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Biodegradable Magnetic Silica@Iron Oxide Nanovectors with Ultra-Large Mesopores for High Protein Loading, Magnetothermal Release, and Delivery

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Abstract: The delivery of large cargos of diameter above 15 nm for biomedical applications has proved challenging since it requires biocompatible, stably-loaded, and biodegradable nanomaterials. In this study, we describe the design of biodegradable silica-iron oxide hybrid nanovectors with large mesopores for large protein delivery in cancer cells. The mesopores of the nanomaterials spanned from 20 to 60 nm in diameter and post-functionalization allowed the electrostatic immobilization of large proteins (e.g. mTFP-Ferritin, ~534 kDa). Half of the content of the nanovectors was based with iron oxide nanophases which allowed the rapid biodegradation of the carrier in fetal bovine serum and a magnetic responsiveness. The nanovectors released large protein cargos in aqueous solution under acidic pH or magnetic stimuli. The delivery of large proteins was then autonomously achieved in cancer cells via the silica-iron oxide nanovectors, which is thus a promising for biomedical applications.

Graphical abstract

Introduction

Biodegradability is a crucial feature for the biomedical application of nanotechnology.[1-11] The absence of particle biodegradability prevents almost always the approval of the food and drug administration (FDA) and other regulatory agencies to enter the pharmaceutical market. Non-degradable nanomaterials are indeed raising concerns of toxicity due to their uncontrolled bio-accumulation. The degraded products of biodegradable nanoparticles (NPs) should also to be biocompatible. Biodegradable nanomaterials include various polymer,[6] liposome,[12, 13] silicon,[14, 15] organically-doped silica,[2, 3, 5] and calcium phosphate nanosystems.[16, 17] In addition, nano-objects of hydrodynamic diameter (HD) below 10 nm are excreted by the body via the renal clearance route which prevents the toxicity associated with the bio-accumulation of particles. As a result, large biodegradable particles which can be degraded into sub-10 nm fragments such as calcium phosphate and organosilica NPs as well as renal-clearable NPs such as quantum dots have attracted increasing attention in recent years.[1] Some nanomaterials successfully reached human clinical trials such as liposomes[18] and silica C dots.[19]

The delivery of large proteins is required for the treatment of several pathologies and yet remains a persistent challenge in nanotechnology.[20-26] Proteins are often not able to be delivered selectively due to several limitations such as membrane impermeability, short lifetimes, and their susceptibility to enzymatic degradation.[23, 27] A plethora of nanocarriers has therefore been developed to deliver more

efficiently proteins under various stimuli.[23, 28] Protein carriers are grouped into four categories: lipid-based, polymer-based, protein-based, and inorganically-based nanomaterials.[22] The main advantages of the first three categories are the biodegradability of the nanocarrier and the biocompatibility of their degraded products (though not always), but typical drawbacks are often the relatively low protein loadings and the uncontrolled leakage. The protein could be either covalently attached or physically adsorbed onto the surface of the carrier, or alternatively loaded into the nanocarrier.[22] Another disadvantage of many non-porous nanocarriers is their specificity. For example, carriers covalently linked with proteins, or specific carrier-proteins combinations which allow the formation of NPs. Disadvantages of the porous nanocarriers, mostly inorganic NPs, are often twofold: a low biodegradability and limited mesopore diameters. Among the largest proteins transported by NPs, human serum albumin proteins (66 kDa, ~4 nm) were loaded into polymethacrylate-hydroxyapatite[29] and ferritin proteins (450 kDa, ~12 nm) were loaded into biodegradable polylactide NPs.[30]

Mesoporous silica nanoparticles (MSN) and mesoporous silica hybrid nanomaterials have attracted a lot of attention in a variety of research fields.[31-36] MSN are promising nanoplatfroms for nanobiomedicine due to their biocompatibility, high specific surface areas, tunable pore sizes, and known silicon chemistry.[37] Silica NPs are slowly degraded in water by dissolution into biocompatible and bioadsorbed silicic acid products,[38] and silica NPs are renally excreted.[39-41] The unique properties of MSN and silica hybrid NPs have been implemented in catalysis,[37, 42] separation,[43-45] bio-imaging,[33, 46-48] and in cargo delivery applications,[34-37, 49-51] to name a few. Large-pore silica and silica hybrid NPs have also been designed for biomedical applications.[52] Silica NPs with pore sizes ranging from 5 nm up to 40 nm have been utilized to load proteins of various molecular weights from cytochrome C (12 kDa) to immunoglobulin G (150 kDa).[53-59] Iron oxide@mesoporous silica NPs with 5-9 nm[60] and 10-15 nm[61] pores were also developed and loaded with biomolecules of sizes below 4 nm.[52] The large pore sizes available with the elaboration of novel inorganic

nanocarriers is necessary because the HD of large proteins can be significantly higher than their theoretical size, and to enable the transportation and delivery of larger cargos. One main drawback of mesoporous silica particles is their slow biodegradation which takes several weeks or months.[38, 62-64]

Herein, we describe the elaboration of biodegradable mesoporous silica-iron oxide nanohybrids with ultra-large mesopores for the high loading of large proteins and their delivery in cancer cells (Figure 1). The size, morphology, composition and the structure of the nanovectors were characterized via different techniques which revealed uniform spherical 100 nm nanocomposites with large internal cavities of 20 to 60 nm with iron oxide nanophases homogeneously incorporated within the silica walls. The novelties and advances of our study are summarized as follows: (1) we report the loading of the largest protein, mTFP-Ferritin (~534 kDa, HD of ~20 nm), in the mesoporous silica and silica hybrid family, (2) the first inorganic mesoporous NPs for large protein delivery which was demonstrated to be biodegradable (in only 3 days), (3) a high and stable protein loading (up to 23 wt%) which mainly occurred inside the nanovectors and thus may protect the proteins against degradation processes, (4) the magnetically-actuated release of large cargo in solution, (5) a multifunctional magnetic nanosystem for bio-imaging and therapy, specifically applied in-vitro for the autonomous pH-triggered delivery of large proteins into cancer cells.

Figure 1. Representation of silica-iron oxide-propylamine nanocomposites, the loading of large proteins, and the proposed protein-NPs interactions (a). Large protein delivery via silica-iron oxide NPs (b).

Results and Discussion

The silica-iron oxide-propylamine nanovectors were first synthesized by a previously reported method of our group.[65] The procedure involved the initial preparation of MSN followed by repeated infiltrations with iron chloride hexahydrate complexes, and eventually a thermal annealing step in order to form iron oxide nanophases into silica NPs while expanding the pore sizes. Using annealing temperatures of 380 or 430 °C respectively led to silica-iron oxide nanocomposites with pore sizes of 10 and 20 to 60 nm (Figure S1). Large-pore nanocomposites annealed at 430 °C were then surface-functionalized with aminopropyltriethoxysilane to electrostatically attach negatively-charged proteins into the nanovectors (Figure 1a).

The size and the morphology of the nanocomposites were then assessed via electron microscopy (Figure 1a-d). Transmission electron microscope (TEM) of silica-iron oxide nanovectors displayed uniform spheroid 100 nm nano-objects with large internal cavities connected to the particles surface with 20 to 60 nm-large mesopores (Figure 1c). Scanning transmission electron microscopy (STEM) and TEM images at various magnifications confirmed the general uniformity of the shape and of the morphology of the particles (Figures 1 and S2a-c), though a minority of small pore nanocomposites and larger nanocomposites were observed (Figure S2d). Observing these elegant nanovectors with high magnification STEM images also validated the presence of the iron oxide nanophases, as depicted by the variation of the imaging contrast (Figure 2e). Dynamic light scattering (DLS) measured a single population with a hydrodynamic diameter of 130 nm (Figure S3).

The composition of the nanocomposites was then investigated by various techniques. The successful incorporation of iron oxide nanophases and the aminopropyl functionalization of the silica nanostructure were investigated by STEM-electron energy-loss spectroscopy (EELS, see a typical spectrum in Figure S4). The homogeneous distribution of iron oxide NPs within the mesoporous silica-aminopropyl framework was demonstrated by STEM-EELS elemental mappings of silicon, oxygen, iron and carbon atoms (Figure 2e,g-j). Energy dispersive x-ray (EDX) spectroscopy confirmed the presence of silicon, oxygen, carbon, and iron atoms (Figure S5). The content of iron oxide was estimated to be of 48 wt%. The crystallinity of the iron oxide nanophases was shown by the diffraction spots on the fast Fourier transform (FFT) (Figure 2f). X-ray diffraction (XRD) revealed that the iron oxide is a combination of the α -Fe₂O₃ and γ -Fe₂O₃ phases (Figure 3a).[66] The size of iron oxide NPs that formed within the silica NPs was estimated to be 4.5 nm in average via the XRD diffractogram using the Debye-Scherrer equation. High magnification TEM supports this conclusion (Figure S6) in good agreement with Figure 2e,h.

The surface modification of silica-iron oxide NPs was confirmed by the presence of the $\nu_{\text{N-H}}$ and $\nu_{\text{C-H}}$ stretching mode of the amine groups at 1430 and 2980 cm^{-1} , respectively, in addition to the presence all the characteristic peaks of silica such as the $\nu_{\text{Si-O}}$ mode at 1080-1050 cm^{-1} typical of well-condensed networks (Figure S7). The mode of vibration observed around 600 cm^{-1} was attributed to the iron oxide $\nu_{\text{Fe-O}}$ stretching. The zeta potential of the nanocomposites was consistently reversed from -23 mV to +33 mV after the amine grafting (Figure S8). Note that, the surface functionalization of the particles was also suggested by the merged elemental mappings of Si, Fe, and C atoms (Figure S9).

The mesoporous structure of the particles was then characterized with the nitrogen sorption analysis. Figure 3b shows the isotherm of silica-iron oxide NPs and the corresponding pore size distribution curve. The nanocomposites displayed a type IV isotherm,[67] which is indicative of the mesoporosity of the material of low porosity. A surface area of 167 $\text{m}^2 \text{g}^{-1}$ for silica-iron oxide NPs was derived from the Brunner–Emmett–Teller (BET) theory. Given the high content of iron oxide, it worth noting that this would correspond to about 330 $\text{m}^2 \text{g}^{-1}$ with the density of pure silica. The presence of magnetic particles incorporated into the siliceous matrix increased the average Barrett–Joyner–Halenda (BJH) pore size ranging from 2.2 nm in MSN to 20-60 nm in the nanocomposites (Figure 3b, inset).

Figure 2. Representation of silica-iron oxide-propylamine nanocomposites (a). STEM (b,d-e) and TEM (c) micrographs of the NPs. FFT of the NPs depicting the iron oxide nanocrystals diffraction pattern (f). Elemental mapping of silica-iron oxide-propylamine NPs (g-j) from the spectrum image of (e).

Figure 3. Large angle XRD pattern of silica-iron oxide nanocomposites demonstrating the presence of α - and γ -Fe₂O₃ nanocrystallites inside the large-pore silica matrix (a). Sorption isotherm of silica-iron oxide nanocomposites (b). Inset is the pore size distribution of the NPs.

The biodegradability of silica-iron oxide nanovectors was then investigated in bio-relevant conditions. Inspired by two recent studies on iron-doped silica NPs which preferentially degraded in protein-containing media,[68, 69] we compared the stability of silica-iron oxide nanocomposites in deionized water and in fetal bovine serum (FBS) (Figure 4a). The nanomaterial was incubated at 37 °C for three days and then analyzed by TEM and DLS (Figure 4b-g). The structure silica-iron oxide NPs remained intact in water after three days, and the aggregation of the carriers was observed by TEM and confirmed by DLS data (Figure 4c,f). On the other hand, when the particles were dispersed in FBS, which contains transferrin proteins, the collapse of the structure and the formation of fragments of HD

below 50 nm occurred (Figure 4d,g). Note that the FBS medium displayed a HD of 4.8 nm presumably arising from various proteins, and thus did not interfere with the analysis of the degraded NPs (Figure S10). The drastic difference of HD before and after the particle degradation was attributed to the high binding constant of transferrin proteins with iron centers ($\sim 10^{20}$), which led to an increase of the porosity in the silica by forming “defects” which in turn accelerated the silica dissolution and degradation. It is noteworthy that, silica-iron oxide interfaces necessarily involved lower degrees of silica condensation, leading to more hydro-reactive surfaces which also promoted the degradation of the nanocarriers. The high content of iron oxide in the silica framework thus produced the first biodegradable large-pore mesoporous silica nanohybrid which is essential for safe biomedical applications and potential clinical translation. We validated the protein-mediated degradation mechanism with the evaluation the particle degradation in various media such as PBS and deionized water with or without transferrin proteins and carbonates ligands (see truth Table S1 and TEM micrographs in Figure S11). The degradation did not occur after three days in media containing only transferrin proteins or only carbonates which chelate iron ions. However, it was observed that NPs significantly disassembled in PBS with both transferrin and NaHCO_3 , and partially degraded in deionized water with both transferrin, and NaHCO_3 . The combination of transferrin and carbonates was previously reported to enhance the chelating properties of transferrin, which in this case suggested that the chelating ability of carbonates may cause the removal iron centers from transferrin, thus allowing the transferrin to further remove iron centers from the silica-iron NPs. The higher degradation in the PBS containing transferrin and NaHCO_3 also suggested that the other ions or the ionic strength have a significant role on the degradation kinetics.

Figure 4. Representation of the degradability of large-pore silica-iron oxide NPs in water and in FBS (a). TEM images of the nanovectors before (b) and after three days of dispersion in water (c) or in FBS (d), and the corresponding DLS analyses (e-g). The DLS distributions were plotted by the number of nano-objects as a function of their hydrodynamic diameter.

The potential of the nanocomposite vectors for magnetically-actuated biomedical applications was also studied. The magnetic properties of the NPs were first measured by using a superconducting quantum interface device (SQUID) magnetometer. As shown in Figure 5, the NPs exhibited a superparamagnetic behavior[70] with a saturation magnetization value of 1.65 emu g⁻¹ at 10 000 Oe and 300 K. The relaxivity r_2 was determined at 500 MHz to be 44 mM⁻¹ s⁻¹ (Figure 5b). The magnetothermal actuation of the nanovector was demonstrated by the temperature-time curves of the NP suspensions with the various particle concentrations under an alternative magnetic field (AMF) of 425 kHz (Figure S12). Higher particle concentrations resulted in greater temperature elevations. The magnetic properties of the NPs were also visualized by placing a magnet close to a dispersion of the magnetic nanovectors (Figure 5c). Hence, the nanovectors could potentially be used to magnetically actuate diagnosis and

therapeutic applications, such as magnetic resonance imaging (MRI), hyperthermia, and magnetically-actuated release of cargos.

Figure 5. Magnetization curve of silica-iron oxide nanocomposites at 300 K (a). Determination of relaxivity r_2 at 9.25 MHz (b). Photographs of a dispersion of silica-iron oxide nanocomposites after 0 and 10 minutes with a magnet.

The release of large proteins from the hybrid nanovectors in solutions was then monitored upon pH and magnetic actuations. Ferritin proteins (450 kDa) have a theoretical outer diameter of 12 nm.[71] Alexa-Ferritin (453 kDa, HD ~ 16 nm, pI 5.56) and mTFP-Ferritin (~534 kDa, HD ~ 20 nm, pI 6.33) model large proteins were selected to demonstrate the proof of principle of the delivery of large cargos requiring a nanocarrier approach. The amination of the large pore nanovectors allowed the electrostatic attachment of both proteins in aqueous solution. The successful loading of proteins was demonstrated by an array of analyses. Following loading the NPs with mTFP-Ferritin or Alexa-Ferritin protein, zeta potentials decreased from +33 mV to +23 mV and +27 mV, respectively, consistently with the slightly negative charges (-3 to -1 mV) of the proteins (Figure S13). The absorption peaks of Ferritin proteins and their conjugate fluorophores also appeared in the spectra of protein-loaded silica-iron oxide nanocomposites, indicating the successful loadings (Figure S14). STEM-EELS also validated the protein

loading by elemental analysis (Figure S15) and the quantification of Si, O, C, and Fe elements before and after loading, which followed the expected trend of the decrease of the Si and Fe percentages associated with the carrier, and the increase of the C and O percentages consistent with the additional atoms of the proteins (Figure S16). The protein payloads were calculated according to the Beer-Lambert law and UV absorbance measurements, and high loadings were obtained (227 mg Alexa-Ferritin per gram of NPs; 140 mg mTFP-Ferritin per gram of NPs). Since it was proposed that the protein attachment was mainly governed by electrostatic interactions, one hypothesis would be that the release of proteins could be actuated at a lower pH. The mTFP-Ferritin-loaded nanovectors were thus dispersed in water at physiological pH in PBS (pH 7.4) and then the pH was adjusted with HCl aliquots to pH 5.0 to mimic the lysosome, and pH 6.5 to mimic the tumor microenvironment, then the protein releases were monitored by a fluorospectrophotometer (ESI). Successfully, the rapid release of large proteins occurred efficiently at pH 5 (Figure 6a,c). In physiological conditions (pH 7.4), however, only a negligible amount of mTFP-Ferritin leaked from the NPs thanks to the electrostatic interactions between the protein and surface of the pore of the silica-iron oxide-propylamine NPs which could thus act as protective nanocarriers. The same results were obtained for Alexa-Ferritin-loaded NPs (Figure S17). Note that, the release of mTFP-Ferritin could also be triggered at pH 6.5 due to its pI of 6.33 (Figure S18).

The magnetic field was used as another stimulus to release the protein cargos (Alexa-Ferritin/mTFP-Ferritin) from the silica-iron oxide-propylamine nanovectors. This system exhibited the controllable mTFP-Ferritin protein release by switching between the on and off modes of the AC magnetic field (Figure 6b,d, see Figure S17b,d for Alexa-Ferritin). The proposed mechanism for the protein release is based on the conversion of the electromagnetic energy into thermal energy by the nanovectors, which in turns disrupts the electrostatic interactions between the loaded proteins and the pores of the superparamagnetic nanovectors. The protein release was higher and faster when the NPs

were exposed to the magnetic field at pH 5. Comparing the pH-triggered and the magnetically-triggered releases, the latter displayed a full release at pH 5 after only 3 h, which was much faster than that without the magnetic field, which occurred after 70 h. Importantly, after both the magnetically-actuated and the pH-actuated release of the proteins, TEM analyses revealed that the silica-iron oxide nanovectors remained intact (data not shown). This suggests that the protein-loaded nanovectors could first achieve their biomedical therapeutic application and then be rapidly biodegraded.

Figure 6. Representation of the pH-triggered (a) and magnetically-triggered (b) release of mTFP-proteins from the loaded silica-iron oxide nanocomposites. Release profile of mTFP-proteins from the loaded NPs via pH (c) or magnetic actuation (d). Blue rectangles indicate the magnetic actuation periods (d).

The delivery of mTFP-protein via silica-iron oxide nanocomposites in HeLa cancer cells was then performed. The good biocompatibility of the silica-iron oxide nanovectors was first confirmed in

HeLa cells (Figure S19). The loading of large proteins into the hybrid nanovectors was then expected to transport the cargos and to deliver them autonomously by the internal pH change associated with the lysosomal compartment. Incubating the loaded nanovectors into the cells, the delivery of the mTFP-Ferritin proteins was demonstrated by confocal laser scanning microscopy (Figure 7). The nuclei were stained by DAPI dyes, the cell membranes appeared in red via CellMask™, and the green color associated with the fluorescence of mTFP-Ferritin protein was observed in the cytoplasm (Figure 7b). In order to confirm the role of the nanovectors, fluorescence-activated cell sorting (FACS) was performed on the free mTFP-Ferritin and on the mTFP-Ferritin-loaded nanovectors covalently linked with rhodamine X dyes (Figure S20). As expected, silica-iron oxide nanovectors allowed the internalization of mTFP-ferritin into cells, while free mTFP-Ferritin proteins were not internalized (Figures S21-22).

Figure 7. CLSM images of mTFP-proteins from the loaded silica-iron oxide nanocomposites in HeLa cells. Nuclei were stained in blue with DAPI dyes (a), mTFP-Ferritin protein appear with the green fluorescence (b), cells membranes in red with CellMask™ (c), and merged images (d).

Conclusion

We described a multifunctional biodegradable magnetic hybrid nanovectors capable of protecting and delivering large proteins (~534 kDa, HD ~20 nm) into cancer cells. The hybrid nanomaterials morphology, composition, and structure were extensively studied through various techniques. The unique large cavities of the particles were used to electrostatically immobilize high contents of large proteins up to 23 wt%. The loaded proteins were stable in neutral conditions and could be released at pH 5 or with a reversible 'on/off' magnetic actuation. The hybrid nature of the particles combined the advantages of both the silica and the iron phases, which were respectively the control of the morphology, porosity as well as functionalization, and, the magnetic responsiveness which could potentially be used for MRI and the magnetically-actuated delivery of large cargos. Additionally, synergistic features were found with the combination of the silica and the iron phases such as the enhanced biodegradability of the carrier, due to the removal of iron centers in physiological conditions and the subsequently accelerated silica dissolution. The nanosystem thus provides the proof of concept of the use of mesoporous silica-iron oxide nanocomposites for the delivery of large cargos with a biodegradable nanovector for the next generation of biomedical applications.

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