by different concentrations (10, 2, 1, 0.5 mM) of glutathione (GSH). The higher rate for NO release was achieved with 0.5 mM of GSH, resulting in a maximum flux of 1429 ppb NO mg^{-1} s^{-1} after 45 min. In addition, reperfusion experiments performed with GSH (0.5 mM) and G4-SNAP together (varying the concentration between 23 pM–31 nM) resulted in an optimal therapeutic dose of 0.23 nM of G4-SNAP (*i.e.*, 15 nM of SNAP). This result represents a 133 fold lower dose in comparison to the free SNAP optimal dose (2 mM), and led to only 2.34 ± 0.9 % infarct (Johnson *et al.*, 2010).

Benini, McGarvey and Franco (2008) evaluated the relaxation effects of ruthenium nitrosyl dendrimers (**11** and **13**) in denuded normotensive rat aortic rings (precontracted with noradrenaline). Slow delayed relaxation started after 15 min (14.1 ± 6.2% induced by compounds **11** and **13**, at a concentration of 3 mM of the ruthenium nitrosyl) and the maximum relaxation (36.8 ± 6.5%) was achieved in the second hour. This is on agreement with the behavior observed for $[Ru(edta)NO]^-$ in solution, for which the maximal relaxation effect in the second hour was 62.3 ± 16.5%. Compound **11** was also evaluated regarding to its trypanocidal activity *in vitro* against drug-resistant strain (Y strain) of *Trypanosoma cruzi.* At the concentration of 1.0 mM (in relation to ruthenium nitrosyl), 100% of typomastigote forms of the parasite were killed, in comparison to the 89% of activity exhibited by $[Ru(edta)NO]^-$ not attached to the dendrimer (Benini, McGarvey, Franco, 2008). The trypanocidal effect was attributed to NO release, once the non NO-releasing complex $[Ru^{III}(edta)(H_2O)]^-$ did not exhibited any activity (Benini, McGarvey, Franco, 2008).

Sun (2012) evaluated the antibacterial activity of compounds **18**, **21**, **22**, **25**, **26**, **29** (see Figure 6 and Table 1) and each respective precursor (dendrimer without diazeniumdiolate moiety – non NO-releasing dendrimers, described for simplicity as **18a**, **21a**, **22a**, **25a**, **26a**, **29a**) against *Pseudomonas aeruginosa* (Gram-negative), standard *Staphylococcus aureus* (Gram-positive) and antibiotic-resistant (methicillin) *S. aureus* (MRSA). In general, the concentration of NO-releasing dendrimers needed to kill completely *P. aeruginosa* was lower than the one required by the respective non NO-releasing dendrimers. Similar results were observed for Gram-positive *S. aureus*, except for the dendrimer functionalized with PO, in which bactericidal efficacy of compound **22a** (non NO-releasing dendrimer) was greater than for **22** (NO-releasing dendrimer). This behavior was attributed to the negative zeta potential (ζ) value for the NO-releasing **22** (-14.0 ± 5.4 mV) in comparison to the positive value for **22a** (7.1 ± 0.6 mV) (Sun, *et al.*, 2012). Once bacterial membrane is negatively charged, interactions with molecules with more positive ζ are expected to be enhanced, improving the efficiency of these compounds (Sun *et al.*, 2012). NO-releasing dendrimers (**18**, **21**, **22**, **25**, **26**, **29**) also presented greater bactericidal efficacy against antibiotic-resistant *S. aureus* (MRSA) than the respective non NO-releasing species (**18a**, **21a**, **22a**, **25a**, **26a**, **29a**) (Sun *et al.*, 2012). Nitric oxide bactericidal activity was attributed to NO byproducts such as peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3) which would drive the oxidative and the nitrosative stress (Sun *et al.*, 2012).

The same authors (Sun *et al.*, 2012) also evaluate the antibacterial activity of compounds **18**, **21**, **22**, **25**, **26**, **29** on respect to the dendrimer size (G2 *vs.* G5) and exterior functionality (PO, SO and PEG) (Figure 6). In general, dendrimer G5 (**21**, **25**, **29**) were more effective

than G2 (**18**, **22**, **26**), and this behavior was attributed to the high density of diazeniumdiolates groups, which led to NO release close to the bacteria cells. In respect to the exterior functionality, dendrimer with SO on the surface (Figure 6) were the most effective, followed by PO and PEG (bacterial activity SO > PO > PEG) (Sun *et al.*, 2012). Authors attributed this result to the association between the dendrimer surface and the cytoplasmic membrane of the bacteria (negatively charged), which is dependent on hydrophobic and electrostatic interactions, and thus favoring SO groups (more positive zeta potential and hydrophobicity) on the surface of dendrimers relative to PO and PEG (Sun *et al.*, 2012).

According to the data described above, in general, the NO-releasing dendrimers exhibit low cytotoxicity and the biological assays showed that the activity of the different NO-donors was similar or improved after the attachment to dendrimers.

CONCLUSION

The combination of classical and new NO-donors with different materials allows to expand the field of possible applications for the NO molecule, constituting an attractive therapeutic option. In general these strategies aim to solve the main challenge in NO-donor therapy: release a desired amount of NO at specific targets.

In this context, dendrimers are molecules which hold interesting properties to be used on the development of NO-carrier systems. The possibility of surface functionalization of dendrimers with one or more molecules of interest can improve biocompatibility, solubility and specificity of these systems, allowing the delivery of nitric oxide to targeted cells or tissues. Furthermore, according to the type of NO-donor conjugated with the dendrimer (metal nitrosyl, diazeniumdiolates, *S*-nitrosothiols and new ones), the mechanism of NO release can be modified to fit a desired situation, like fast and large amount of NO released, or a low and continuous NO flow. Also, the mechanism involved in the nitric oxide release can be chosen according to the purpose, from spontaneous to triggered.

The diverse NO-releasing dendrimers described in this review offers advantages and disadvantages, and exhibit inherent characteristics regarding to the NO-release mechanism. This fact allows the choice of the most appropriate NO-donor dendrimer and acts as a guide for the development of new NO-donors carriers. Also, the diverse biological applications tested so far with NO-releasing dendrimers show the potential uses of this system in different therapies and the new possibilities.

Another aspect that deserves attention is on regard to NO scavengers dendrimers. This topic has only briefly mentioned here as a consequence of a lack of literature on this regard. Certainly, synthetic aspects and biological experiments aiming septic shock control will be quite welcome.

In this context, the use of dendrimers as NO carriers is a quite promising subject of research to be explored.

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