Apart from their generic transporting ability, hollow and porous inorganic nanomaterials enriched with multifunctionality can potentially open diverse avenues in developing a new technology in nanomedicine.

Inorganic hollow nanoparticles and nanotubes in nanomedicine Part 1. Drug/gene delivery applications

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Recent cytotoxicity studies on carbon nanotubes have shown that the biocompatibility of nanomaterial might be determined mainly by surface functionalization, rather than by size, shape, and material. Although the cytotoxicity for individual inorganic hollow nanomaterials should be extensively tested *in vitro* and *in vivo*, potential safety concerns about the use of inorganic nanomaterials in biomedical applications could be alleviated with proper surface treatment. Inorganic hollow nanoparticles and nanotubes have attracted great interest in nanomedicine because of the generic transporting ability of porous material and a wide range of functionality that arises from their unique optical, electrical, and physical properties. In this review, we describe recent developments of hollow and porous inorganic nanomaterials in nanomedicine, especially for drug/gene delivery.

Recently, nanotechnology has been rapidly emerging in biomedical and biotechnological applications, including drug/gene delivery carriers, disease diagnosis, and cancer therapy. In most applications, bio-nanomaterials such as liposomes and biodegradable polymers have played key roles in nanomedicine because they are considered to be safe in the human body [1,2]. In contrast, the use of inorganic nanomaterials such as carbon nanotubes, metals, and metal oxide nanoparticles has been limited by safety issues in biomedical applications.

Since recent cytotoxicity studies on carbon nanotubes (CNTs) have shown that biocompatibility of nanomaterial might be mainly determined by surface functionalization rather than by size, shape, and material [3,4], inorganic nanoparticles are attracting great interest in the field of nanomedicine. Inorganic nanomaterials have fundamental advantages over bioorganic nanomaterials such as liposome, dendrimer, and biodegradable polymer in terms of their size and shape control and surface functionalization [5,6]. In addition, since inorganic nanomaterials permit a wide range of functionality arising from their unique optical, electrical and physical properties, they may provide a solution for many physical barriers of the cell that limit biomedical applications.

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Nanotube, nanoshell, and mesoporous nanoparticle are attractive vehicles for drug/gene delivery because of their hollow and porous structures and facile surface functionalization. The inner void can take up a large amount of drug, and the open ends of pores serve as gates that can control the release of drug/gene. Furthermore, the hollow tubular nanoparticle can be differentially functionalized between the inner and outer surfaces, which can provide a platform of multifunctionality into the nanoparticle through sequential and sectionalized surface modifications. Most importantly, unlike spherical nanoparticles, since hollow structures isolate drug/gene payload from the environment, they can transport the payloads safely into the cell without hydrolytic degradation of biological payload or aggregation of nanomaterials caused by many hydrophobic drug molecules during the delivery into the cell. In this review, we describe recent developments of hollow and porous inorganic nanoparticles and nanotubes in nanomedicine, especially for drug/gene delivery.

Drug/gene delivery applications

Carbon nanotube

Carbon nanotubes are the leading inorganic nanomaterial for biomedical application, and their toxicology and pharmacology were extensively reviewed by Kostarelos's group [7,8]. Although

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carbon nanotubes (CNTs) have attracted increasing attention as new vectors for the delivery of therapeutic molecules, because of the easiness of translocation across cell membranes and low toxicity [9–12], issues about the ultimate biocompatibility of CNTs have limited their widespread use in biomedical applications [13–15]. However, a recent study reported by Singh *et al.* revealed that water-soluble functionalized carbon nanotubes (f-CNTs) can be rapidly cleared from a systemic blood circulation through the renal excretion route [3]. In order to trace nanotubes *in vivo*, the group functionalized water-soluble, single-walled carbon nanotubes (SWNTs) with the diethylenetriaminepentaacetate (DTPA)/indium (^{111}In) complex. After intravenous (i.v.) administration of nanotubes was performed through the tail vein of a mouse, biodistribution of nanotubes was investigated by radioactivity tracing. After three hours, less than 1% of injected nanotubes were detected in all organs measured. From the *in vivo* excretion study, Singh *et al.* found that nanotubes were excreted via urine. This result indicates that CNTs can be made safe by increasing water solubility through chemical modification, which alleviates some concerns over the safety of CNTs and is expected to accelerate the development of CNTs for biomedical use.

Bianco *et al.* reported that functionalized carbon nanotubes (f-CNTs) can be used for presentation and delivery of antigens and for gene delivery [9]. The group prepared soluble f-CNTs using 1,3-dipolar cycloaddition of azomethine ylides [16]. The resulting amine groups attached to the sidewall of the CNTs were linked with peptide antigens (B cell epitope from the foot-and-mouth disease virus (FMDV)). This was done to study their immunogenic properties and was also used to condense the pCMV-βgal plasmid DNA. In the antigenicity and immunogenicity studies, the peptide-CNT was recognized by antibodies equally well as the free peptide and immunization of mice with the peptide-CNT clearly enhanced anti-FMDV peptide antibody responses. Moreover, no immune response to CNTs was detected, which is an important issue in view of epitopic suppression when peptide antigen carriers are used. Gene expression efficiency offered by DNA-CNT was about ten times higher than that of DNA alone.

Alternative strategies for the introduction of two different and orthogonal functionalizations to CNTs were investigated as well by Bianco *et al.* [17]. The orthogonal methodology allows the selection and control of attachment of active molecules to sidewalls and tips of CNTs. To achieve this result, Boc and phthalimide groups were used to protect amine groups on the tips/sidewalls of CNTs. The phthalimide group is particularly useful because it is stable in harsh acidic conditions and orthogonal to the Boc group, thus fluorescein can be attached to the tips of the CNTs and amphotericin B (AmB) to the sidewalls of the CNTs. Fluorescein and AmB were chosen for tracking the uptake of material and as an antibiotic moiety, respectively. AmB is considered to be the most effective antibiotic, but is highly toxic to mammalian cells because of the formation of aggregates as a result of its lower solubility [18]. From a Human Jurkat lymphoma T cell viability test, Bianca *et al.* observed that the conjugation of AmB to CNTs remarkably reduced the toxic effects. In addition, maximum fluorescence was observed only after one hour of incubation, indicating fast cell uptaking of FITC-AmB-MWNTs. Most conjugates were found in the cytoplasm and around the nuclear membrane. For the mechanism of cell membrane penetration, they proposed a spontaneous mechanism: MWNT behave like nanoneedles and pass through the cell membrane without causing cell death [19]. They explained the reason for reduced toxicity as the ability of CNTs to internalize AmB rapidly into the cytoplasm of Jurkat cells, thus reducing the possibility of disruption of the cell membrane structure.

SWNTs have strong optical absorbance in the near infrared (NIR) range [20]. This property can be used to trigger drug/gene release from the SWNT by illumination of NIR light. The absorbance of NIR light can produce localized heat that stimulates the release of drugs or genes from the nanotube surface. Kam *et al.* have designed folate-conjugated SWNTs [21]. Selective internalization of SWNTs inside cells labeled with folate receptor tumor markers has been observed. Upon irradiation of NIR to HeLa cells after Cy3-DNA-SWNT uptake, they detected the colocalization of fluorescence of Cy3-DNAs in the cell nucleus, indicating that DNAs were released from SWNT transporters to the nucleus after the laser pulses.

Hollow nanoshells and porous nanospheres

Hollow carbonaceous capsule

Despite the promising results of CNTs for drug delivery [9–12], the wide size distribution of CNTs can be a drawback for biomedical applications, since the size distribution and functionalization will influence biodistribution, targetability, and drug release. Sun and Li have recently prepared hollow carbonaceous capsules with reactive surface layers via hydrothermal methods with the anionic surfactant sodium dodecyl sulfate (SDS) and glucose as starting materials. The void size within the capsule and the shell thickness can be tuned by adjusting the amount of SDS and the hydrothermal parameters, such as time, temperature, and glucose concentrations [22]. These capsules can have potential biomedical applications such as drug delivery and clinical diagnostics.

Calcium phosphate nanoshell

Calcium phosphate, the main component of the skeletons and teeth of vertebrate animals, is biocompatible and biodegradable. Solid calcium phosphate [$Ca_x(PO_4)_yOH_z$] particles have been widely investigated for their applications in biotechnology. Roy *et al.* [23,24] synthesized calcium phosphate nanospheres around 80 nm in diameter to encapsulate DNAs for a targeted gene delivery. Studies were carried out both *in vitro* and *in vivo*. The results show promising transfection efficiency. The nanoshell form of calcium phosphate was reported by Schmidt and Ostafin [25]. The nanoshells were produced with a liposome as a template. The lipid was the phosphatidic acid 1,2-dioleoyl-SN-glycero-3-phosphate (DOPA). The nanoshells with a shell thickness of 2–10 nm and a liposome/water core of 45–100 nm in diameter were obtained. *In vivo* expression of beta-galactosidase in different body tissues has been detected after administration of pSVbGal plasmid DNA encapsulated in the calcium phosphate nanoparticles. Nanoshells, unlike nanospheres where the drug is dispersed through out the particles, are vesicular systems where the drug can be stored and protected from a premature inactivation. As a result, calcium phosphate nanoshells have potential as sustained drug delivery vehicles.

Mesoporous silica nanosphere for controlled drug release

Botterhuis *et al.* [26] have synthesized and characterized hollow silica spheres with an ordered pore structure via a microemulsion method. Encapsulation and subsequent release of different dye molecules have suggested that the materials could have potential as drug carriers. Vallet-Regi *et al.* [27] have synthesized the ordered mesoporous material MCM-41 and have studied the application of the materials as a drug delivery system. The materials are synthesized by a self-assembly of a0020silica surfactant, C16-trimethylammonium bromide and C12-trimethylammonium bromide (TAB), and their condensation in water. The pore size distribution is centered at 2.5 and 1.8 nm for the samples prepared from C16-TAB and C12-TAB, respectively. Ibuprofen was introduced into these two MCM-41 materials. The weight percentage ratio of drug/MCM-41 was 30%. The *in vitro* drug release profiles in a simulated body fluid show that the entire drug incorporated into the MCM-41 matrix can be released into a solution over a period of three days.

Lai *et al.* [28] developed an MCM-41 type mesoporous silica nanosphere (MSN)-based carrier system with chemically removable cadmium sulfide (CdS) nanoparticle caps for stimuli-responsive controlled release of neurotransmitters and drug molecules. The MSN is modified with 2-(propyldisulfanyl)ethylamine. The average size of MSNs is 200.0 nm with a pore size of 2.3 nm. The mesopores of the MSN were used as drug reservoirs, and the openings of the mesopores were capped by photoluminescent CdS nanocrystals with a diameter of 2.0 nm via an amidation reaction between the MSN surface modified amine groups and the water-soluble mercaptoacetic acid derivatized CdS nanocrystals. The disulfide linkages are chemically labile and can be cleaved by disulfide reducing agents. The CdS nanoparticle caps can, therefore, be released from the drug loaded MSNs, and the process can be regulated. The drug releasing profiles and biocompatibility of ATP-loaded MSNs with neruoglial cells have been studied *in vitro*. An Attofluor system in conjunction with a Zeiss microscope was used to detect the ATP-induced increases of calcium transients. The increase is represented by the color changes in pseudo-color images of the cells. The data indicate that the releasing rate depends on the rate of CdS cap removal.

Radu *et al.* [29] have developed a gene transfection system on the basis of the same mesoporous silica nanosphere materials. Figure 1 shows schemes for the controlled gene delivery of MSN. MSN materials were first functionalized with 3-isocyanatopropyl (ICP), loaded with pEGFP-C1 DNA, and then capped by second-generation polyamidoamine (G2-PAMAM) dendrimer via a urea linkage between the amine group of PAMAM and ICP. The plasmid DNA encodes for an enhanced green fluorescence protein. The transfection efficacy of G2-MSNs with pEGFP-C1 was studied with neural glia, human cervical cancer cells, and Chinese hamster ovarian cells. Both fluorescence confocal microscopic and TEM images illustrate cellular uptake of a large number of the G2-MSNs with pEGFP-C1 into the eukaryotic cells. In a biocompatibility study, the growth profiles of HeLa cells with and without G2-MSNs (0.1 mg/ml) were compared. The similar growth profiles within six days for these two trials indicated the low cytotoxicity of G2-MSNs. Flow cytometry analysis on 48 hours post-transfected cells showed an efficiency of 35% with the gene delivery system.

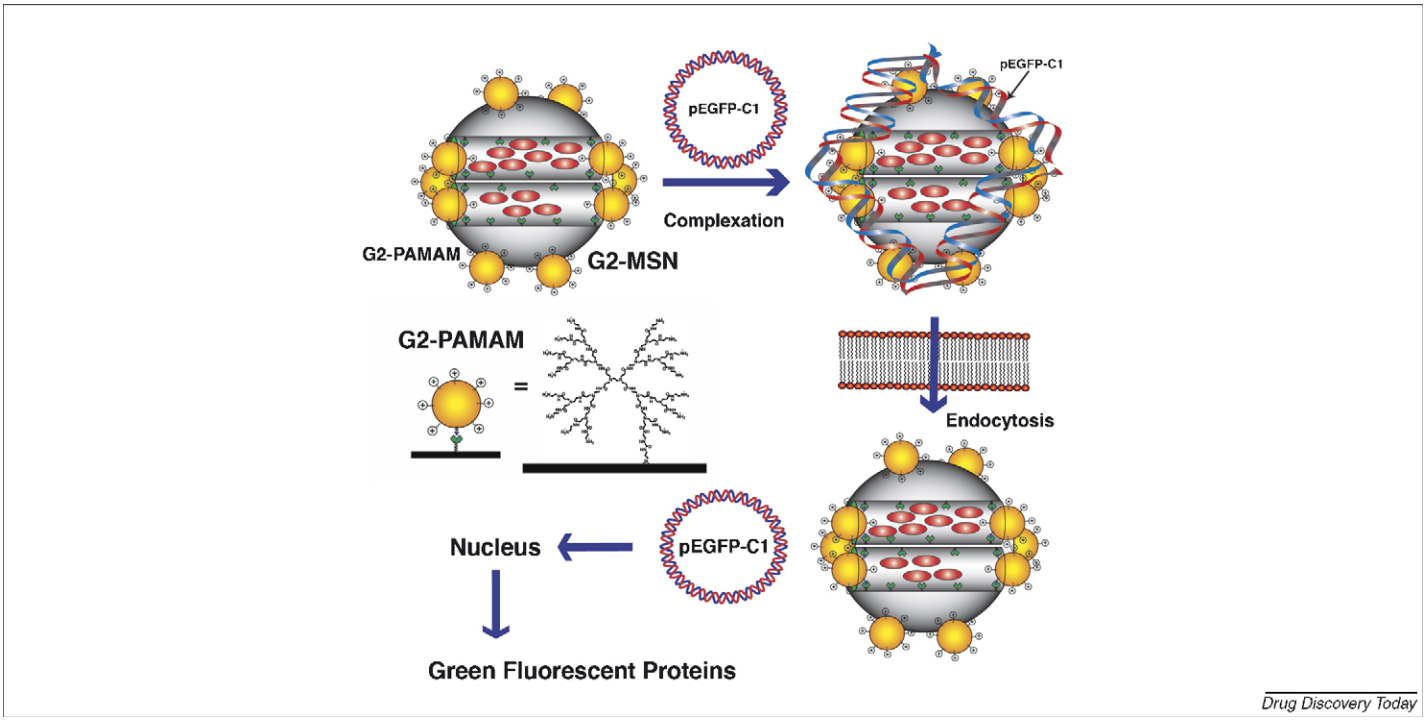

FIGURE 1

Schematic illustration of a nonviral gene transfection system based on capped MCM-41 type mesoporous silica nanosphere (MSN), as reported by Radu *et al.* [29]. MSN were loaded with pEGFP-C1 DNA and then capped by polyamidoamine dendrimer. The dendrimer cap protects DNA from an enzymatic degradation during a gene delivery and regulates DNA transfection as a labile cap that releases after internalization into a cell.

The efficiency is higher than that of other commercial transfection reagents, such as PolyFect (15% efficiency), SuperFect (10%), and Metafectene (16%) under the same experimental conditions.

Giri *et al.* [30] developed a superparamagnetic nanoparticle-capped MSN system using disulfide bonds for stimuli-triggered release. The internalization of the Fe_3O_4-capped MSNs was observed. They proposed that the MSN system could be a promising nanodevice for a site-selective interactive sensory and a controlled-release drug delivery.

However, it could be difficult to apply the system to a controlled release triggered by disulfide reducing agents *in vivo*. In addition, the modification of the inner voids for pore caps can limit the drug loading capacity and/or the diversity of compatible drugs that can be loaded.

Nanotubes prepared from template synthesis

Although nanoparticles have been successfully used in most nanomaterial-involved biomedical and biotechnological applications, spherical nanoparticles still need to be improved in terms of a surface modification and an environment compatibility, especially when a multifunctionality is required, since a sphere has only one surface and as a result every surface functionalization takes place on the same surface, which may lead to interference between various functional groups. In this regard, nanotubes (NTs) can offer attractive alternatives for some applications which require multifunctionality.

NTs have inner and outer surfaces, which can be functionalized differently depending on their roles (e.g. the inside with drugs or imaging agent and the outside with targeting moieties and antifouling agents). The inner void of the NT, for example, can provide a space to load a large amount of drug and its open end can serve as a gate for drug uptake/release. Because of these unique attributes of NTs, compared with that of spherical nanoparticles, NTs are considered ideal nanovectors for nanomedicine [6].

Of the various synthetic methods for nanotube production, template synthesis (developed by Martin [31]) provides the easiest way to control nanotube size and shape. This method offers a general approach for preparing nanomaterials that involves the synthesis or deposition of a desired material within the cylindrical and monodispersed pores of a nanopore membrane or other solid surfaces [32]. Figure 2 shows an alumina template that has cylindrical nanopores with monodispersed diameters and lengths. Porous alumina templates can be obtained by a well-established electrochemical anodization method on aluminum plates. The dimensions of the pores can typically be adjusted from five to a few hundred nanometers in diameter and from tens of nanometers to hundreds of micrometers in length [33,34].

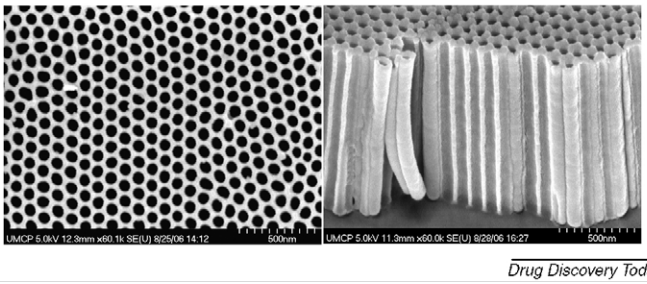

FIGURE 2

Field emission scanning electron micrographs (FESEM) of a home-made alumina template (60-nm diameter) after silica "surface sol–gel" template synthesis; top-viewed (left) and cross-sectional viewed image (right). The cross-sectionally viewed image reveals that silica nanotubes were synthesized within the pores of the template.

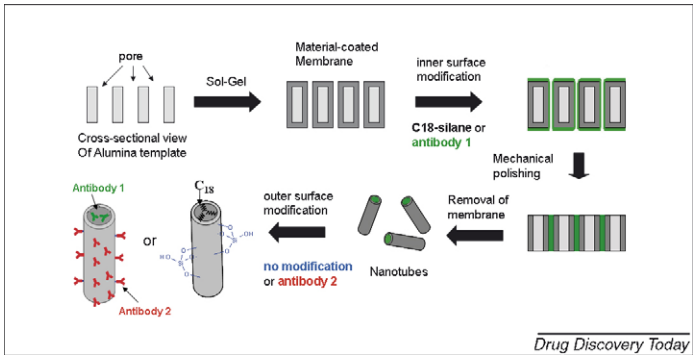

FIGURE 3

Schematic illustration of a differential functionalization, as reported by Son *et al.* [40]. The first surface functionalization is performed while nanotubes are still embedded in the pores of template so that the inner surface of the nanotubes can be selectively modified. Since template walls protect the outer surfaces of the nanotubes from being accessed by a surface modifier, the outer surface remains intact. After dissolving the template, the second functionalization can be performed to modify the outer surfaces of the nanotubes.

Another virtue of template synthesis is that the differential functionalization between the inner and outer surfaces of nanotubes is possible and was recently reported by the Martin's group [35]. Figure 3 shows schemes for the differential functionalization. The first surface modification reaction to functionalize the inner surface of NTs selectively is performed when they are still embedded in the pores of the template. In this step, the outer surface of the NT remains intact because the template wall protects the outer surface of the NT. After obtaining freestanding nanotubes by dissolving the template, the second functionalization produces the desired functionality on the outer surface. Differential functionalization between the inner and the outer surfaces of NTs can provide a facile and effective way to integrate multifunctionality in NTs by separating surface modification chemistry depending on the role.

Silica nanotube

Wu *et al.* [36] reported fluorescent silica nanotubes (SNTs) for gene delivery. SNTs were prepared inside pores of 200-nm diameter commercial alumina membrane via a sol–gel process utilizing tetraethyl orthosilicate. The inside of the SNT was then modified with 3-aminopropyl silane (APTS) to generate a polycationic surface required to hold CdSe/ZnS core–shell quantum dots (QDs) or DNAs through the electrostatic forces. For cell membrane permeability of SNTs, cultured mammalian cells such as monkey kidney COS-7 cells were treated with green fluorescent silica nanotubes (gfSNTs). Confocal microscopy revealed that the gfSNTs entered about 60–70% of the cells by endocytosis and were mostly localized in the cytoplasm. In cytotoxicity tests, approximately 80% of the cells were still viable after the treatment of gfSNT, indicating that silica nanotubes are not especially toxic under these experimental conditions. For the gene delivery experiment, the plasmid DNA was inserted into the nanotube to form a DNA/SNT complex,

and the complex was added to COS-7 cells. The cytoplasmic GFP expression complex was observed with DNA/SNT but not with free DNA. Although the efficiency of SNT-mediated DNA transfection (ca. 10–20%) is less than that of conventional calcium phosphate (ca. 60–70%), the advantage of this strategy is that cargo biomolecules carried by SNTs can be any other biomolecules such as RNA or proteins.

Magnetic nanotube

Son *et al.* have reported magnetic nanotubes (MNTs), silica nanotubes embedded with magnetite nanoparticles [37]. The main advantage of the drug carrier having a magnetic property is that it allows the use of a powerful imaging technique, magnetic resonance imaging (MRI), to track drug delivery. In addition, movement of the drug carrier can be controlled by an external magnetic field, and hence directed to specific anatomical sites *in vivo*. The combination of attractive magnetic properties with a tubular structure makes the MNT an ideal candidate as a multifunctional nanomaterial used for biomedical applications. For the synthesis of MNTs, silica nanotubes were first prepared by the ''surface sol–gel'' (SSG) method [38]. The resulting template embedded with silica nanotubes was dip-coated with a 4:1 mixture solution of 1 M $FeCl_3$ and 2 M $FeCl_2$, dried and treated with 1N NH_4OH [39].

The inner void of MNTs can be used to control the release of drug molecules into a solution. To achieve this result, the inside of the MNTs were differentially functionalized with amino-silane (aminopropyltriethoxysilane, APTS). 5-Fluorouracil (5-FU), 4-nitrophenol, and ibuprofen (Ibu) molecules were tested as model drug molecules for the controlled drug release experiment. As seen in Figure 4, depending on pK_a values (Ibu, 4.8; 4-nitrophenol, 7.2; 5-FU, 8.1), different release patterns were observed. For example, 10% of the ibuprofen was released in 1 hour and 80% was released after 24 hours, whereas more than 90% of the 5-FU and the 4-nitrophenol were released in 1 hour. Son *et al.* proposed that the strength of ionic interactions between drug molecules and amine groups of the nanotube inner surface is the main factor for the drug release from the inside of the NT. This suggests that the drug release rate can be controlled by regulating the modification of the inner surface of NTs on the basis of molecular interactions, such as ionic or hydrophobic interactions.

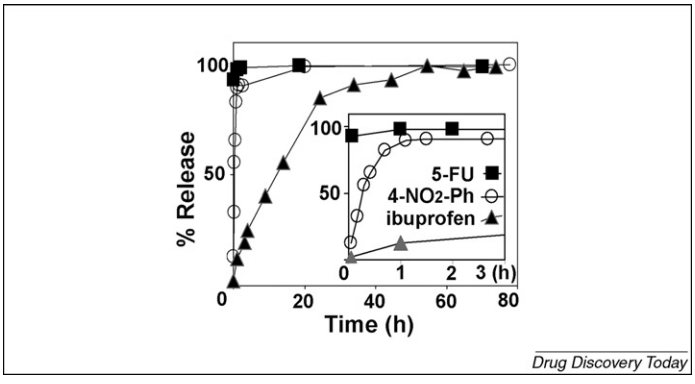

FIGURE 4

In vitro release of ibuprofen, 4-nitrophenol (4-NO_2-Ph), and 5-fluorouracil (5-FU) from an amine silane-treated magnetic nanotube (MNT, 60-nm diameter, 250-nm length) at pH 7.4, as reported by Son *et al.* [37]. In case of ibuprofen that has the lowest pK_a value and thus mostly exists as a negative charged form at pH 7.4, sustained release pattern was observed due to a strong interaction with the positively charged nanotube inner surface.

Cytotoxicity of these MNTs against the human metastatic breast cancer cell line MDA-MB-231 was recently investigated by Son *et al.* [40], who studied the effect of varying size and surface functionalization of MNTs on cytotoxicity. Toxicity was highly dependent on the concentration of MNTs rather than on the size or surface functionalization. At the lower concentrations (0.05 and 0.005 μg/ml), the toxicity of all the nanotubes was low. However, positively functionalized MNTs were more toxic than non-functionalized MNTs, possibly because of increased cellular association and uptake of the nanotubes, and the interaction between positive functionalized MNTs and the negatively charged cell surface.

Another type of MNTs heterostructured MNTs produced through layer-by-layer (LbL) deposition of polyelectrolytes and nanoparticles in the pores of a track-etched polycarbonate membrane have been prepared [41]. They are composed of alternating multilayers of cationic poly(allylamine hydrochloride) and anionic poly(styrene sulfonate), and then a multilayer of magnetite nanoparticles (NPs). The content of magnetite NPs was calculated to be 20% of the total mass of the MNTs on the basis of the saturation magnetization. Large uptake of anionic molecules was observed when MNTs were treated with acid (pH < 2.5). Lee *et al.* proposed that the increased capacity arises from the protonation of free amine groups within the multilayers and a pH-sensitive swelling of the polyelectrolytes. For their use as drug carriers, three anionic molecules (ibuprofen, acid red 8 and rose Bengal) were tested in a release study using acid-treated MNTs. It turned out that the molecular structure, particularly the size of the anionic molecules, played a key role in sustained drug release. In the case of bulky rose Bengal, sustained release was observed, whereas small ibuprofen was immediately released.

Nanotube corked with a nanoparticle cap

For NTs to be used for controlled drug release, surface functionalization has been used [6,42]. However, this approach does not offer a full range of drug release control. An alternative payload-release strategy was explored by Martin and co-workers by corking nanotubes with a chemically labile cap [42]. Open ends of NTs functionalized with amino groups were spontaneously corked with anionic aldehyde-functionalized latex NPs via Schiff base linkage when the NTs were embedded in the alumina template. More than 80% of liberated NTs from the template remain corked, indicating that NP caps are not attached to the ends of NTs by an electrostatic interaction but by covalent imine linkages. Schiff's bases are known to be thermodynamically unstable in the presence of water, but they proposed that the meta-stable condition of the assembled structure is possible because of the multiple points of contact between the NTs and NPs. They suggested that if NTs could be loaded with a payload, such as drugs or genes, before corking the open ends with a chemically labile cap, these NTs could be used for a universal delivery vehicle.

Another approach to cap NTs with gold nanoparticles was attempted by Lee and co-workers, using controlled Au NP diffusion in nanotubes. In this method, negatively charged 2-nm Au NPs were selectively immobilized on the positively charged inside of nanotube through attractive electrostatic interaction. Since the

non-functionalized outer surface bares partial negative charges and the repulsive forces produced by Au NPs occupying the open ends block further diffusion of Au NPs into the channel of the NTs, the Au NPs were localized at the open end of nanotube and were found neither on the outer surface of NT nor on the deep inner surface. Pre-trapped Au NPs can serve as a seed for ''seed-mediated gold growth'' reaction to make caps at the open end of the NTs [43]. In this reaction, $HAuCl_4$ was exclusively deposited onto the pre-trapped Au NPs after reduction with ascorbic acid. The group proposed that their capping procedure can be potentially adopted for a general *in situ* encapsulation of biomolecules (e.g. DNA or enzyme).

Conclusion

In this article, we have reviewed hollow and porous inorganic nanomaterials in nanomedicine, focusing on the drug/gene delivery. The unique properties of a variety of inorganic materials such as mesoporous silica, quantum dot, CNT and gold nanoshell make it possible to enhance, or even surpass, the capabilities of conventional delivery. However, more research is needed before the hollow and porous inorganic nanomaterials are able to be used outside the laboratory. For controlled drug/gene release, as suggested by Martin [6,32], open end gates of pores will require labile caps that reversibly response to local signals. Although long-term safety studies have to be thoroughly examined, recent studies show that the characteristics being used for judging or criteria for assessing inorganic material safe for *in vivo* use are changing from the type, size, and shape of material to its level of environmental friendliness [3,4,9]. This change could cause researchers to consider that inorganic nanomaterials are more appropriate for biomedical use because proper surface treatment will be able to alleviate potential safety concerns.

As seen in the examples discussed in this review and mentioned by Ferrari [5], the fundamental advantage of the use of hollow and porous inorganic nanomaterials in biomedicine lies in multifunctionality. Apart from the generic transporting ability of porous material, other characteristics such as magnetism and NIR-absorbance, which are useful properties in biomedical applications, can be easily integrated in a single unit. Enriched with multifunctionality, hollow and porous inorganic nanomaterials can potentially open diverse avenues in developing a new technology in nanomedicine.

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