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Nitric oxide donor superparamagnetic iron oxide nanoparticles

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ABSTRACT

This work reports a new strategy for delivering nitric oxide (NO), based on magnetic nanoparticles (MNPs), with great potential for biomedical applications. Water-soluble magnetic nanoparticles were prepared through a co-precipitation method by using ferrous and ferric chlorides in acidic solution, followed by a mercaptosuccinic acid (MSA) coating. The thiolated nanoparticles (SH-NPs) were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscopy (TEM), and vibrating sample magnetometry (VSM). The results showed that the SH-NPs have a mean diameter of 10 nm and display superparamagnetic behavior at room temperature. Free thiol groups on the magnetite surface were nitrosated through the addition of an acidified nitrite solution, yielding nitrosated magnetic nanoparticles (SNO-NPs). The amount of NO covalently bound to the nanoparticles surface was evaluated by chemiluminescense. The SNO-NPs spontaneously released NO in aqueous solution at levels required for biomedical applications. This new magnetic NO-delivery vehicle has a great potential to generate desired amounts of NO directed to the target location.

1. Introduction

It is well known that the endogenous molecule 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, immune response, neuronal communication, and wound healing [1]. On the other hand, several pathologies are associated to dysfunctions in endogenous NO production. NO is considered a unique biomolecule due to its chemical nature: small size, lack of charge and high lipophilicity, which makes NO a highly diffusible molecule capable of diffusing through cell membranes to interact with intracellular targets, without the action of membrane channels or receptors. [2]. As a free radical in the biological medium, NO can readily react with biomolecules, leading to its inactivation. Therefore, there is great interest in the development of NO-releasing drugs and matrices which are capable of stabilizing and releasing NO locally, directly into different tissues and organs [3].

In recent decades, many different systems have been proposed for drug delivery, such as micelles [4–6], nanoparticles [7,8] and polymers [9]. In these systems, the drug may be captured, attached, adsorbed, or encapsulated in or on nanomatrices [10]. Among the nanostructured materials, magnetic nanoparticles (MNPs) appear as good candidates for drug delivery [11–17], in part due to their low toxicity, and mainly because they can be guided in vivo to the specific target sites by an external magnetic field [18]. In this context, iron oxide particles, such as magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3), are the most common MNPs used for biomedical applications. These NPs are of comparable size to important biological systems (at diameters smaller than 100 nm), presenting superparamagnetic behavior at room temperature [19–22], high effective surface areas, low sedimentation rates, and improved diffusion through tissues [1,23].

In general, in vitro studies of iron oxide MNPs demonstrated little or no toxicity of this material, indicating the biocompatibility of this nanovector [24,25]. Moreover, coated nanoparticles are found to be less toxic compared to uncoated nanoparticles, due to the presence of the biocompatible coating, which also lower protein adsorption on nanoparticle surface [26]. Similarly, in vivo studies based on intravenous/intraperitoneal administrations of iron oxide MNPs coated with different biocompatible ligands showed no long-term implications of their use when administered at clinically relevant concentrations [27]. In vivo, iron oxide nanoparticles are reported to be metabolized to iron ions, which are incorporated to the biological iron storage pool, such as erythrocytes, indicating the safe use of this nanomaterial [27]. Moreover, these nanoparticles have already been tested in phase I trial in patients with prostate cancer and no systemic toxicity was reported after several months post application [28]. Taken together, these results indicate that iron oxide nanoparticles are safe for several biomedical applications.

Specific interactions with biomolecules usually require chemical modifications on the MNP surface. To our best knowledge, this is the

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first work that describes the synthesis and characterization of surface-modified MNPs as vehicles to carry and deliver NO molecules.

2. Materials and methods

2.1. Materials

Mercaptosuccinic acid (MSA), sodium nitrite ($NaNO_2$), iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), iron(II) chloride tetrahydrate, ammonium hydroxide (Sigma Aldrich Ch. Co., Inc., USA) and hydrochloric acid (12 mol/L, Synth, USA) were used as received. Aqueous solutions were prepared using analytical grade water from a Millipore Milli-Q Gradient filtration system.

2.2. Methods

2.2.1. Synthesis of magnetic nanoparticles

The NPs were synthesized by using a co-precipitation method, as previously reported [29]. In brief, 4.0 mL of $FeCl_3 \cdot 6H_2O$ and 1.0 mL of $FeCl_2 \cdot 4H_2O$ (molar ratio 2:1), prepared in 1.0 mol/L HCl, were mixed and stirred, while a volume of 50 mL of NH_4OH (0.7 mol/L) was added as precipitator. At this stage, the solution was centrifuged and the precipitate was decanted, followed by the addition of 6.0 mL of oleic acid. This mixture was then stirred for 20 min. The solution was centrifuged several times and the new precipitate was washed several times with ethanol and acetone, leading to a NP covered with oleic acid.

2.2.2. Adsorption of mercaptosuccinic acid

Oleic acid coated-NPs (~10.0 mg) were suspended in 1.0 mL of toluene while MSA was dissolved in dimethyl sulfoxide (DMSO). The two solutions were mixed and vigorously stirred for 14 h producing a black powder that was isolated by centrifugation. This procedure led to ligand exchange and, hence, to the formation of water stable thiol-containing NPs (SH-NPs).

2.2.3. Nitrosation of MSA-coated MNPs

An aqueous suspension of MSA-coated NPs was filtered in a Microcon centrifugal filter device containing ultrafiltration membranes (MWCO 10-kDa molar mass cut-off filter, Millipore, Billerica, MA, USA). The SH-NPs were washed with water and centrifuged 5 times for 10 min at 13,400 g in a Micromax RF centrifuge (Thermo IEC, Milford, MA, USA). The thiol groups present on the surface of MNPs were nitrosated through the addition of an acidified sodium nitrite solution. In this step, an amount of 4.6 mg of filtered SH-NPs was suspended in 1.0 mL of deionised water containing 5 μL of 6.0 mol/L aqueous HCl. A volume of 200 μL of aqueous sodium nitrite (60 mmol/L) was added to the SH-MNPs. After 15 min of incubation at room temperature, the nanoparticles suspension was filtered by centrifugal ultrafiltration and washed with deionised water, as described above.

2.2.4. Fourier transform infrared (FTIR) spectroscopy

Dry Fe_3O_4-MSA (1:40) nanoparticles were mixed with pure potassium bromide (KBr) powder using a w/w sample:KBr ratio of 1:100. These mixtures were ground into fine powders, pressed in a mechanical press to form translucent pellets and analyzed using a Bomen B-100 spectrometer (Hartmann & Braun, Baptiste, Quebec, Canada). A pure KBr pellet was used as background. The FTIR spectra were registered from 700 to 4000 cm^{-1} at a resolution of 2 cm^{-1}.

2.2.5. X-ray diffraction (XRD)

The diffractograms were obtained with approximately 200 mg of powdered Fe_3O_4-MSA onto a glass substrate of 2×2 cm. The measurements were performed in reflection set-up, with a conventional X-ray generator (CuKα radiation of 1.5418 Å and a graphite monochromator) coupled to a scintillation detector. The angular scanning performed on all samples ranged from 20 to 70º with 0.05º step-width at 5 s per angle. The average size of the nanoparticles was calculated from the full width at half maximum of the (311) reflection (spinel structure) using the Scherrer's equation [30].

2.2.6. Transmission electron microscopy (TEM)

Photomicrographies of the nanoparticles were obtained using a Philips CM200 transmission electron microscope (TEM) with an energy dispersive spectrometer, operating at 160 kV.

2.2.7. Vibrating sample magnetometry (VSM)

A VSM was used to obtain the magnetization versus magnetic field loop (M versus H) at room temperature up to H = 20 kOe. The apparatus was calibrated with a Ni pattern. The magnetization measurements were carried out on a known quantity of powdered sample, slightly pressed and conditioned in cylindrical Lucite holders.

2.2.8. Detection of total free NO released from nitrosated ultra filtered MNPs

The total amount of NO released from nitrosated MNPs (SNO-NPs) was measured by chemiluminescence using a Sievers chemiluminescence NO analyzer® (NOA 280i, GE Analytical, Boulder, CO, USA). Aliquots of 10 and 100 μL of aqueous suspensions of SNO-NPs (3.8 mg of nanoparticles/mL), and controls (aqueous suspension of SH-MNPs, and aqueous solution of sodium nitrite) were injected into the sampling compartment which contained 5.0 mL of an aqueous solution of ascorbic acid (160 mmol/L at pH 11). This condition allowed the detection of free NO released from S-NO groups present on nanoparticles surface, without the influence of nitrite. Calibration curves were obtained with aqueous solutions of S-nitrosoglutathione, which were freshly prepared and immediately analyzed (data not shown).

2.2.9. Kinetics of NO release from SNO-NPs in aqueous solution

The NO release profile from nitrosated nanoparticles was obtained in real time by chemiluminescence. SNO-NPs were freshly prepared by adding 2.20 mg of SH-MNPs to 200 μL of deionized water and homogenized. After homogenization, aliquots of 10 μL of this suspension were added to 3.0 mL of deionized water that contained 10 μL of aqueous HCl (0.6 mol/L). A volume of 30 μL of aqueous $NaNO_2$ (50 mmol/L) was added to the SH-NPs suspension. The final suspension was homogenized, protected from light with an aluminum foil, and kept at room temperature (25 °C) for kinetic measurements. The stability of S-NO groups present on the surface of the MNPs was kinetically monitored at 25 °C, in the dark, by injecting 5.0 μL of the sample into the NO analyzer, at different time intervals over 6.7 h. Before each injection, the samples were vigorously homogenized in a vortex. The experiments were performed in duplicate.

3. Results and discussion

The transfer of NPs from the organic to the aqueous phase provides biostability to the particles at physiological pH. Synthetic MNPs are generally surface-modified with hydrophobic ligands, becoming unstable in aqueous suspension. In this work, the nanoparticles were initially coated with oleic acid and were therefore insoluble in water. Replacement of oleic acid by MSA led to MNPs containing sulphydryl (SH) groups on the surface, as identified by FTIR spectroscopy. This technique is useful to identify the most important stretching vibrations of the MSA ligand attached on the particle surface [31].

Fig. 1 shows the IR spectrum of the synthesized NP coated with MSA at the molar ratio 1:40. The sharp band at 580 cm^{-1} is a fingerprint of Fe–O–Fe bond [32,33]. On the other hand, the peaks at 3437 cm^{-1} (νOH), 1707 cm^{-1} (νCO), 1611 cm^{-1} (ν_{assym}COO) and 1408 cm^{-1} (ν_{sym}COO) are assigned to the carboxylic group. It is possible to observe that there are two small peaks at 2845 and 2924 cm^{-1} that can be are attributed to the νSH of the free thiol group of the MSA