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COMMUNICATION

NO-Responsive Vesicles as Drug Delivery System

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A rationally designed amphiphile containing a hydrophobic Hantzsch ester and a hydrophilic phosphate ester was able to form vesicles in aqueous solution, and resulted in the first example of a NO-responsive drug delivery system.

Drug delivery systems (DDSs) are extremely valuable in the treatments for various diseases. The main advantages of DDSs are the protection of reactive drugs under physiological conditions, improvement of drug solubility, fewer side effects, and better efficacy against resistant diseases. Generally, to design an effective DDS, two basic aspects should be considered: the type of the system, and the mechanism of the trigger-release. Concerning the structure of DDSs, commonly reported examples include dendrimers, micelles, vesicles, gels, etc.[1-5]Among them, vesicles have attracted increasing attention due to their unique structures and outstanding abilities, such as encapsulation and release of both hydrophilic and hydrophobic drugs in the aqueous compartments and lipid membranes. The trigger-release strategies have been extensively studied during the past years. For instance, pH, enzyme activity, redox conditions, temperature, competitive binding, magnetic field effects, light, etc.[6-14] However, NO-responsive DDSs have never been reported.

Nitric oxide (NO) is one of the smallest gaseous molecules found in nature. It is a highly reactive and multifunctional free radical, and was primarily accepted as an environmental pollutant in early years.[15,16] Recently, researchers have discovered that NO also serves as an indicator for many biological processes in the human immune system, nervous system, epithelium system and cardiovascular system.[17,18] NO is synthesized from L-arginine, NADPH and oxygen by NO synthase (NOS). There are three isoforms of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS and nNOS are referred to as constitutive NOS (cNOS), and their activity is mainly dependent on the concentration of calcium ions. The concentration of NO produced by cNOS generally ranges from fM (10^{-15} M) to pM (10^{-12} M). Unlike cNOS, various factors, including diseases, have a big impact on the activity of iNOS, leading to a concentration of NO higher than nM (10^{-9} M). As a result, NO relates to various diseases, including cancer, Parkinson's disease, hypertension, heart disease, etc.[19-21] Most of all, moderate-to-high concentrations of NO produced by iNOS are thought to cause DNA damage and promote neoplastic transformations, and become an important factor which results in tumorigenesis.[19] For instance, genetic disruption of iNOS produced an 80% reduction in urethane-induced lung tumor formation.[22] Therefore, a NO-responsive DDS would possess potential value for treating these diseases, especially cancer.

Various probes for NO detection have also been studied.[23-26] Recently, our group reported several fluorescence probes for NO detection based on the 4-substituted Hantzsch 1,4-dihydropyridines (DHP).[27-29] According to the former work, DHP is not only able to quantitatively react with NO, but also exhibits a good selectivity for NO against to other interfering reactive oxidative species (ROSs). Interestingly, when the substituent in the 4-position is an isopropyl or benzyl group, it will break away from the pyridine ring when the DHP is treated with NO.[26,30]

Taking the above information into consideration, we herein report on a rationally designed amphiphile which combines a hydrophobic Hantzsch ester and a hydrophilic phosphate ester via a tyrosol linkage (Scheme 1, **VNO**), **VNO** successfully proved to form NO-responsive vesicles for the first time according to our knowledge. 4-Benzylmodified 1,4-dihydropyridine is the key switch unit, its oxidation to pyridine by NO would result in the breakage of the benzyl linkage in the amphiphile and the rupture of the vesicles formed. The potential applications of the **VNO** vesicles in NO-responsive drug delivery were also investigated *in vivo* and *in vitro*.

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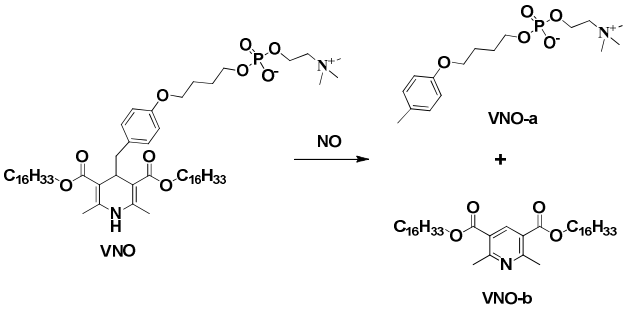

Scheme 1. Structure of **VNO** and its decomposition in the presence of NO.

The synthesis of **VNO** started from 4-bromobutoxy(*tert*-butyl)-dimethylsilane by the reaction with tyrosol in the presence of K_2CO_3, further oxidation with Dess-Martin periodinane and reaction with hexadecyl 3-oxobutanoate in the presence of ammonia, resulting in **VNO-H**. Removal of the TDMS protecting group in **VNO-H**, further phosphoration with 2-chloro-1,3,2-dioxaphospholane-2-oxide, and the final formation of the quaternary ammonium group afforded the target compound **VNO**. The details of the synthesis and full characterization of the new compounds can be seen in the supporting information.

According to the former work,[26-29] Hantzsch ester, a dihydropyridine derivative, is not only able to quantitatively react with NO, but also exhibits a good selectivity for NO against other interfering ROS, such as peroxynitrite ($ONOO^-$), superoxide ($\cdot O_2^-$), hydroxyl radicals ($\cdot OH$), etc. To evaluate the reactivity and selectivity of **VNO** with NO, ^1H NMR spectroscopy was applied to monitor the reaction process. Due to the purification problem with **VNO-a**, the standard product of **VNO**, the precursor **VNO-H** was used in the assay. The standard products **VNO-b** and **VNO-c** were synthesized by the reaction of **VNO-H** with NO in high yields (Fig. 1). **VNO-b** was also confirmed by the reaction of **VNO** with NO in dichloromethane (See the supporting information).

Upon the addition of the NO stock solution (1.9 mM in deionized water) to the solution of **VNO-H** (100 μM, DMSO-d_6 containing 20% of H_2O), the intensity of peaks of H1, H2 and H3 gradually decreased, while the intensity of peaks of H1a, H2a gradually increased (Fig. 1A). As the proportion of NO increased to 3.5 equiv., the peaks of H1, H2 and H3 totally disappeared. However, due to the insolubility of **VNO-b** in the tested solvents, the peak of H4 was never been found. Furthermore, the responses of **VNO-H** to NO and other ROSs were also compared (Fig. 1B). Except for NO, no new peaks appeared upon the addition of H_2O_2, ClO^-, NO_3^- or any other ROSs, which exhibited a superb selectivity of **VNO-H** for NO. We can infer that **VNO** also shares the same character.

The aggregation behavior of **VNO** in aqueous solution was confirmed through the following experiments including dynamic light scattering (DLS), transmission electron microscopy (TEM). Critical micelle formation (CMC) is a good indicator that describes the aggregation behavior and the stability of the aggregates formed by **VNO**, which was determined to be about 18.2 mg/ L (18 μM) by the fluorescence probe technique with pyrene as a polarity sensor. To a certain extent, the value suggested the resulting aggregates have a reasonable stability which may be suitable for other practical applications.

Particle size is an important parameter to evaluate the potential application in the encapsulation of drugs and cellular uptake. The size of the aggregates derived from **VNO** was determined by the results of DLS (Fig. S3). The measurements showed that the averaged diameters of the aggregates were around 138 nm with a narrow size distribution in aqueous solution, which were in the desired aggregate size range for the encapsulation and delivery of hydrophilic drugs.[31]

The appearance of **VNO** aggregates was further supported by TEM (Fig. 2). The black outline, which represent the lipid bilayer, indicating the aggregates are vesicles. The averaged

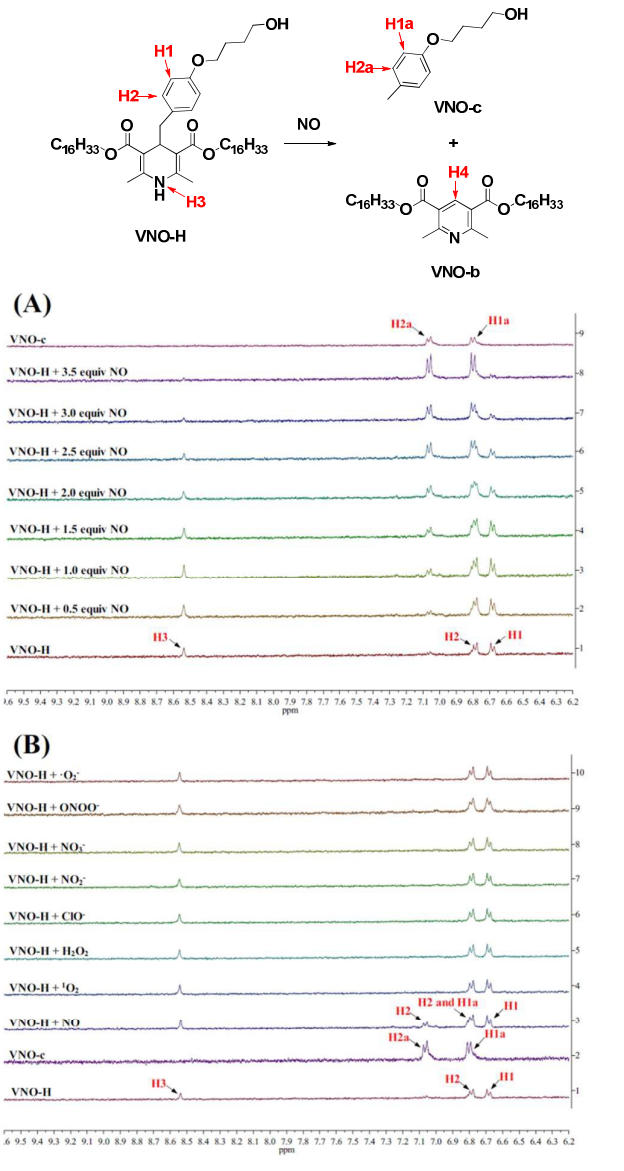

Fig. 1 ^1H NMR spectra of the **VNO-H** (100 μM, DMSO-d_6 containing 20% of H_2O) with (A) different concentrations of NO and (B) different ROSs (1 equiv.).

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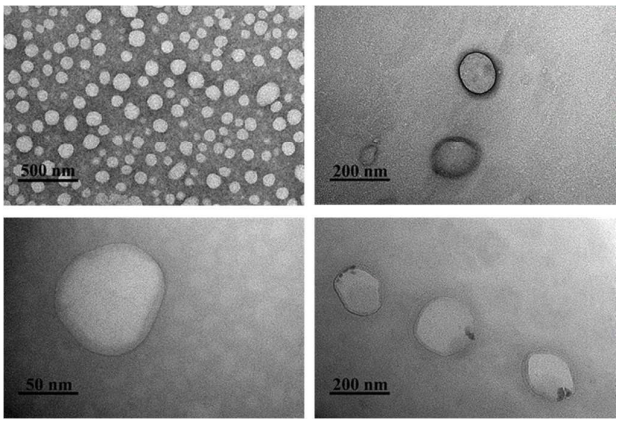

Fig. 2 TEM images of the **VNO** vesicles.

diameter of spherical vesicles is in accord with the data of DLS measurements. However, some vesicles seem to be smaller in size than the average level obtained from DLS measurements. This is probably due to collapse of vesicles during solvent evaporation in vacuum.

To investigate the encapsulation and controlled release ability of the vesicles, carboxyfluorescein (CF), a common fluorescent dye for biological assays as a model drug, was encapsulated in vesicles at a self-quenching concentration (200 mM).[31] After treated with Triton X-100, a detergent which could destruct vesicles, the dye was completely released and diluted; the increase in the fluorescence intensity at 520 nm was measured as the final release value of 100% (Fig. 3A). The release behavior proves the existence of the aqueous compartments, which is also a strong evidence indicating the aggregates are vesicles.

Then the vesicle stock suspension was treated with the NO stock solution (1.9 mM)[32] at various concentrations to evaluate the NO-triggered release of CF from the vesicles (Fig. 3B, C). Not surprisingly, as the concentration of NO increased from 0 to 50 μM (5 equiv. to the concentration of **VNO**), the fluorescence intensity at 520 nm was enhanced therewith; implying the release of CF was increasing, due to the particular reaction of DHP with NO. That was the main phenomenon to exhibit the NO-triggered drug release in vitro. CF release tended to be complete after treatment with 5.0 equiv. of NO for about 20 min at 37 °C, and over 80% fluorescent intensity was recovered. As a supplement, the reaction mixture of the vesicles with NO (1 equiv.) was characterized with MS (Fig. S2), and the dominant peak (m/z = 644.5286) given by the [M + H^+] ion of the major product **VNO-b** revealed that the trigger-release mechanism of the vesicles was indeed due to the cleavage of the benzyl linkage in **VNO**.

The vesicle stock suspension was irradiated by a laser to exhibit Tyndall effect (Fig. 3D), indicating the presence of colloidal particles. These particles disappeared after treatment with 5.0 equiv. of NO for 30 min, and the optical path of laser light was no longer visible. These observations also implied the disruption of **VNO** vesicles in the presence of NO stimulus.

To evaluate the cytotoxicity of **VNO** and its product **VNO-b**, the cell viability test on four kinds of cells, including Hela,

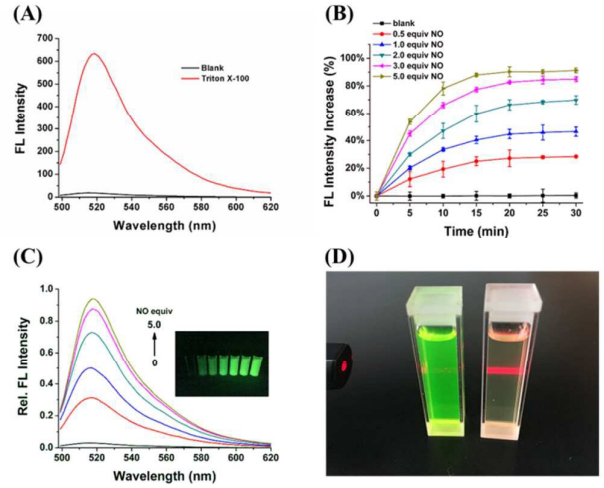

Fig. 3 (A) Fluorescent spectra of vesicle stock suspension before and after treated with 0.5 % of Triton X-100; (B) Time-dependent fluorescent spectra of vesicles in the presence of NO with different concentrations; (C) Fluorescent spectra of vesicle stock suspension after treated with NO at different concentrations for 30 min; (D) **VNO** vesicle solutions exposed to laser irradiation in the presence (left) and absence (right) of 5.0 equiv. of NO.

A549, Hek293T and Raw264.7 by the MTT assay was applied at different concentrations. Both compounds exhibited low cytotoxicity to all the four kinds of cells (Fig. S3). These results suggest compound **VNO** and its reaction product **VNO-b** are harmless to various cells, and vesicles prepared by **VNO** can act as drug carriers in biomedical applications.

The mouse macrophage cell RAW264.7 was chosen to evaluate the feasibility of NO-triggered drug release from the vesicles in living cells. Cells were first treated with different inducers, and then vesicle stock suspensions (10 μM for the concentration of **VNO**) were added and kept for another 20 min. iNOS can be induced by lipopolysaccharide (LPS) to produce large amount of intracellular NO[33]. N^G-methyl-L-arginine (L-NMA) is often used as an inhibitor to block the production of intracellular NO[34]. It can be seen that strong fluorescence in the green emission channel was observed as there is intracellular NO in cells (Fig. 4B), or exogenous NO

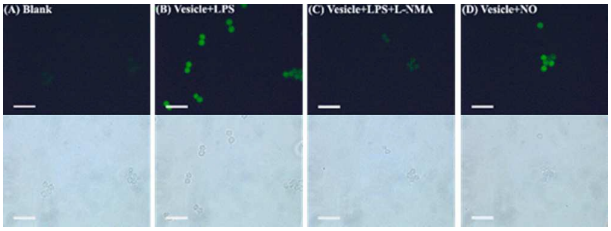

Fig. 4 Fluorescence (top) and bright field (bottom) microscopic images of RAW264.7 cells treated with (A) vesicles only; (B) LPS (1 μg/ mL) for 4 h, then loaded with vesicles; (C) NOS inhibitor (L-NMA, 2 mM) 1.5 h and incubated with LPS (1 μg/ mL) 4 h, then loaded with vesicles; (D) NO (30 μM) for 10 min, then loaded with vesicles. Scale bars: 50 μm; vesicles stock solution: 10 μM.

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produced by the addition of NO stock solution (Fig. 4D). In a sharp contrast, barely fluorescence enhancement was observed for the control group(Fig. 4A), neither in the existence of L-NMA (Fig. 4C).Taken together, the vesicles formed by **VNO** showed the possibility to be a NO-triggered drug delivery system in living cells.

In conclusion, a rational designed amphiphile **VNO** was successfully synthesized through the combination of a hydrophobic Hantzsch ester and a hydrophilic phosphate ester via tyrosol linkage and showed high reactivity and selectivity to NO. The vesicles formed by **VNO** in aqueous solution were investigated by DLS, TEM and fluorescent measurements. The disassembly of vesicles and the release of CF were fluorescently evaluated in the presence of NO. Moreover, the intracellular trigger-release of the encapsulated simulate drug was observed directly using fluorescence microscopy. This is the first example of NO-responsive drug delivery system with the potential applications in biomedicines. Further work to improve the sensitivity and put into practical applications is in progress.

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