Designed Multifunctional Nanocomposites for Biomedical Applications

By Humphrey H. P. Yiu, Hong-jun Niu, Ellen Biermans, Gustaaf van Tendeloo, and Matthew J. Rosseinsky*

The assembly of multifunctional nanocomposite materials is demonstrated by exploiting the molecular sieving property of SBA-16 nanoporous silica and using it as a template material. The cages of the pore networks are used to host iron oxide magnetic nanoparticles, leaving a pore volume of 0.29 cm$^3$ g$^{-1}$ accessible for drug storage. This iron oxide–silica nanocomposite is then functionalized with amine groups. Finally the outside of the particle is decorated with antibodies. Since the size of many protein molecules, including that of antibodies, is too large to enter the pore system of SBA-16, the amine groups inside the pores are preserved for drug binding. This is proven using a fluorescent protein, fluorescein-isothiocyanate-labeled bovine serum albumin (FITC-BSA), with the unreacted amine groups inside the pores dyed with rhodamine B isothiocyanate (RITC). The resulting nanocomposite material offers a dual-targeting drug delivery mechanism, i.e., magnetic and antibody-targeting, while the functionalization approach is extendable to other applications, e.g., fluorescence–magnetic dual-imaging diagnosis.

1. Introduction

Multifunctional nanomaterials are highly sought after in biomedical sciences because of their potential in areas such as diagnosis,[1] drug delivery,[2] hyperthermia,[3] stem cell therapy,[4] and tissue engineering.[5] In particular, introduction of physical functionality, such as magnetic,[6] optical,[7] and dielectric properties,[8] into nanomaterials is desirable for many advanced techniques in biomedicine. For example, functionalized magnetic nanoparticles have been developed into new transfecting agents[9] while biocompatible quantum dots have been used for immunoassay applications.[10] Nonetheless, assembling multifunctional nanomaterials is not straightforward as several functional entities have to be built inside a small space.[11] Given recent advances in nanomaterials synthesis, it is now timely to address the assembly of multifunctional nanomaterials. Here our target is to synthesize nanomaterials for drug delivery with combined magnetic and antibody-targeting properties.

Drug carriers with magnetic nanoparticles respond to an external magnetic field and can be used as a magnetic–targeting agent for drug delivery. Use of magnetic nanomaterials for drug delivery has been widely regarded to be a feasible targeting mechanism.[12] Magnetic targeting has already shown some success in animal testing[13] and clinical trials (Phase I/II) for cancer treatments.[14] On the other hand, targeting using antibodies and antigens is also a hot topic in drug delivery.[15] Attachment of antibodies onto the drug carriers for cancer cell targeting has been reported since 1995.[16] As a result, a combination of these targeting mechanisms in one drug carrier may achieve drug delivery with enhanced efficacy. However, research in developing materials for dual-targeting is still limited, and only a few recent reports have been focused on materials with the combination of magnetic and antibody-targeting capability.[17] This is because assembly of magnetic and antibody entities, and drug storage capacity, into one single particle can be problematic.

To make this design feasible, a base material with a sophisticated structure is needed to carry the multifunctional entities. Mesoporous molecular sieves with long-range ordered porous structures, such as the MCM series[18] and the SBA series,[19] are ideal template materials for assembling these dual-targeting carriers and many reports have already described the use of these materials for delivery of various drugs in vitro[20] and for other medical applications in vivo.[21] Several key unique characters of these molecular sieves are 1) a high surface area (400–1000 m$^2$ g$^{-1}$) to tether a large number of functional groups for drug binding; 2) a large pore volume (0.5–1 cm$^3$ g$^{-1}$) to provide a high drug loading capacity and space for nanoparticles of materials such as Fe$_3$O$_4$; and 3) a narrow pore size distribution acting as a molecular selector and excluding large protein molecules from entering the pores. In this way, the protein molecules are solely tagged to the outside of the surface for maximum targeting while the internal cavity is reserved for drug binding. Since antibodies are a subcategory of proteins, the same tagging procedure applies, provided the antibody molecules are too large to enter the pores of SBA-16. Among the many mesoporous molecular sieves, SBA-16 was chosen because of its 3D porous structure to give a high diffusion rate of drug molecules, its large cages for impregnation of Fe$_3$O$_4$.
nanoparticles with a minimum size of 6 nm, and its pore window size of ca. 3 nm for molecular sieving purposes. Composite materials such as dextran–iron oxide are also magnetic and can be functionalized to store drugs. However, these materials usually have a poorly defined structure with large pores (>18 nm).[22] Without the molecular sieving property, protein molecules will be bound to the amine groups inside the pores, and no available functional groups will be available for drug binding after protein-tagging. Also use of biodegradable polymers (e.g., dextran) in drug binding is usually associated with poor control of drug release, and crosslinking is commonly required.[22,23]

In this paper, our aim is to introduce a protocol to assemble complex nanocomposite materials with a number of functionalities by exploiting the physical and chemical properties of some basic materials. To demonstrate such an assembly, we have designed a porous, nanostructured drug carrier with combined magnetic and antibody-targeting capability, with a step-by-step method to prepare multifunctional porous materials, using SBA-16 as a template (see Fig. 1). First, magnetic iron oxide particles were impregnated into the SBA-16 cages by thermal decomposition, followed by reduction. This iron oxide–silica nanocomposite was then functionalized at low temperature with amine groups using 3-(aminopropyl)triethoxysilane in order to create a drug storage capacity and functionality for binding antibodies. Finally, antibody rabbit immunoglobulin G (IgG) was tagged on the outside of the particle in order to establish a generic binding mechanism for antibodies onto these nanocomposite particles. An amine-functionalized magnetic iron oxide–silica particle tagged with fluorescent protein was also prepared in this work—the fluorescent labeling allows the particles to be tracked during in vivo experiments. A combination of antibody-tagged and fluorescently labeled particles is also possible using similar assembling protocols. The method introduced in this work can also be extended to assemble materials with other properties, such as catalytic (for enzymatic therapies or in vivo drug synthesis) and conducting (for new diagnostic applications), thus expanding the range of multiple-function materials available for biomedical applications.

2. Results and Discussion

2.1. Characterization of SBA-16 Particles

The use of ordered mesoporous silica SBA-15 as a template for the synthesis of iron oxide–silica nanocomposites has been demonstrated previously.[24] In this work, SBA-16 mesoporous silica was chosen as the template because of its 3D interconnected porous structure (1m3m), with six windows of around 3 nm arranged as a cube on one cage.[25] With cages of around 6 nm, it is an ideal host material for iron oxide nanoparticles large enough to possess a sufficient magnetization for magnetic targeting. It is known that, for nanoparticles, the magnetization decreases as the particle size decreases and Fe3O4 nanoparticles of a >5 nm diameter have been commonly used for biomedical applications. Moreover, blockage may be caused in the 2D hexagonal porous structure of SBA-15 (5–7 nm in pore size) by impregnation of iron oxide nanoparticles.

This may limit the use of these composite materials for applications such as drug storage and delivery. Other ordered mesoporous silicas, such as SBA-1 and MCM-48, also have 3D porous structures, but their small pore diameters would inhibit the growth of the iron oxide nanoparticles to <2.5 nm, which is likely to produce composite materials with a low overall magnetization value. SBA-1, prepared using cetyltrimethylammonium bromide as surfactant template, has the Pm3n symmetry with a much smaller pore size (2–2.5 nm).[26] MCM-48, prepared with cetyltrimethylammonium bromide (CTAB) as surfactant template, belongs to the Ia3d space group with a pore size of 2.5–4 nm.[26] Considerations of the nanoscale structures of the templates strongly impact the carrier material selection, as high magnetization values and facile diffusion are key characteristics of the targeted composite. However, the required magnetization of particles varies depending on the targeting environment. For example, higher targeting efficiency is likely to be observed in regions of slower blood flow.[27] and the flux densities at the target site need to be on the order of 2 kOe. In general, Fe3O4 nanoparticles of 5–10 nm in diameter are used for magnetic targeting. Research into magnetic nanoparticles for targeted drug delivery in vivo has been reported on a mouse model.[17] In this work, γ-Fe2O3 particles of 5–10 nm with a magnetization of 22 emu g−1 (or 1.76 emu mmol−1 Fe) at 20 kOe were used for drug delivery of the anticancer agent platin. If larger iron oxide particles are required, mesoporous silicas with a large cage size can be used as a template for preparing these composite materials.

![Figure 1. Schematic illustration of the design of antibody-tagged NH2-FeOx-SBA-16.](image-url)
materials. For example, Kruk and Hui recently reported a FDU-12 material (closely related to SBA-16) with 16–26 nm cages.\[28\]

In general, spherical particle shapes are widely accepted as best, though other morphologies can be desirable for certain applications. We have used spherical particles here as many researchers have reported that sharp edges of a solid may cause inflammatory response.\[29\] Crystalline mesoporous silica materials tend to have sharp edges, which may not be ideal for medical applications. For the synthesis of mesoporous silica, the use of a cosolvent, such as isopropanol, is a common protocol to prepare spherical silica particles.\[30\] Figure 2a,b show the scanning electron microscopy (SEM) images of the SBA-16 particles synthesized for this work using isopropanol as a morphology controlling agent. These particles are homogeneous in size (5 to 8 \(\mu\)m in diameter, which is comparable with the size of human red blood cells) and of an “aggregated sphere” morphology.

The transmission electron microscopy (TEM) image shown in Figure 2c depicts the highly ordered pore structure of SBA-16. The nitrogen adsorption and desorption isotherm at 77 K for the calcined SBA-16 sample prepared with the use of isopropanol shows a typical type IV isotherm with a hysteresis loop for \(0.4 < P/P_0 < 0.7\) (Fig. 3a). The Brunauer–Emmett–Teller (BET) specific surface area, measured from the nitrogen adsorption isotherm, was found to be 705.7 \(\text{m}^2\ \text{g}^{-1}\), with a Barrett–Joyner–Halenda (BJH) pore volume of 0.59 \(\text{cm}^3\ \text{g}^{-1}\). These data, in conjunction with the TEM study, suggest that the use of isopropanol did not affect the long-range order of the SBA-16.

![Figure 2](image-url)

**Figure 2.** Electron microscopic analysis of a–c) SBA-16, d–f) Fe\(_2\)O\(_3\)-SBA-16, and g–i) FeO\(_x\)-SBA-16. For SBA-16 with isopropanol as morphology modifier, the SEM images in (a) and (b) show that the particles are of aggregated spheres morphology; and the TEM image (c) of these SBA-16 particles reveals its cubic porous structure. For Fe\(_2\)O\(_3\)-SBA-16, the high-resolution (HR)-TEM image (d) with fast Fourier transform (FFT, inset) shows that the particle size is around 5–7 nm. TEM images show that the cubic porous structure (e) has been preserved while the iron(III) oxide particles in (f) are arranged in an ordered pattern. The inset magnified an area of well-arranged nanoparticles (scale bar = 20 nm). When compared with FeO\(_x\)-SBA-16, no significant change in particle size was observed from HR-TEM (g) with FFT (inset), and the cubic porous structure is still retained as shown in the TEM image (h). In (i), after reduction, the FeO\(_x\) particles were still in an ordered pattern with the magnified area shown in the inset (scale bar = 20 nm).
2.2. Magnetic FeO\textsubscript{x}-SBA-16 Nanocomposite Particles

Use of the large cavity of nanoporous silica for hosting metal oxide or metal has already been exploited by scientists and is widely available in literature.\cite{31} For example, impregnation of various forms of iron oxides inside nanoporous silica SBA-15 has been reported.\cite{24} In this work, in order to ensure all iron oxide nanoparticles are inside the porous network of SBA-16, a two-solvent system was adopted to prepare the composite material.\cite{32} Unlike the conventional wet impregnation procedure, calcined SBA-16 was suspended in an nonpolar solvent (\textit{n}-hexane in our case) and the iron precursor Fe(NO\textsubscript{3})\textsubscript{3}/C\textsubscript{19}H\textsubscript{2}O was dissolved in a small amount of a polar solvent (acetonitrile), which must be immiscible with the other solvent. When the Fe(NO\textsubscript{3})\textsubscript{3} solution was added to the SBA-16 suspension, Fe(NO\textsubscript{3})\textsubscript{3} will be adsorbed inside the porous network of SBA-16. To ensure that Fe(NO\textsubscript{3})\textsubscript{3} was not adsorbed on the outside, the maximum amount of polar solvent used has to be calculated from the pore volume of SBA-16 (0.59 cm\textsuperscript{3} g\textsuperscript{-1}). After adsorption of Fe(NO\textsubscript{3})\textsubscript{3} onto the SBA-16 particles, the composite was heated to 500 °C at a low heating rate (2 °C min\textsuperscript{-1} suggested by Delahaye et al.), and Fe\textsubscript{2}O\textsubscript{3}-SBA-16 is formed after thermal decomposition.\cite{32} However, such a thermal decomposition is likely to form a mixture of ferromagnetic γ-Fe\textsubscript{2}O\textsubscript{3} and paramagnetic α-Fe\textsubscript{2}O\textsubscript{3}.\cite{33}

In Figure 4b, the powder X-ray diffraction (XRD) pattern of Fe\textsubscript{2}O\textsubscript{3}-SBA-16 shows two broad peaks at 2\textdegree = 20°–35° and 70°–80° with a shoulder at around 40°. The broad peak at 20°–35° corresponds to the amorphous silica of the SBA-16 template (see Fig. 4a). The shoulder at around 35°–40° was due to the broadened reflections (104) and (110) of α-Fe\textsubscript{2}O\textsubscript{3} and (331) of γ-Fe\textsubscript{2}O\textsubscript{3}. The particle size of iron oxide in the sample FeO\textsubscript{x}-SBA-16 was estimated to be 6.1 nm from the (331) and (440) reflections using the Scherrer equation.

Figure 3. Nitrogen adsorption isotherm of a) SBA-16 (○ and ●); b) Fe\textsubscript{3}O\textsubscript{4}-SBA-16 (Δ and △); and c) FeO\textsubscript{x}-SBA-16 (● and ○). The black symbols denote the adsorption points, and the white symbols denote desorption points.

Figure 4. Powder XRD of a) calcined SBA-16; b) Fe\textsubscript{3}O\textsubscript{4}-SBA-16; c) α-Fe\textsubscript{2}O\textsubscript{3} powder (indexes with reference pattern 01-086-0550), and d) γ-Fe\textsubscript{2}O\textsubscript{3} nanoparticles (indexes with reference pattern 00-024-0081); e) FeO\textsubscript{x}-SBA-16; f) Fe\textsubscript{3}O\textsubscript{4} nanoparticles as reference materials (indexes with reference pattern 01-077-1545). The particle size of iron oxide in the sample FeO\textsubscript{x}-SBA-16 was estimated to be 6.1 nm from the (331) and (440) reflections using the Scherrer equation.
γ-Fe$_2$O$_3$, while the broad peak at 70°–80° was due to the broadened reflections (214) and (300) of α-Fe$_2$O$_3$ and (400) of γ-Fe$_2$O$_3$. Such broadening of peaks is due to the small particle size and poor crystallinity. For example, the (311) reflection at 2θ = 41.5° of a 5–6 nm (size of the cage in SBA-16) particles gives a broadening of 1.97°–1.64° at half peak height. Although it is well-known that thermal decomposition of Fe(NO$_3$)$_3$ forms mixed phase Fe$_3$O$_4$,[34] it is difficult to identify the precise phase assemblage from the pattern recorded for Fe$_2$O$_3$-SBA-16 due to this line broadening. In Figure 2d, fast Fourier transforms (FFT) of the high-resolution (HR)-TEM images of the iron oxide nanoparticles correspond to diffraction along zone-axis [111] and [143] of α-Fe$_2$O$_3$ iron oxide. Interplanar distances, 2.25, 2.28, 1.51, and 1.74 Å, measured in the second FFT correspond with known interplanar distances along (111), (212), (301), and (123) of α-Fe$_2$O$_3$: $d_{111} = 2.151$ Å, $d_{212} = 2.323$ Å, $d_{301} = 1.514$ Å, and $d_{123} = 1.692$ Å. This result show that the nanoparticles are α-Fe$_2$O$_3$ iron oxide with a trigonal rhombohedral structure with cell parameters $a = b = c = 5.03$ Å and $\alpha = 60° = 55.3°$. To remove the paramagnetic α-Fe$_2$O$_3$ phase and increase the magnetization at low magnetic field, the Fe$_2$O$_3$-SBA-16 was reduced using 5% H$_2$ in N$_2$ at 350 °C, as suggested in the literature.[35] In Figure 4, the powder XRD patterns of the iron oxide–silica composite materials before and after reduction reveals that the majority of α-Fe$_2$O$_3$ had been converted to Fe$_3$O$_4$, part of which has been possibly re-oxidized to γ-Fe$_2$O$_3$. Therefore, we denoted the material as Fe$_2$O$_3$-SBA-16. It is difficult to quantify the extent of reduction from α-Fe$_2$O$_3$ to Fe$_3$O$_4$, because of the size broadening of peaks for nanoparticles. The re-oxidation from Fe$_2$O$_3$ to γ-Fe$_2$O$_3$ is also difficult to quantify as the XRD patterns of these two species are similar due to their closely related spinel structures. On the other hand, the magnetic behavior of these two iron oxides is also similar. Therefore the amount of re-oxidation does not have a significant effect on the overall magnetization of the Fe$_2$O$_3$-SBA-16, and the targeted functional properties. The key point is that the initially formed low-magnetization α-Fe$_2$O$_3$ is further transformed into the high-magnetization spinel-related phases in this second step.

In Figure 2g–i, the TEM images of FeO$_x$-SBA-16 show that the iron oxide particles are embedded inside the nanoporous network of the SBA-16 template while the cubic porous structure of the SBA-16 was retained after the impregnation and reduction treatments. The particle size of the iron oxide nanoparticles was around 5–7 nm, which is consistent with the estimation (6.1 nm) calculated from the XRD pattern using Scherrer equation, as shown in the HR-TEM image (Fig. 2g). FFT of the HR-TEM image of the iron oxide nanoparticles within this reduced material correspond to diffraction along zone-axis [111] of a face-centered cubic structure (Fig. 2g inset). This reveals that the measured interplanar distances along (220) and (224) are 3.06 and 1.77 Å respectively, which are in good agreement with the known interplanar distances $d_{220} = 2.96$ Å and $d_{224} = 1.713$ Å of Fe$_2$O$_3$. The results confirmed that the nanoparticles in the reduced sample are Fe$_3$O$_4$ iron oxide with a cubic face-centered structure with cell parameter $a = 8.39$ Å. The reduction treatment therefore forms face-centred iron oxides, which give an enhanced magnetization. Careful examination of the location of the iron oxide particles suggests that these nanoparticles are locked inside the cages of SBA-16 as the distance between the particles is very uniform at around 5 nm (Fig. 2i). In general, the particle size of many nanoparticles is likely to increase after high-temperature (350 °C) post-synthetic treatment due to sintering to reduce the surface area. Here, SBA-16 acts as a cage to keep the iron oxide nanoparticles apart as depicted in Figure 2e,f, and hence sintering is avoided. As a result, there is no increase in particle size of the iron oxide nanoparticles observed after the reduction process, since the cage structure of the host permits reduction without sintering.

The magnetic properties of Fe$_2$O$_3$-SBA-16 and FeO$_x$-SBA-16 were studied using a superconducting quantum interference device (SQUID) magnetometer. Figure 5 shows the magnetization (M) versus the applied field (H) plot for the samples at 300 K; in both cases, saturation was not reached at 50 kOe. At 50 kOe, the magnetization of Fe$_2$O$_3$-SBA-16 and FeO$_x$-SBA-16 was found to be 1.2 and 2.3 emu g$^{-1}$ with an increase of over 90% due to the reduction of α-Fe$_2$O$_3$ to Fe$_2$O$_3$. At 300 K, the magnetization of Fe$_2$O$_3$ at saturation is 92 emu g$^{-1}$ (7.11 emu mmol$^{-1}$ Fe) for bulk material[36] and only 11 emu g$^{-1}$ (0.85 emu mmol$^{-1}$ Fe) for 5 nm nanoparticles.[37] In comparison, Fe$_3$O$_4$-SBA-16, which has a Fe:9 Si atomic ratio, also shows a magnetization value of 1.42 emu mmol$^{-1}$ Fe, which is comparable with the value for 5–10 nm γ-Fe$_2$O$_3$ nanoparticles from the literature.[38] For Fe$_3$O$_4$ nanoparticles and other nanomaterials, it is known that the magnetization value at saturation decreases as the particle size decreases.[39] Despite the increase in magnetization from the reduction treatment, it is likely that some of the α-Fe$_2$O$_3$ was not reduced. Based on the magnetization data, the amount of γ-Fe$_2$O$_3$ can be estimated to be 18.5% before reduction, and the total magnetic iron oxide (both γ-Fe$_2$O$_3$ and Fe$_2$O$_3$) has been increased to 37.5% upon reduction. To increase the magnetization value of FeO$_x$-SBA-16, increasing the iron oxide content is a logical solution. However, that has to be balanced against the reduction of the internal space for drug storage. The other solutions are to increase the crystallinity of the iron oxide nanoparticles or to further reduce them to metallic iron nanoparticles, which have a higher magnetization value than iron oxide nanoparticles but would be unstable in vivo.

The nitrogen adsorption and desorption isotherm of FeO$_x$-SBA-16 shows a typical mesoporous structure with a hysteresis loop at around $P/P_0=0.4$ to 0.6. The specific BET surface area of
FeO$_x$-SBA-16 was measured to be 444 m$^2$ g$^{-1}$, which means that a large surface area for carrying amine functional groups remains. The BJH pore volume of 0.29 cm$^3$ g$^{-1}$ shows that a high internal volume is available for drug storage.

It is essential to study the stability of FeO$_x$-SBA-16 nanocomposite particles if they are to be used in vivo. Results from dissolution experiments suggest that these nanocomposite particles are likely to decompose inside the human body over a long period of time. In Figure 6, about 25% of the total silica was dissolved in water at 37°C after 3 weeks while the dissolution was even faster in phosphate buffered saline (PBS) or simulated body fluid (SBF), up to 80% and 60%, respectively after 3 weeks. However, only 5% of the iron was detected in PBS, and no detectable amount of iron was found in double deionized (dd) water or SBF. In PBS, it is possible that a detectable amount of iron oxide nanoparticles was released to the solution when the SBA-16 was dissolved, while this is less likely in SBF when only 60% of the silica was dissolved.

Nanoporous silicas such as SBA-15 and MCM-41 are known to be biocompatible and biodegradable. Results from our dissolution experiments suggested that the impregnation of iron oxide did not change their biodegradable nature. As a result, after several weeks, these nanocomposite materials will break down, disperse throughout the body, and finally be excreted in the urine. The slow dissolution of FeO$_x$-SBA-16 suggests that drug can be released not only by ion exchange, which is a commonly used mechanism, but also through degradation of the silica carrier. In this case, target drugs are not limited to charged molecules, and they can also be chemically bound onto the silica. As a result, the delivery and release of drugs can be more controlled with slow degradation. Also, this dissolution property makes FeO$_x$-SBA-16 suitable not only for drug delivery, but also for other biomedical applications such as diagnosis, as the particles will not be permanently inside the body. However, the nature of the iron material after dissolution is difficult to confirm due to its small quantity, and the metabolism of iron from this composite needs further research.

![Figure 6](image)

**Figure 6.** Experimental results from dissolution of FeO$_x$-SBA-16 in deionized water (black), phosphate buffered saline PBS (white) and simulated body fluid SBF (gray). The % Si dissolved was calculated from amount of Si measured in the supernatant using inductively coupled plasma–atomic emission spectroscopy (ICP-AES) and the maximum Si content from the sample weight.

### 2.3. Amine-Functionalization of FeO$_x$-SBA-16 Particles for Drug Storage

In order to exploit the high remaining surface area and pore volume of FeO$_x$-SBA-16 nanocomposite particles for drug storage, organic functional groups need to be tethered so that the drug molecules bind onto the multifunctional carrier through strong drug-to-surface interaction. For example, to bind a drug such as negatively charged ibuprofen or aspirin, the surface of the particles needs to carry positive charges. The introduction of amine groups is widely used in preparing drug carriers (see Fig. S1, Supporting Information). A standard silylation process using triethoxy- or trimethoxysilylane is commonly used to functionalize silica materials. We adopted the same procedure to prepare amine-functionalized FeO$_x$-SBA-16. It is worth noting that such functionalization has to be carried out after impregnation of iron oxide, or any inorganic entities, as the high temperature involved in the impregnation process will decompose any organic functionality.

From the results of the elemental C, H, and N analysis, amine-functionalized particles NH$_2$-FeO$_x$-SBA-16 were found to have 1.91 mmol amine groups per gram of sample on the surface. The Fourier transform (FT) IR spectrum of NH$_2$-FeO$_x$-SBA-16 also confirms the amine functionality on the surface (Fig. S2, Supporting Information). This amine functionalization introduced a large number of positive charges. As a result, the zeta potential (measured in dd H$_2$O with no buffer) of the particles increased from $-0.3$ to $+66.3$ mV. Such an increase suggests that negatively charged drug molecules will be bound to the surface of the NH$_2$-FeO$_x$-SBA-16. In a drug binding experiment, NH$_2$-FeO$_x$-SBA-16 was found to bind 0.25 mmol of ibuprofen and 0.21 mmol aspirin. These values are equivalent to 13% and 11% coverage of the amine groups. The zeta potential of the ibuprofen-loaded and aspirin-loaded NH$_2$-FeO$_x$-SBA-16 was recorded as +20.4 and +38.1 mV, respectively. The decrease was due to the negatively charged drug binding onto the positively charged amine sites. The residual positive charge was due to the unbound amine sites. Higher coverage can be difficult to achieve, as some amine groups may not be accessible to the drug molecules while others may cause steric hindrance to each other and be prevented from interacting with amine groups. In addition, the FeO$_x$ particles inside the cages are also likely to cause further hindrance.

The drug release profile of ibuprofen from FeO$_x$-SBA-16 and NH$_2$-FeO$_x$-SBA-16 is shown in the supporting information (Fig. S3). Amine functional groups delayed the release due to the electrostatic interaction. However, the release is faster than other amine-functionalized mesoporous materials such as NH$_2$-SBA-15. This is likely to be due to the 3D porous structure of SBA-16 enhancing the rate of diffusion and to shorter pores formed with blockage by the FeO$_x$ nanoparticles in cages. Also, from the dissolution result, we may expect an increase in drug release due to degradation of the carrier particles.

### 2.4. Cytotoxicity of FeO$_x$-SBA-16 and NH$_2$-FeO$_x$-SBA-16 Particles

The toxicity of nanomaterials has been a concern in the research community. Although it has been shown that both nanoporous silicas and iron oxide nanoparticles exhibit no effect on cell cultures and...
are considered to have low toxicity to humans, it is still important to study the cytotoxicity of these new nanocomposite materials.

Cytotoxicity of the FeOx-SBA-16 materials was tested using Cell Counting Kit-8 (CCK-8) on mouse embryonic stem (mES) cells. Both samples showed no toxicity on cells after 4 days of culture at a dosage of 33 microgram per one million cells. In the viability test, cells show a viability of 84% and 86% when cultured with FeOx-SBA-16 and NH2-FeOx-SBA-16. As a result, FeOx-SBA-16 materials can be used as the base materials for developing various biomedical devices.

2.5. Tagging Fluorescent Proteins and Antibodies onto NH2-FeOx-SBA-16 Particles

Tagging protein or peptide molecules onto amine-functionalized surfaces using (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling has been well studied. However, it is a challenge to preserve a significant amount of free amine groups for drug binding after tagging protein molecules onto a solid. Being a molecular sieve with a pore size of 3 nm, large protein molecules >4 nm cannot enter the pores of NH2-FeOx-SBA-16, and therefore the amine groups inside the pores will not react with the protein molecules. The molecular dimensions of many protein molecules can be found from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank database. To demonstrate this concept, bovine serum albumin (BSA; size 5 nm × 7 nm × 7 nm) was first labeled with fluorescein (fluorescein isothiocyanate, FITC) and then bound to the outer surface of NH2-FeOx-SBA-16. The FITC-BSA-tagged NH2-FeOx-SBA-16 sample was studied using 3D fluorescent confocal microscopy. FITC-labeled BSA has to be washed thoroughly with phosphate buffer solution because any residual FITC will react with the amine.

![Figure 7. Confocal image of BSA-tagged particles a) before and b) after binding RITC. The scale bar represents 5 μm.](image-url)
groups inside the pores of the composite material\cite{48} and reduce the availability of the amine groups for drug binding.

In Figure 7a, the confocal images of the FITC-BSA-tagged NH$_2$-FeO$_x$-SBA-16 show that the green fluorescence signals are on the outer surface of the particles, and there is no fluorescence inside. This suggests that the FITC-BSA molecules did not react with the amine groups inside the pores due to their large size. In order to prove the existence of free amine groups inside the pores, the FITC-BSA-tagged NH$_2$-FeO$_x$-SBA-16 was reacted with rhodamine B isothiocyanate (RITC). The confocal images of these RITC-dyed particles (Fig. 7b) show that red fluorescence was detected inside the particles because the free amine groups had reacted with the RITC dye. As a result, availability of amine groups inside the pores was not affected by the protein tagging using EDC as a coupling agent.

Since BSA was successfully tagged onto the outer surface of NH$_2$-FeO$_x$-SBA-16 particles, the same principle can be applied to tag the whole antibody. Rabbit IgG-tagged NH$_2$-FeO$_x$-SBA-16 was prepared to examine if the tagged antibodies are still active after EDC coupling. Zeta potential measurements showed that the IgG-tagged NH$_2$-FeO$_x$-SBA-16 sample has a potential of $-1.6$ mV, which is significantly lower than that of FITC-BSA-tagged NH$_2$-FeO$_x$-SBA-16, $-27.9$ mV. This is because BSA has an isoelectric point at pH 4.7, or is negatively charged in deionized water, while that of IgG varies from pH 6.1–8.7.\cite{49} To test the IgG activity, rabbit IgG-tagged NH$_2$-FeO$_x$-SBA-16 particles were suspended in a dilute solution of secondary antibody anti-rabbit IgG-FITC.\cite{50} Such a test provides a fast, visual result for proving that the material was tagged with IgG antibody. The green fluorescence observed in the confocal images (Fig. 8) suggests that the rabbit IgG-tagged NH$_2$-FeO$_x$-SBA-16 particles were capable of conjugating with anti-rabbit IgG-FITC. This result means that other antibodies can be bound to the NH$_2$-FeO$_x$-SBA-16 particles, and a dual-targeting agent can be prepared for drug delivery with high specificity.

Use of small molecules such as folic acid in targeting cancer cells has shown some success in vitro, and sometimes it is preferred to the use of antibodies.\cite{51} However, binding these molecules usually requires specific chemistry such as click chemistry and does not universally apply to other targeting agents, including peptides and carbohydrates.\cite{52} As a result, multi-agent targeting in a single particle using small molecules is unlikely. Since cancer cells express not only one receptor, but a range of gene and protein products, multitargeting provides an advantage for precise targeting. To assemble particles with multitargeting capacity, binding several antibodies may be the only solution because the binding chemistry for antibodies, as for other proteins, is generic. The binding strategy demonstrated in this work can be generally applied for all antibodies.

Drug delivery studies using functionalized nanoporous silica such as SBA-15, MCM-41, and MCM-48 have been well-documented in literature.\cite{20} These reports provide in depth investigations in the binding chemistry and release kinetics of drugs, including ibuprofen, taxol, and doxorubicin. Reports on the preparation of iron oxide–nanoporous silica nanocomposites have been available since 1999,\cite{53} but literature on grafting functional groups onto these materials are still relatively rare; only one reference was found directly related to this type of functionalization in 2009.\cite{54} In this work, only the functionalization process was reported and no applications of the materials was presented and discussed. There are very few research works on using iron oxide–nanoporous silica nanocomposites for drug storage and delivery.\cite{55} In contrast, our work presents a protocol to enhance this magnetic nanocomposite material further by tagging the antibody, which provides a different dimension to this already sophisticated material. In order to leave the majority of the amine functional groups available for drug binding, antibody molecules have to be kept away from the channels. This is made possible by the unique molecular selecting

**Figure 8.** Confocal images of rabbit IgG-tagged NH$_2$-FeO$_x$-SBA-16 after conjugation with FITC-tagged anti-rabbit IgG secondary antibody. The scale bar represents $5 \mu$m.
property of nanoporous silica. Also, the choice of SBA-16 is based on their cage size being large enough to host iron oxide nanoparticles of \( > 5 \) nm and the small channel size to block antibody molecules from entering the channels. Such an assembly of multifunctional nanomaterials with magnetic and antibody-targeting is novel.

Although we have shown a protocol for assembling the multifunctional nanomaterials with a dual-targeting property, the future challenge is to study these materials in vivo. Also, since there are many categories of drugs with various chemical properties and many antigens for targeting, there is no generic material for all drugs and all antigens. For example, paclitaxel, a common anticancer drug for ovarian cancer, breast cancer, lung cancer, does not carry any negative charges and does not bind to amine groups, like ibuprofen or aspirin does. Therefore, a different surface property of the magnetic carrier than that reported here is needed to store paclitaxel. Nonetheless, the FeO\(_x\)–SBA-16 composite provides us with a platform to explore the dual-targeting mechanism. Further functionalization of NH\(_2\)-FeO\(_x\)-SBA-16 and other related materials should be feasible and materials can therefore be prepared according to the surface chemistry of the specific drugs.

3. Conclusions

The use of an SBA-16 nanoporous silica to assemble a multifunctional nanomaterial for biomedical applications has been demonstrated by making use of its unique molecular sieving property. Magnetic iron oxide nanoparticles have been impregnated inside the cages of the nanoporous network and compositionally tuned to maximize magnetization while avoiding sintering, while both the internal and external surfaces of the iron oxide–silica nanocomposite FeO\(_x\)-SBA-16 have been functionalized with amine groups. With these amine groups, the NH\(_2\)-FeO\(_x\)-SBA-16 was found to have a drug storage capacity for ibuprofen and aspirin. These amine groups remained available after the NH\(_2\)-chemistry of the specific drugs.

In the amine-functionalization of FeO\(_x\)-SBA-16, (3-aminopropyl)triethoxysilane (APTES, 99%, Gelest) was used as the silylation agent with toluene (AnalaR grade, BDH) as solvent. Ibuprofen sodium salt (99%, Aldrich) and aspirin (99%, Aldrich) were used as model drugs for drug capacity measurement.

Fluorescent dyes (FITC and RITC), CCK-8, EDC, BSA (lyophilized powder), rabbit immunoglobulin G (IgG), and FITC-labeled anti-rabbit IgG were all purchased from Sigma.

Preparation of SBA-16 and FeO\(_x\)-SBA-16: Preparation procedure for nanoporous silica SBA-16 can be found in literature [19]. SBA-16 belongs to a family of ordered nanoporous silica prepared by using liquid crystal templating with coblock polymer surfactants. Synthesis of these materials involves a gelling and a hydrothermal stage. A pH regulator, HCl in this case, is normally required. Details of the formation mechanism of these materials are also available in literature [58]. In a typical experiment, 2.0 g of Pluronic F127 surfactant and 4.0 g of KCl were dissolved in 100 cm\(^3\) of dd H\(_2\)O. The solution was then acidified with 20 cm\(^3\) of 35% HCl. When all the surfactant was dissolved, 10 cm\(^3\) of isopropanol and 8.6 g of TEOS were added to the reaction solution, and the mixture was stirred for 24 h at 30 °C. After 24 h, the reaction mixture was transferred to a polytetrafluoroethylene (PTFE) bottle and heated to 100 °C for 3 days. The white precipitate was then filtered, washed, and air-dried at room temperature. Finally the white solid was then calcined at 550 °C for 6 h in air. This step is necessary to remove all surfactant template in order to reveal the nanoporous network of the SBA-16 particles. The calcined SBA-16 samples were then characterized using TEM, SEM, nitrogen adsorption isotherms.

Iron oxide was impregnated inside the SBA-16 particles with two steps: 1) thermal decomposition of Fe(NO\(_3\))\(_3\) to Fe\(_2\)O\(_3\) at 500 °C, and 2) partial reduction of Fe\(_2\)O\(_3\) to Fe\(_3\)O\(_4\) at 350 °C. Reduction of Fe\(_3\)O\(_4\) to Fe\(_2\)O\(_3\) was then performed at 500 °C for 2 h. The particles were cooled down immediately by removing them from the furnace after heating. This sample was denoted as FeO\(_x\)-SBA-16. To reduce samples, 1.0 g of Fe\(_2\)O\(_3\)-SBA-16 was submitted to a tube furnace and heated to 350 °C at 2 °C min\(^{-1}\) under a flowing stream of 5% H\(_2\) in N\(_2\) for 4 h. Particles of dark brown color were formed and denoted as FeO\(_x\)-SBA-16.

In this two-solvent procedure, in order to give FeO\(_x\)-SBA-16 an atomic ratio of Fe:Si:9, iron precursor 0.72 g Fe(NO\(_3\))\(_3\)·9H\(_2\)O was dissolved in 0.5 cm\(^3\) acetonitrile. Calcined SBA-16 (0.9 g) was suspended in 5 cm\(^3\) of hexane. The Fe(NO\(_3\))\(_3\) solution was then added to the SBA-16 suspension dropwise and adsorbed by the SBA-16 particles. After 16 h, the particles were recovered, dried, and heated at a heating rate of 2 °C min\(^{-1}\) to 500 °C for 2 h. The particles were cooled down immediately by removing them from the furnace after heating. This sample was denoted as FeO\(_x\)-SBA-16.

In a dissolution experiment, 5 mg of FeO\(_x\)-SBA-16 was suspended in 50 cm\(^3\) of dd H\(_2\)O, phosphate buffered saline (PBS), or simulated body fluid (SBF) in a polypropylene centrifuge tube, and the suspensions were kept at 37 °C for 1, 2, and 3 weeks. At the time interval, the supernatants were collected by filtration, and the Si and Fe content was quantified using an ICP-AES spectrometer (Spectro Ciros CCD).

Preparation of NH\(_2\)-FeO\(_x\)-SBA-16 and Drug Binding Capacity: NH\(_2\)-FeO\(_x\)-SBA-16 was prepared by silylation using APTES; the procedure is widely available in literature [38]. During this reaction, the alkoxysilane reacts with the silanol groups (Si–OH) on the surface to form a Si–O–Si linkage at a high temperature. This reaction is usually catalyzed in presence of a base, and the APTES also acts as a catalyst. In a typical experiment, FeO\(_x\)-SBA-16 (0.5 g) was suspended in 50 cm\(^3\) of dry toluene, and 0.5 g of APTES was added to the suspension. The reaction mixture was then heated

4. Experimental

Materials: All chemicals were used as received from the suppliers without further purification. For the preparation of SBA-16 and FeO\(_x\)-SBA-16, tetraethylorthosilicate (TEOS, Aldrich, 98%) was used as the silica source and triblock copolymer surfactant Pluronic F127 poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) (EO\(_{106}\)PO\(_{70}\)EO\(_{106}\), BASF) was used as the structure-directing agent. Potassium chloride (KCl, Analar grade), concentrated hydrochloric acid (HCl, 36%), and isopropanol (HPLC grade), hexane (GPR grade), acetonitrile (GPR grade) were purchased from BDH. Iron nitrate (Fe(NO\(_3\))\(_3\)·9H\(_2\)O, 98%, Aldrich) was used as the iron precursor.

In the amine-functionalization of FeO\(_x\)-SBA-16, (3-aminopropyl)triethoxysilane (APTES, 99%, Gelest) was used as the silylation agent with toluene (Analar grade, BDH) as solvent. Ibuprofen sodium salt (99%, Aldrich) and aspirin (99%, Aldrich) were used as model drugs for drug capacity measurement.

Fluorescent dyes (FITC and RITC), CCK-8, EDC, BSA (lyophilized powder), rabbit immunoglobulin G (IgG), and FITC-labeled anti-rabbit IgG were all purchased from Sigma.
to reflux for 24 h with stirring using an overhead stirrer at 175 rpm. The solid was finally recovered using a NdFeB magnet, washed with acetone, and air dried. The nitrogen content of NH₂-FeOₓ-SBA-16 was quantified using elemental C, H, and N microanalysis (Thermo EA1112 Flash CHNS-O Analyzer), and the zeta potential of the particles in dd H₂O was measured using a Malvern Zetasizer Nano ZS instrument.

**Measurement of Drug Binding Capacity:** The drug binding capacity of NH₂-FeOₓ-SBA-16 was measured using ibuprofen sodium salt and aspirin as model drugs. In a typical experiment, 5 mg of NH₂-FeOₓ-SBA-16 was suspended in an ethanolic solution of the drug at a concentration of 100 mg cm⁻³. The solid was then recovered by centrifugation at 2000 rpm and dried at room temperature under vacuum. The amount of drug adsorbed onto NH₂-FeOₓ-SBA-16 was quantified using thermogravimetric analysis (TGA, Perkin Elmer) under flowing air. The samples were heated at 5 °C min⁻¹, and the weight loss at the temperature range of 120–550 °C was used for the calculation of drug binding capacity, with blank NH₂-FeOₓ-SBA-16 as a reference material.

**Characterization of SBA-16, FeOₓ-SBA-16, FeOₓ-SBA-16: High-angle annular dark field-scanning transmission electron microscopy (HAADF-STEM) images were recorded using a JEM 3000 F operating at 300 kV while a Tecnai microscope at 100 kV was used for TEM imaging. Samples for TEM examination were suspended in ethanol and deposited on a copper specimen grid supported by a holey carbon film. The morphology of the SBA-16 was studied using a Hitachi S-4800 cold-field-emission SEM instrument at 20 kV. XRD was carried out on a PANalytical Xpert system using Co Kα radiation. Scherrer analysis was carried out using the Xpert Highscore Plus software. Nitrogen adsorption isotherms were recorded using a Micromeritics ASAP 2000 nitrogen absorption analyzer. Samples were outgassed overnight at 100 °C under vacuum. The surface area of samples was calculated using the BET equation. Magnetic properties were measured using a Quantum Design MPMS SQUID magnetometer. M versus H measurements were performed at 300 K in the field range of −50 000 ≤ H/Ω ≤ 50 000. FTIR spectra were obtained from powder samples on a JASCO FT/IR-4200 type A spectrometer equipped with a standard source and a TGS (triglycine sulfate) detector. Two hundred spectra in the region from 600 to 3800 cm⁻¹ were accumulated with a resolution of 4 cm⁻¹.

**Cytotoxicity Assays for FeOₓ-SBA-16 and NH₂-FeOₓ-SBA-16:** The cytotoxicity of FeOₓ-SBA-16 and NH₂-FeOₓ-SBA-16 was tested using WST-8 assay (Sigma). E14 mouse embryonic stem (mES) cells were used in this cytotoxicity assay. Cells were grown and maintained using high-glucose advanced medium culture supplemented with 2% fetal calf serum (ES-grade), 1000 U cm⁻³ Leukemia Inhibitory Factor (LIF), 5 µM mercaptoethanol, and 2 mM l-glutamine. For cytotoxicity assays, cells were seeded at 6000 cells/well in a 96-well tissue culture plate in 0.1 cm³ medium and cultured overnight. The BSA solution, and the reaction mixture was covered with aluminum foil rotated at 4 °C overnight. The solid was washed with PBS to remove the excessive secondary antibody and recovered using a NdFeB magnet. Again, the 3D fluorescent images were recorded with the Leica confocal microscope system.

**Acknowledgements**

The authors acknowledge EPSRC (EP/C511794) for financial support. The authors also acknowledge Mr. Gary Evans (SEM), Dr. Duong Giap (SQUID), Dr. Iryna Palona (cytotoxicity and confocal microscopy), Miss Cristina Olaire (cytotoxicity), Dr. Laurent Bouffier (FTIR spectroscopy), and Dr. Alec Simpson (confocal microscopy) of the University of Liverpool for their support in various experiments. Dr. James Long (IOTA, Liverpool) for his assistance in zeta potential measurement, and Prof. David Fernig (Liverpool) for discussion on antibody targeting. Supporting information is available online from Wiley InterScience or from the authors.

Received: November 10, 2009
Revised: February 13, 2010
Published online:

[15] C. C. Berry,