

Utilizing Model Nanostructured Porous Inorganic Compounds Such as Silica to Beneficially Confine “Bio”-Relevant Molecules and Demonstrate their Potential in Therapeutics

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Abstract | The importance of well-defined inorganic porous nanostructured materials in the context of biotechnological applications such as drug delivery and biomolecular sensing is reviewed here in detail. Under optimized conditions, the confinement of “bio”-relevant molecules such as pharmaceutical drugs, enzymes or proteins inside such inorganic nanostructures may be remarkably beneficial leading to enhanced molecular stability, activity and performance. From the point of view of basic research, molecular confinement inside nanostructures poses several formidable and intriguing problems of statistical mechanics at the mesoscopic scale. The theoretical comprehension of such non-trivial issues will not only aid in the interpretation of observed phenomena but also help in designing better inorganic nanostructured materials for biotechnological applications.

1. Introduction

With the emerging trends and recent advances in nanotechnology,¹⁻³ it has become increasingly possible to employ nanomaterials to address various shortcomings typically associated with conventional methods used for tackling different issues or problems in biotechnology. For example, by using nanoscale drug delivery vehicles, the pharmacological properties (e.g., solubility and circulating half-life) of various drugs can be dramatically improved leading ultimately to the discovery of optimally safe and effective drugs. Current nanotechnology-based therapeutic products have been mainly validated through the improved effectiveness of approved drugs. Following initial successes, many novel strategies,

such as devices based on nanotechnology relevant to different areas of biotechnology have been conceived and are in advanced stages of development. Some of these areas are in vitro diagnostics, in vivo imaging, therapy techniques, biomaterials and tissue engineering. In this review, we discuss the applications of nanotechnology focusing specifically on controlled drug delivery and biomolecular sensing. Both these issues are discussed in detail utilizing model porous nanostructured inorganic materials such as silica (SiO₂). It is supposed that these model studies would effectively lead to the design of better inorganic and hybrid multifunctional nanostructured host materials for diverse biotechnological applications.

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2. Drug Delivery

Research breakthroughs in designing pharmaceutical drugs for different diseases have greatly aided in advancing the knowledge of the physicochemical properties of drug molecules and the mechanisms of cellular uptake. This has led to numerous effective therapeutic strategies. In the treatment of cancer by chemotherapy, for example, prevalent therapies primarily employ cytotoxic drugs. These drugs when administered via conventional methods have limited utility and adverse side effects. To overcome this hurdle, a widely pursued strategy is to design a drug delivery system (DDS) that can transport an optimised dosage of drugs to the targeted cells and tissues. The success of this approach depends largely upon the ability to synthesize a biocompatible vehicle that allows high amounts of drug loading and prevents any premature drug release prior to reaching the target. A DDS in general should possess the following characteristic properties:

- biocompatibility and biodegradability
- high loading/encapsulation of desired drug molecules without any significant structural or morphological changes
- minimum premature release
- controlled release rate so as to obtain concentrations within the permissible pharmaceutical window of the drug

Though several biodegradable materials have been tried as drug carriers. However, every drug carrier exhibits some shortcomings. However, since the advent of biological nanostructures such as liposomes⁴ as carriers of proteins and drugs for the treatment of diseases, nanotechnology has made a significant impact on the development of drug delivery systems. They were considered “smart” drug delivery systems as they could be tuned to release pharmaceutical drugs in a controlled manner under physiological conditions in an aqueous solution. The release takes place following the structural degradation of the carrier which is triggered by various chemical factors, such as pH. Based on this principle of drug carrier degradation, a variety of nanomaterials, mainly organic (polymeric), have been utilised as delivery vehicles to serve various therapeutic modalities. So far, there have been over a few dozen nanotechnology-based therapeutic products approved by the Food and Drug Administration (FDA) of the United States of America for clinical use and several are in clinical trials. Compared to conventional drug delivery, nanotechnology based systems provide a number of advantages. In particular, they can

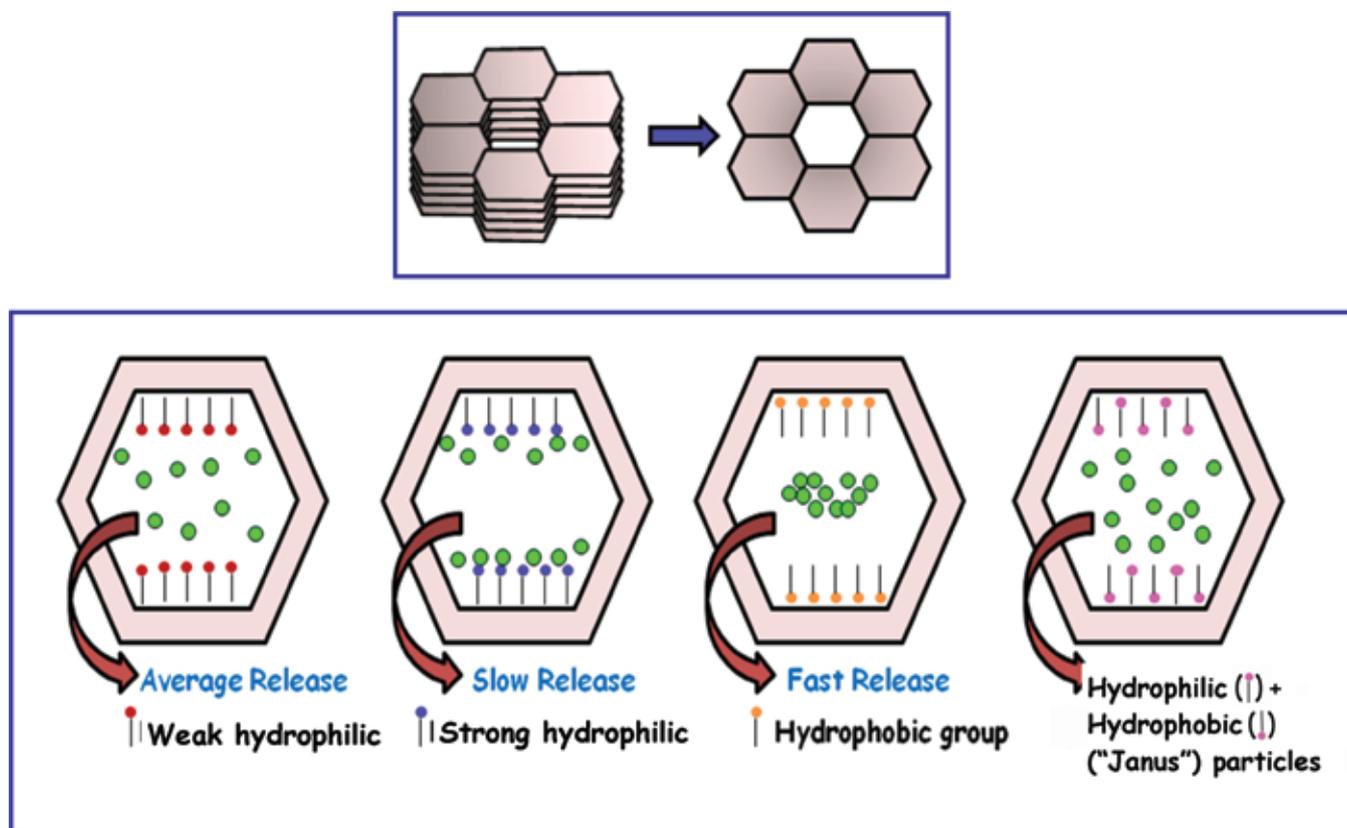
enhance therapeutic activity by prolonging drug half-life, improving the solubility of hydrophobic drugs, reducing potential immunogenicity and releasing drugs in a sustained manner. The drug can also be released in a controlled manner under an external stimulus. In this way, the toxic side effects of drugs can be reduced as well as their administration. In addition, nanoscale particles can passively accumulate in specific tissues e.g., tumors, due to enhanced permeability and retention (EPR) properties.⁵ Beyond displaying clinical efficiency, nanotechnology has also been utilized to bring about new therapies and develop next generation nanosystems for “smart” drug delivery. In the following sections, we discuss various modes of drug delivery utilizing inorganic nanostructured drug vehicles (section #3). We also discuss the implications of confinement at the nanoscale on the structure and function of “bio”-relevant molecules such as drugs, proteins and enzymes (section #4).

3. Porous Nanostructured Inorganic Materials as Hosts for the Controlled Delivery of Various “Bio”-Relevant Molecules

As discussed previously, for the complete effectiveness of a drug, a suitable delivery vehicle is always desirable. Polymer-based materials, although biodegradable and biocompatible, are unstable and also lack the desired circulation times within the blood. Generally, polymer-based drug delivery vehicles undergo rapid chemical degradation (such as hydrolysis) upon dispersion in water and thus display burst release kinetics of the encapsulated compound. Polymeric systems generally require usage of organic solvents for drug loading and these can trigger undesirable modifications of the host structure and/or function of the encapsulated molecules such as protein denaturation and aggregation. Additionally, drug loading amounts are low in polymer DDS. There are several excellent reviews on polymer-based drug delivery vehicles² and the reader may refer to them for more details. The discovery of highly-ordered mesoporous silica materials by scientists at the Mobil Corporation in 1992 (Mobil Crystalline Materials, abbreviated as MCM), was quickly recognized as a major breakthrough that could lead to a variety of important applications.^{6,7} The implications of mesoporous materials in the field of drug/enzyme delivery as well as other biotechnological applications also came into the limelight (figure 1). Mesoporous materials have a uniformly connected pore network with the pore diameter

Mesoporous materials: Porous materials with interconnected pores (size range: (2-50) nm) arranged in a definite geometrical arrangement. The high pore surface area is particularly useful for a wide variety of applications.

Figure 1: Schematic diagram showing drug release from inorganic mesoporous materials such as alumina (Al_2O_3) and MCM-silica (SiO_2). The drug loading and release kinetics can be effectively controlled by the tuning of pore characteristics and surface chemistry. The decoration of the pore surface with two different chemical functionalities enables the mesoporous host (also known as "Janus" particles) to deliver two different drugs.



Janus particles: The term is used to describe spherical particles with one hemisphere hydrophilic and the other being hydrophobic.

Nanostructured materials: General terminology used to refer to nanomaterials varying in geometrical dimensions, shapes and internal morphology. It encompasses both inorganic and organic materials.

ranging from 2–50 nm, and possess high surface areas $1500 \text{ m}^2/\text{g}$ (pore volumes $\sim 1 \text{ cm}^3 \text{ g}^{-1}$). This property, along with high chemical and thermal stability, and easy surface functionalization, makes mesoporous silica an ideal candidate for supporting adsorption, catalysis, chemical separations and biotechnology devices.^{8–11} The chemical stability of silica or, in general, inorganic compounds, prevents the premature release of drug molecules or any unnecessary interaction of the guest molecules (inside the host) with the analytes in solution. Significant research efforts have been directed to achieving control over the pore size and morphology of mesoporous silica. This has led to the development of new silica materials such as Santa Barbara Amorphous (abbreviated as SBA),¹² Michigan State University (abbreviated as MSU)¹³ and folded sheet mesoporous (abbreviated as FSM) materials¹⁴ with characteristic pore morphologies. The discovery of mesoporous silica also triggered

the development of various inorganic, organic and even inorganic/organic hybrid materials with mesoporous structures. At SSCU, IISc, we performed systematic studies on the influence of the host nanostructure morphology and surface chemistry on drug release (figures 2–6). Based on our studies using model DDS based on silica (SiO_2), titania (TiO_2), and alumina (Al_2O_3) the drug release kinetics has been observed to depend strongly and also on several parameters of the nanostructured host such as pore size, volume, configuration as well as on the surface chemistry and dimension of the host. Mesoporous systems are not the only inorganic structures which have biotechnological applications. Other nanostructured materials such as nanospheres, nanotubes (for e.g., figure 6) and hierarchical structures have also received considerable attention. Even in such cases the release kinetics has also been observed to depend strongly on the host morphology and chemistry.

Figure 2: Release kinetics of ibuprofen (IBU) from alumina (Al_2O_3)-X (X: -OH, $-\text{CH}_3$, $-\text{NH}_2$, -DN: 2,3-dihydroxynaphthalene) into simulated body fluid (SBF, pH = 7.4) at 25°C.²⁵ Release kinetics observed via monitoring the characteristic peak at 264 nm of IBU by UV-vis spectroscopy. The plot clearly shows a correlation between release kinetics and the hydrophobicity/hydrophilicity of the oxide surface. Hydrophobic surfaces showed faster release of IBU in aqueous simulated body fluid compared to hydrophilic oxide surfaces.

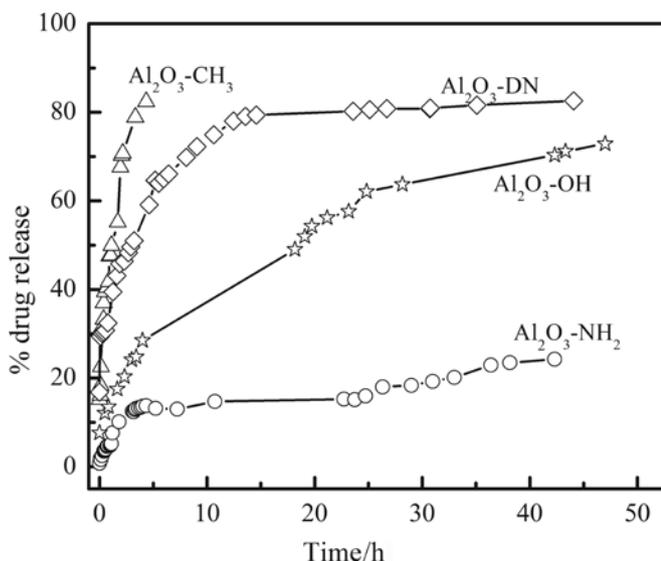
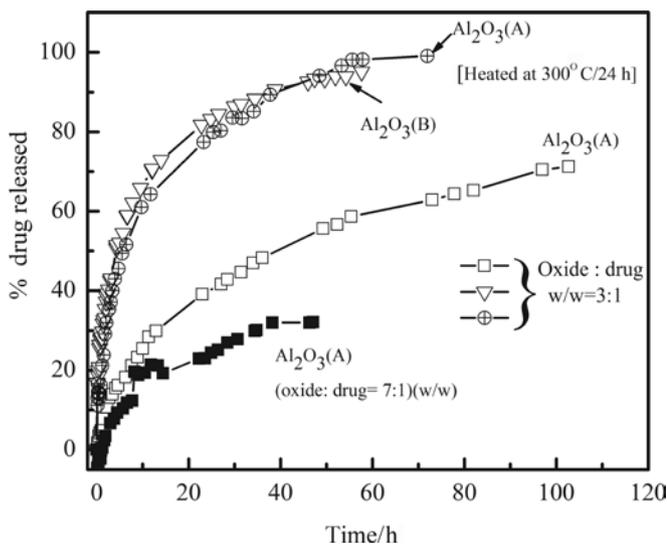


Figure 3: Kinetics of ibuprofen (IBU) release in simulated body fluid (SBF, pH=7.4) from alumina (Al_2O_3): X samples (X: acidic (A), basic (B)). Release kinetics observed via monitoring characteristic peak at 264 nm of IBU by UV-vis spectroscopy. The plot clearly shows correlation between density of surface silanol groups and drug loading (not shown here) and release kinetics. Higher [-OH] density (i.e. higher acidity) results in stronger drug host interaction and hence slower release of IBU.²⁴



3.1 Mesoporous inorganic nanoparticles as drug carriers

The drug delivery applications take advantage of the high surface area ($\geq 1000 \text{ m}^2 \text{ g}^{-1}$) and pore volume ($\geq 1 \text{ cm}^3 \text{ g}^{-1}$) of mesoporous materials such as MCM silica. Guest drug molecules can be suitably adsorbed on to the mesopore surface. The drug loading and release are governed by pore morphology and surface chemistry (figures 2 and 3). The group of Vallet Regi at Madrid were the first to demonstrate the utility of mesoporous oxides such as MCM-41 silica for drug delivery. Several reports¹⁵ from the Madrid group demonstrated the importance of pore morphology and their configurations on drug delivery. Victor Lin's group at Iowa State University has also contributed considerably towards understanding the effect of the pore and particle morphology of mesoporous silica.¹⁶ A room-temperature ionic liquid (RTIL) template mesoporous silica nanoparticle (MSN) system, abbreviated as RTIL-MSN, with various particle morphologies including spheres, ellipsoids, rods and tubes for the controlled release of antimicrobial agents, was developed. By changing the RTIL template, the pore morphologies could be tuned from the MCM-41-type hexagonal mesopores to the rotational Moiré-type helical channels and to wormhole-like porous structures. It was observed that the spherical/hexagonal mesopore RTIL-MSN exhibited superior (1000-fold) antibacterial activity compared to the tubular/wormhole RTIL-MSN. The results were accounted for by the fact that the rate of release via diffusion from the parallel hexagonal channels was faster than that from the disordered wormhole pores. Thus, the role of texture and structure host matrices was clearly demonstrated. In addition, Lin's group has also studied the effect of pore enlargement and chemical functionalization of mesoporous silica on the target-specific delivery of proteins into plant cells^{17,18}

Hybrid silica-based mesoporous materials can also be employed to serve specific clinical needs. In the simplified sense, hybrid silica particles can be obtained by modifying the mesoporous silica pore surface with covalent attachments of organic functional groups.¹⁹ The organic functional groups can be used to anchor several types of drugs. The attachment of the drug to the matrix is either through electrostatic attractive interactions, hydrophilic-hydrophobic forces or electronic interactions.¹⁸⁻²⁰ Vallet-Regi and his co-workers observed that the loading of L-tryptophan was greatly enhanced when the surface of SBA-15 was modified using quaternary amines with different alkyl lengths (methyl and octadecyl).²³ The importance of surface chemistry is also duly

RTIL: Ionic liquids are salts in the liquid state. Room temperature ionic liquid abbreviated as RTIL are mainly organic salts containing of bulky and asymmetric organic cations such as alkyl-imidazolium/pyridinium with anions such as halides existing in the liquid state at room temperature.

demonstrated in refs.^{9–11} where MCM-41 was functionalised with an aminopropyl group. The amino group is protonated during the loading process either by its reaction with the acid group of the drug in a non-polar aprotic solvent or by loading the drug at a pH below the pK_a value for primary amines (9–11). The release takes place at a physiological pH of 7.4, at which value the amino groups remain protonated. It was found that the MCM-41 (pore diameter: 3.8 nm; surface area: 1157 m²/g) and SBA-15 (pore diameter: 9 nm; surface area: 719 m²/g) surfaces when modified with aminopropyl groups, resulted in an increase of alendronate by almost 3 times compared to unmodified MCM-41.²¹ Further, a remarkable decrease of the initial burst effect was noticed. Almost 55% of the alendronate loaded into the unmodified SBA-15 was released in the medium during the first 24 h of assay, while the amino-functionalised SBA-15 showed a release of only 11%. The different release kinetics between unmodified and amino modified SBA-15 proves beyond doubt the importance of surface chemistry on drug loading and release. The drug release rate can be retarded/accelerated by appropriately tuning the hydrophobicity/hydrophilicity of the pore surface. The presence of hydrophobic functional groups does not allow the aqueous solution inside the pores²⁴ thus slowing down the drug release. The release of macrolide antibiotic erythromycin from SBA-15 was modulated by organically modifying the surface with C₈ (octyltrimethoxysilane) and C₁₈ (octadecyltrimethoxysilane) hydrocarbon chains respectively. Functionalization resulted in a decrease of surface area and effective pore size of mesoporous matrices which led to a decrease in the amount of drug loading in functionalized SBA-15 compared to bare SBA-15. Functionalization also led to a reduction in the wetting ability of the SBA-15 surface by aqueous solution. This resulted in a marked decrease in drug release kinetics from functionalised samples compared to bare SBA-15. The effect of surface hydrophobicity/hydrophilicity on drug release rates has also been systematically studied by Kapoor et al²⁷ (figure 2). The Decoration of mesoporous alumina-neutral (abbreviated Al₂O₃-N) with various types of silane (such as 3-aminotrimethoxysilane-APTMS, methylmethoxysilane-MTMS) and other groups (such as 2, 3-dihydroxynaphthalene-DN) leads to different release kinetics. The hydrophobic functionalization of the Al₂O₃ surface resulted in a low degree of drug loading (~20%) but faster release rates (85% in 5 hours). The functionalization of the Al₂O₃-N surface with hydrophilic groups on the other hand showed

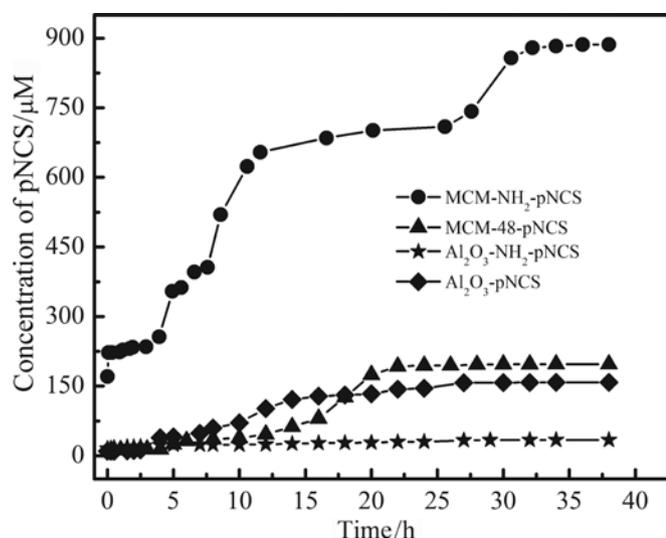
higher drug loading (21–45%) but release rates comparatively slow (12–40%). The drug release from mesoporous Al₂O₃ was found to be diffusion controlled ($\propto \text{time}^{0.5}$). Thus, we can conclude that both the chemical character of the various functional groups lining the alumina surface and the pore characteristics (including the wide pore size distribution of (2–8) nm) played a key role in altering the nature of drug release kinetics.

Das et al ref²⁶ presented a systematic study of drug loading and release kinetics from commercially available mesoporous alumina (Al₂O₃) nanoparticles with varying densities of surface silanol functional groups (figure 3). The density of the silanol groups determined the acidity/basicity of the surface. Acidic-Al₂O₃ (i.e. Al₂O₃ with the highest amounts of OH groups) exhibited the highest drug loading capacity of 26% while basic-Al₂O₃ (i.e. Al₂O₃ with low amounts of OH groups) was the lowest with 22%. Through systematic variation in several parameters, it was found that the release of IBU from basic-Al₂O₃ was significantly faster compared to acidic-Al₂O₃. This is accounted for by the stronger interaction between the drug and its mesoporous host in the case of the acidic-Al₂O₃ sample compared to the basic-Al₂O₃ sample. The pH of the release medium and the ratio of concentration of Al₂O₃ : IBU also influenced the release kinetics. Further, IBU release kinetics as a function of external stimuli (such as pH) was observed to be strongly dependent on oxide surface acidity.

Mesoporous materials can also be suitably designed to carry multiple payloads. “Janus” mesoporous nanoparticles (figure 1) which have two different surface functional groups can be prospective hosts for the controlled delivery of two different kinds of drugs. The concentration of the surface chemical groups controls both the loading as well as the release kinetics of the individual drug. The other possibility for hosting more than one drug can be achieved via the usage of *dendrimers*. Dendrimers are highly branched monodispersed macromolecules possessing a large number of functionalities. Dendrimers have many bio-medical applications, for example, as contrast agents in magnetic resonance imaging, gene transfection therapies, and in drug delivery.²⁵

The application of mesoporous materials has been recently extended to enzyme-inhibitor delivery²⁸ (figure 4). Studies were carried out using a model protein Chick retinal tyrosine phosphatase-2 (CRYP-2), a protein that is identical to the human glomerular epithelial protein-1, and present in hepatic carcinoma. The inhibition of CRYP-2 was demonstrated through the delivery

Figure 4: Paranitrocathecol sulfate (abbreviated pNCS) inhibitor release profiles into 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 25°C from $\text{Al}_2\text{O}_3/\text{Al}_2\text{O}_3\text{-NH}_2$ and MCM-48/MCM-48- NH_2 .²⁶



SBF: Simulated body fluid abbreviated as SBF is laboratory prototype having inorganic ion concentrations similar to those of human extracellular fluid.

of p-nitrocatechol sulfate (pNCS) from bare and amine-functionalized mesoporous silica (MCM-48) and mesoporous alumina (Al_2O_3). MCM-48 functionalised with an amine moiety exhibited the best release of pNCS and hence inhibition of CRYP-2 with a maximum speed of reaction ($v_{max} = 160 \pm 10 \mu\text{mol}/\text{mnt}/\text{mg}$) and an inhibition constant $K_i = 85.0 \pm 5.0 \mu\text{mol}$. This value was close proximity to the values obtained for protein tyrosine phosphatase inhibited via conventional methods. This strongly supports our view of the employment of inorganic nanostructured media for the inhibition of enzymatic activity.

3.2 Drug release using silica nanotubes (SNT) and the effect of external stimuli

The use of porous nanostructured materials of different configurations and dimensions for drug delivery has also been a subject of considerable interest. Kapoor et al used silica nanotubes to demonstrate the controlled drug delivery of Ibuprofen (IBU)²⁹ (figures 5 and 6). SNTs were synthesised using the standard sol-gel template method. The diameter of the SNTs could be suitably tailored from (50–200) nm depending on the guest molecule size. The effects of chemical functionalization as well as external stimuli on drug release kinetics were also discussed. The surface modification and drug loading were carried out using the procedures discussed previously. The SNTs under physiological conditions

exhibited a very poor rate of IBU release (20% in 30 h). This was accounted for by the presence of morphological heterogeneities (e.g., junctions, bends) which posed steric hindrance for the fast diffusion of the solvated drug out of the nanotube. The drug release rate was slow when compared to standard mesoporous materials such as MCM-48 (~90% release within 30 h). The IBU kinetics change significantly as a result of the functionalization of the SNT surface with 2, 3-dihydroxy-naphthalene (DN). The IBU release kinetics from SNT-2, 3-dihydroxy-naphthalene (abbreviated SNT-DN) was intermediate to that of SNT and MCM-48. After ~13 h the IBU release from SNT-DN became higher than that for MCM-48. Nearly 95% of IBU was released from SNT-DN in 24 h compared to 87% for MCM-48 and 20% for SNT. The slow initial release in the case of the SNT-DN was attributed to the dominance of attractive interaction (such as $\pi-\pi$) between the surface naphthalene group and IBU over that of the solvation of IBU (i.e. the COOH group) by the SBF. The faster release of IBU at longer time scales from SNT-DN compared to that from MCM-48 (average pore size ~2.5 nm) was attributed to the difference in morphology of the nanostructured materials. The drug release rate of SNT could also be drastically altered using external stimuli in the form of ultrasound³⁰ impulses. Ultrasound impulses have been used previously in the case of insulin release from the MCM-41/-48 matrix modified with PDMS. Other forms of external stimuli such as electrical field have also been used to release insulin from polymer matrix composite (PEOx and PMMA).³¹ An optimized ultrasound protocol of high frequency, short duration impulses and short waiting times leads to a linear rate of ibuprofen release from SNT. A few tens of percentage enhancements were obtained over approximately 100 min in comparison to bare SNT which exhibited a mere 20% release yield over 30 h (figure 6). The external stimuli created percolation pathways (via the removal of SNT heterogeneities) for the aqueous SBF to leach out IBU from the pores of the nanotube. This led to improved pharmacokinetics of IBU.

3.3 Gated drug delivery systems

As discussed earlier, an important attribute of DDS is to release a drug at a controlled rate at a specific site of interest. Lin et al developed a series of stimuli-responsive mesoporous silica nanoparticle (MSN)-based controlled release systems. They demonstrated a gated MSN system where the drug loaded mesoporous material was capped by mercaptoacetic acid coated CdS nanocrystals³²

Figure 5: A schematic diagram showing drug release from silica nanotubes (SNTs) under ultrasound impulses and from surface functionalized SNTs. The external stimuli and surface chemical functionalization were required to accelerate the release yields of bare SNT.

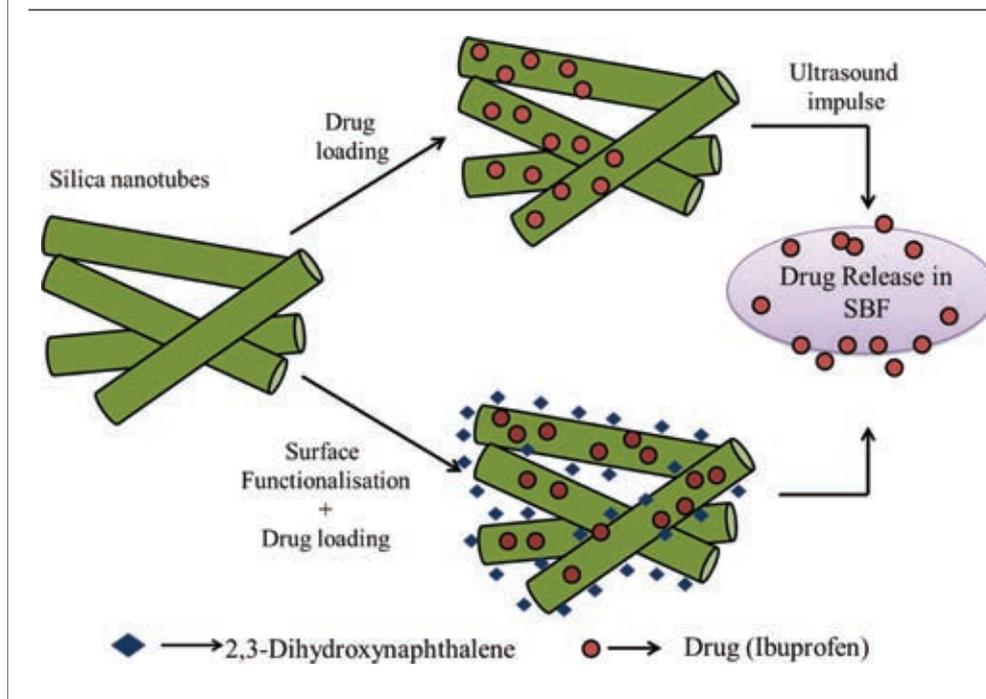
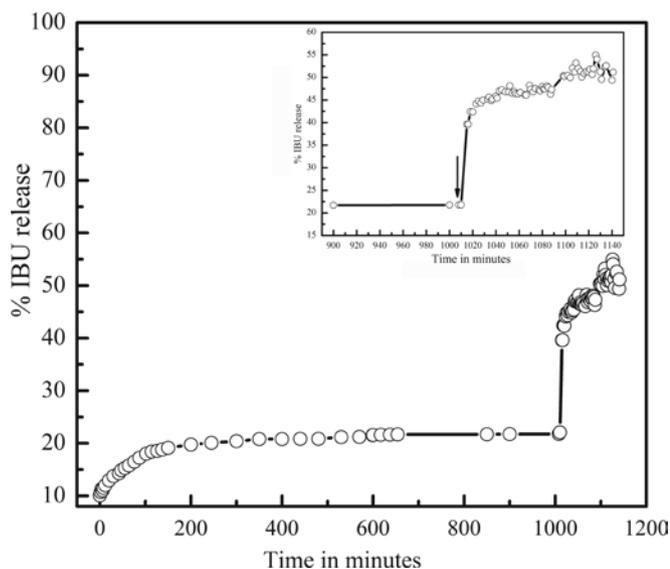


Figure 6: Ibuprofen (IBU) release from silica nanotubes (SNT) with an ultrasound impulse protocol of 0.5 min duration impulses and 2 min rest time. The application of ultrasound results in the enhancement of IBU release by a few tens of percentage over 100 mins compared to a total release of only 20% over nearly 30 h from bare SNT.



through a chemically cleavable disulfide linkage. This was done to prevent premature drug release. The drug was released through the exposure of the capped MSNs to chemical stimulation such as dithiothreitol (DTT) and mercaptoethanol (ME) that cleaves the disulfide linker. Other than chemically stimulated release, Tanaka and his co-workers demonstrated a reversible photocontrolled release system in an organic solvent using coumarin-modified mesoporous silica.³⁴ The release of the steroid cholestane stored within the mesopores was prevented by the coumarin that was covalently attached to the MCM-41 pore walls through a photodimerization reaction that yielded cyclobutane. The release was triggered by exposure to a UV light wavelength of ~ 250 nm to cleave the cyclobutane ring of the coumarin dimmer.³⁴ Zink, Stoddart, and their co-workers developed an electrochemically-controlled delivery system based on a pseudorotaxane cap that could undergo conformational changes triggered by redox chemistry.³⁵ The mesopores were loaded with $\text{Ir}(\text{ppy})_3$ (ppy: 2,2'-phenylpyridyl), a fluorescent molecule. The opening of the gate was stimulated by the addition of an external reducing agent which caused the pseudorotaxane to disassemble.

Drug release under the application of a magnetic field has also been demonstrated. Lin et al designed

Biosensors: A device for detection of biological analytes generally, in solution. Electrochemical biosensors give an electrical signal in response to chemical reaction between the analyte and a bio-relevant molecule in the bio-recognition layer (working electrode-WE) of the sensor. For accurate quantification of the electrical response, two other electrodes are necessary viz.: reference electrode (RE); counter electrode (CE).

a MSN material capped with superparamagnetic iron oxide (Fe_3O_4) nanoparticles.³⁶ The device consisted of rod-shaped MSNs functionalized with 3-(propyl disulfanyl) propionic acid (average particle size of 200 nm \times 80 nm and pore size \sim 3 nm) with the pores bound with 3-aminopropyltriethoxysilyl-functionalized superparamagnetic iron oxide (APTS- Fe_3O_4) nanoparticles. The response to the magnetic impulse was verified by placing the sample between bar magnets. The drug release was triggered by dithiothreitol (DTT).

Another very interesting approach is the concept of dual drug delivery i.e. the simultaneous release of two drugs in response to a stimulus. Lin et al. developed a boronic acid-functionalized mesoporous silica nanoparticle-based drug delivery system for a glucose-responsive controlled release of both insulin and cyclic adenosine monophosphate (cAMP).³⁷ The stimulus for the release was achieved using saccharides. The gluconic acid modified insulin was labelled with a Fluorescein isothiocyanate and this served as the gatekeeper altering the drug release. The decrease in the amount of labelled insulin triggered the release of cAMP. The triggering effect of other saccharides such as fructose, and glucose on the release rates were also studied.

The various studies discussed above have established beyond doubt that drug loading and release from mesoporous and tubular structures can be modulated by controlling the host morphology and surface chemistry as well as the response to external stimuli. It was also shown that numerous pore-capping agents can be incorporated to encapsulate drugs and chemicals in the mesopores of inorganic hosts. Porous inorganic and hybrid structures can suitably adapt to participate in various biological processes including intracellular functions. A suitable selection of materials and processes should be developed for all such applications. Based on the rapid advances in the synthesis and processing of inorganic materials, one foresees applications of biocompatible and biodegradable inorganic nanostructures in the not too distant future.

4. Effects of Confinement Inside Inorganic Host Materials on the Structure and Function of Bio-Molecules and their Implications in Drug Delivery and Sensing

Conditions different from normal physiological temperature and pH often lead to enzyme protein denaturation and hence, loss of reactivity and selectivity. Moreover, the use of free enzymes has several processing difficulties with regard

to re-usage, contamination and separation. One approach to resolve these difficulties is to immobilize enzymes on solid surfaces. The immobilization of proteins from a solution onto solid surfaces of complementary charge has for long attracted much attention due to its potential application in several areas.^{38–40} The adsorption of proteins over controlled porous glass (CPG) and sol-gel derived nanocomposite materials for possible applications such as biosensors and other biological applications has been reviewed by several research groups.^{41–45} The major disadvantage of CPG materials for adsorption studies is their high cost and lower surface area of interaction (pore size \sim (30–200) nm).⁴⁶ Enzyme encapsulation inside silica gels was also attempted.⁴⁷ However, due to small and non-uniform pore size distribution and the lack of a connected pore network as in mesoporous materials, the enzyme usually showed less activity than its free counterparts. However, following the discovery of mesoporous silica materials with controlled pore architecture there has been a vast improvement in enzyme stability especially in extreme environmental conditions, catalytic activity and products specificity. The use of porous materials in bioadsorption and biocatalysis has been extensively reviewed by Hartmann⁴⁸ and Zhao.⁴⁹ These reviews provide a comprehensive account of the application of mesoporous materials to immobilize biological molecules of varying sizes: amino acid to proteins. Other than these mesoporous materials, nanostructured materials in other dimensions (1-D; 2-D) have also been used for bio-molecular encapsulation.

4.1 Mesoporous silica-based materials for bio-molecule encapsulation

Mesoporous silica, in addition to possessing a well defined pore structure, also has a high density of surface silanol groups. These can be suitably modified with a wide range of organic functional groups for immobilizing biomolecules. The immobilization of globular enzymes, cytochrome *c* (bovine heart), papain (papaya latex) and trypsin (bovine pancreas) was carried out in mesoporous molecular sieves such as MCM-41.⁵⁰ The efficiency of enzyme immobilization was found to be pH dependent and it varied for different enzymes. The silanation of pores after trypsin adsorption eliminated the leaching of the enzyme into a solution at high pH. It was also found that entrapped trypsin hydrolysed N- α -benzoyl-DL-arginine-4-nitroanilide (BAPNA) and its stability was also enhanced by its physical entrapment in MCM-41. Similar observations were also reported with SBA-15.⁵¹ Thiol, chloride, amine,

carboxylic acid and phenylsiloxane incorporated SBA-15 were used to immobilise trypsin and were more retention of the enzyme molecules was more than unfunctionalized SBA-15. The resulting encapsulated enzyme was also found to be an active and stable catalyst for the hydrolysis of BAPNA. Additionally, trypsin supported on thiol-functionalised SBA-15 was shown to be recyclable.

In addition to pore size, the surface charges of the protein and of the mesoporous material have to be complementary.⁵² Pepsin at pH 6.5, for example, is negatively charged and does not adsorb onto cyan-modified silicate, whereas subtilisin, which is of a similar size and bears an overall positive charge, is adsorbed. Using Raman spectroscopy, cytochrome c was observed to occur in both high spin and low spin states. In solution, however the protein is predominantly in the low spin state. The presence of the high spin state may account for the enhanced peroxidative activity of the adsorbed protein.

Enzyme immobilization is also influenced by the pore structure of the host. This was demonstrated in the case of the immobilization of cytochrome c into MCM-48, SBA-15 and the layered transition metal oxide Nb-TMS4.⁵³ Greater protein loading was observed for the three-dimensional MCM-48 which had the highest surface area compared to the one-dimensional hexagonal SBA-15 and two-dimensional layered

Nb-TMS4. Cyclic voltammetry studies showed that the entrapped cytochrome c retained its activity upon encapsulation. The beneficial influence of encapsulation on protein function was reported by Vinu et al for cytochrome c⁵⁴ and by Takahashi et al for horseradish peroxidase (HRP).⁵⁵

Eggers and Valentine carried out a circular dichroism study with sol-gel encapsulated proteins to examine the effects of macromolecular crowding and hydration effects on protein stability.⁵⁶ It was concluded that the stability of the proteins in the confined environment was governed by two important factors i.e. excluded volume effects and water structure changes in a confined space. Lysozyme, α -lactalbumin and metmyoglobin retained their native solution structures following sol-gel encapsulation, but apomyoglobin was found to be largely unfolded within the silica matrix under control buffer conditions. Using elastic neutron scattering, Schirò et al studied the effect of hydration on the dynamics of metmyoglobin encapsulated in a sol-gel matrix.⁵⁷ It was observed that the level of hydration in the porous silica matrix strongly affects the mean square displacements of protons in the confined protein, but not in the bulk protein. Confinement also has adverse effects on the protein structure as reported by Sorin and Pande.⁵⁸ Through molecular dynamics simulation, it was shown that the confinement of proteins inside a carbon nanotube denatures protein helices. It was found that both surface chemistry and topography play key roles in determining the structure of the bound proteins.^{59,60}

Kapoor et al recently investigated the influence of confinement on the structure of native Hemoglobin, (abbreviated Hb) and its effect on the ligand binding activity of Hb (figures 7–9). The consequence of confinement inside SNTs on protein structure was further discussed via thermal stability and electrochemical activity studies.⁶¹ The reversible binding of pyridine and its derivatives indicated the absence of any adverse effects on the structure and function of Hb as a result of confinement (figure 8). Additionally, the melting /thermal denaturation temperature of the confined Hb was enhanced by approximately 4°C as compared with that of free Hb in solution (figure 9). Similar thermal stability as a result of confinement was also observed in the case of lysozyme. Contrary to some reports based on carbon nanotubes, confinement inside silica nanotubes did not have any adverse effect on the protein structure and function. Rather, it had more beneficial effects when compared to the protein in the free state. The optimized morphology of the nanostructured host is not expected to impose any restriction on the favourable protein conformations

Figure 7: A schematic diagram showing the effects of confinement investigated using electrochemical techniques (such as cyclic voltammetry) circular dichroism spectroscopy, x-ray diffraction (not shown here).

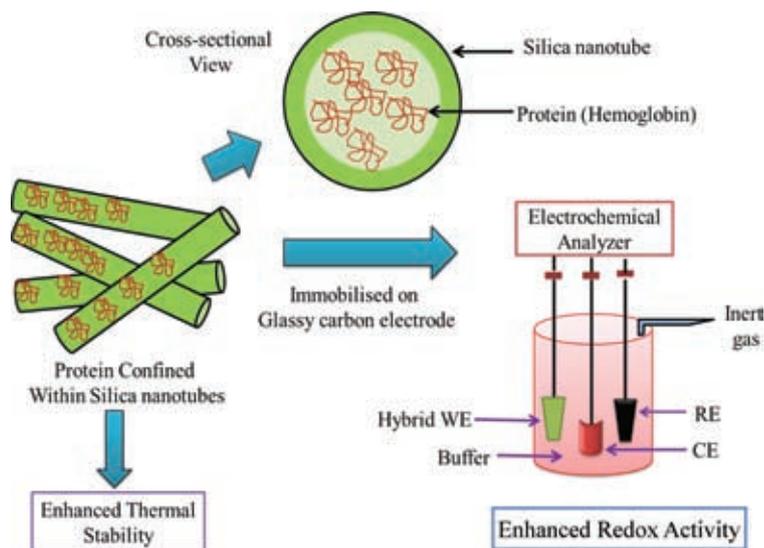


Figure 8: Cyclic voltammogram of (a) silica nanotubes (SNT) (b) native hemoglobin and hemoglobin confined inside SNTs. The SNT-Hb is immobilized on the glassy carbon electrode (GCE). This whole assembly is abbreviated as SNT-Hb/GCE. Scan rate and buffers were respectively 0.01 V s⁻¹ and 0.1 M Phosphate buffered saline (PBS) buffer (pH-7.0). Inset: Transmission electron microscope (TEM) images of SNT (length ~10 μm and diameter 50–200 nm) respectively.

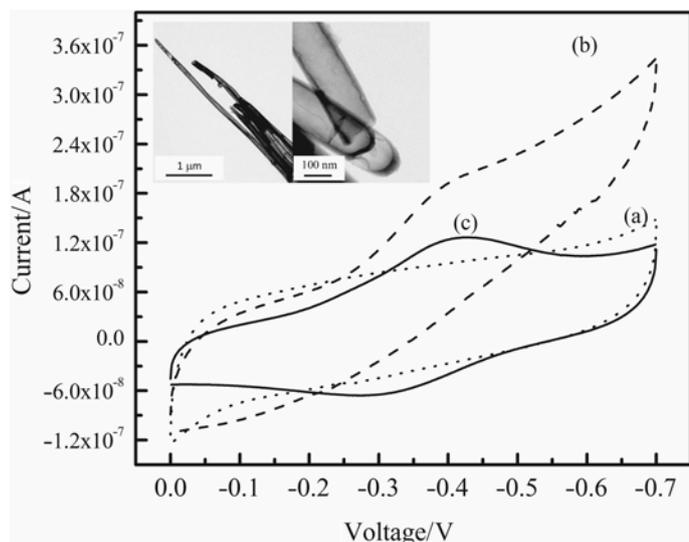
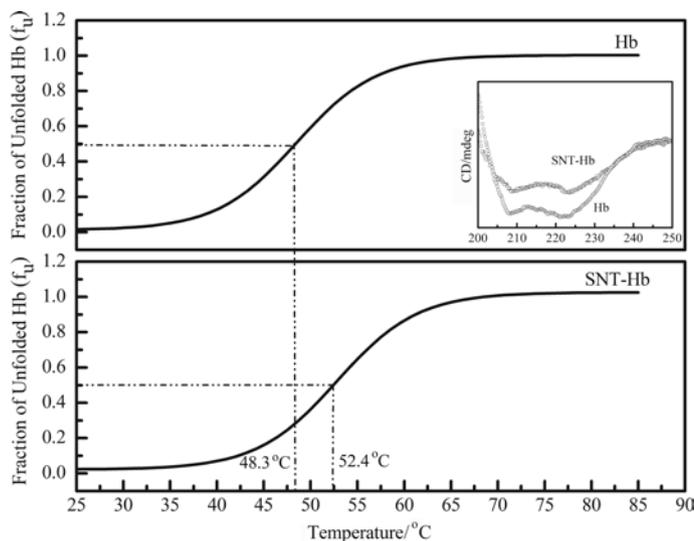


Figure 9: Fraction of hemoglobin, Hb (f_u) in the temperature range 25–85°C (heating rate 1°C/min) for free Hb in solution (0.1 M PBS, pH 7.0) and Hb confined inside SNTs, SNT-Hb obtained by monitoring the CD signal at 222 nm. Inset: Circular Dichroism spectra (CD) at 25°C for Hb and SNT-Hb in 0.1 M Phosphate buffered saline (PBS, pH-7.0).



requisite for primary protein activities such as ligand binding. Confinement may reduce protein dynamics but this may be a blessing in disguise as it would allow the protein to remain in favourable conformations for longer periods of time.

On the basic research front, confinement is an interesting and formidable statistical mechanics problem. Several important issues such as intermolecular interaction, host-guest interactions, the role of water in confined space all have to be taken into consideration for a comprehensive understanding of the confinement phenomenon. Though not discussed explicitly (in section #3), confinement also plays a very important role in drug delivery. The nanostructured host plays a crucial role in determining the molecular conformation during its residence inside the host as well as its release from the host. A recent work by Mandal et al.⁶² reveals the strong dependence of ibuprofen conformation (S or R form) on the morphology of mesoporous titania and titania nanotubes. The host morphology also determined the drug conformation when released into the simulated body fluid buffer under normal physiological conditions.

5. Conclusion and Future Perspectives

We have convincingly shown here that the encapsulation of “bio”-relevant molecules in specially designed nanostructures opens several possibilities of improving the molecular stability, activity and function desired for various biotechnological applications. Nanostructured materials exhibit great versatility and can also be utilized to control regioselectivity, enantioselectivity or even reverse the properties of the native enzyme. The design of a suitable porous host material and formulating a requisite protocol for the encapsulation of “bio”-relevant molecules is a challenging task. However, with the rapid strides made by nanotechnology, it is expected that such hurdles will be overcome with ease in the coming years. The immobilization of molecules inside a space of nanoscale dimensions provides several interesting problems of theoretical interest. Confinement at the nanoscale presents a mesoscopic situation as it demands modelling of various phenomena using much fewer (\ll Avogadro number, $N_a \sim 10^{24}$) molecules. We conclude that a sound theoretical understanding of various complex phenomena at the mesoscale is necessary for the design of better multifunctional inorganic materials. This will effectively lead to a predictable and beneficial confinement of a wide range of “bio”-relevant molecules for diverse biotechnological applications.

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