EMBO reports

viewpoint

Medicine on a small scale

How molecular medicine can benefit from self-assembled and nanostructured materials

Marijeta Kralj & Kresimir Pavelic

Over the past few years, nanotechnology has emerged as a new and exciting research field that deals with the design, synthesis and fabrication of structures at the molecular scale. Smallness is not in itself the prime goal; it is rather the expectation that, by manipulating matter at the molecular level, new intrinsic material properties can be created. Because living matter itself is basically composed of biological 'nanomachines' and nanostructures, researchers recognized quite early on that biology and medicine could be prime fields for the application of nanotechnology. In general, nanomedicine can be defined as the monitoring, repairing, construction and control of human biological systems at the cellular level by using materials and structures engineered at the molecular level. It encompasses much more than just being an extension of 'molecular medicine', and future products and developments may have extraordinary and far-reaching implications for the definition, diagnosis and treatment of disease, and for how medicine is practised (Freitas, 2002). The main interests currently lie in improving diagnostic methods and in developing better drug delivery systems to improve disease therapy. More generally, the scientific community is increasingly focusing its attention on the novel chemical and physical properties of nano-sized materials to develop new applications in regard to human health.

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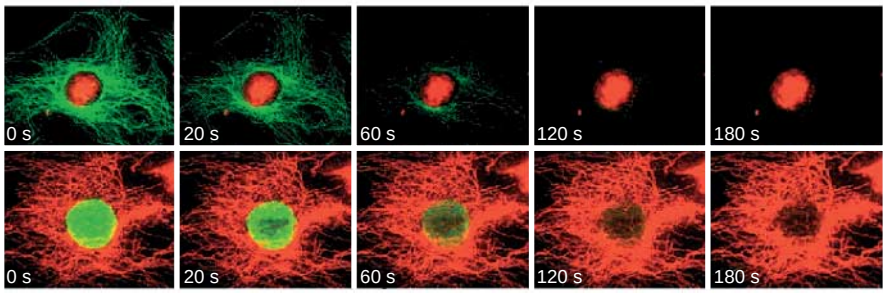

Fig. 1 | Photostability comparison between quantum dots (red) and Alexa 488 (green). Whereas labelling signals of Alexa 488 faded quickly and became undetectable within 2 min, the signals of QD630 showed no obvious change for the entire 3-min illumination period. (Reprinted from Wu *et al.* (2003), with the permission of Nature Publishing Group, www.nature.com).

Nanotechnology today deals mainly with two rather different but complementary types of material: nano-sized structures (or nanoparticles) and nanoporous materials. There are already some exciting developments in the field of diagnostics based on the use of nanoparticles, in particular fluorescent semiconductor quantum dots (QDs). QDs are monodisperse inorganic nanocrystalline particles made from semiconducting material and are typically 2–10 nm in size—about the size of a protein or a short sequence of DNA. They can be linked to biomolecules to form sensitive long-lived probes that target and identify specific cellular compounds (Fig. 1). As fluorescent probes, QDs have several advantages over conventional organic dyes: their emission spectra are narrow and symmetrical on the basis of their size and material composition, and they exhibit excellent photostability. In addition, they display broad absorption spectra, which makes it possible to excite many QDs to different colours with a single excitation light source (Wu *et al.*, 2003). This is certainly an advantage in studying multiple biological targets simultaneously in the cell. The high photostability of QDs also allows real-time monitoring or tracking of intracellular processes *in vivo* over extended periods. Dubertret *et al.* (2002) showed this in a breakthrough experiment by labelling a living frog embryo with more than a billion individual QD particles encapsulated in phospholipid copolymer micelles. Their experiments showed that these QD micelles are non-toxic and stable in biological environments, which suggests the possibility of tracing cell lineages in embryogenesis experiments. Bioconjugated QDs have also been used for DNA hybridization and high-throughput genotyping of single-nucleotide polymorphisms (SNPs), the most common type of genetic variation between individuals. SNPs are very good markers for disease-causing genes, and they hold further potential for personalized medicine as markers for differential drug responses (Xu *et al.*, 2003). As the labelling of individual molecules or cell structures in living cells or tissues is becoming an increasingly important tool in diagnostics, QDs, because of their many

advantages over organic dyes, have a large potential for new and improved diagnostic tests in medicine.

One of the most important problems in medicine is the proper distribution and targeting of drugs and other therapeutic agents within the patient's body. Increasing the efficiency of delivering therapeutic molecules to their final target cells is therefore a priority for the pharmaceutical industry. A large amount of research is being undertaken to develop new 'advanced' materials that incorporate a biologically active substance, transport it to the right place and release it there in a controlled way. This concept is particularly interesting for potent but non-specifically acting drugs, such as anticancer drugs, that cause several severe side effects in healthy tissues or cells. Cell targeting can be achieved either by 'passive' methods, in which a polymer conjugate or particle system is captured by a physiological uptake mechanism of the target cell, or by 'active' targeting—the attachment of a homing moiety, such as monoclonal antibodies or ligands in the form of sugars or lectins—to deliver the drug to the right cell by attaching it to specific receptors on cell surfaces (Davis, 1997).

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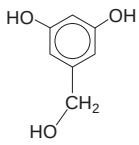
Nanoparticles are promising vessels for drug targeting because their distribution and elimination patterns in the body are dictated mainly by their physicochemical properties, such as particle size, hydrophobicity and surface charge. Generally, a therapeutic molecule can be dissolved, entrapped, encapsulated or attached to a lipid-based or polymer-based carrier system. But scientists are already looking beyond traditional polymer networks to nanotechnology to improve the therapeutic potential of various drugs. Colloidal particles, such as liposomes or emulsions, can overcome the problem of bringing a hydrophobic substance into the aqueous blood compartment. Another benefit of colloidal carrier systems compared with free drugs is their protection of sensitive drugs, such as pure and highly specific peptides and proteins and also synthetic peptide vaccines, against degradation in biological fluids. Association with an appropriate carrier in those cases should confer protection from degradation, particularly when taking the oral route, by decreasing non-specific interactions with food proteins and improving uptake across the intestinal wall. One example of overcoming these problems is the insertion of drugs into biodegradable nanoparticles coated with vitamin B_{12} to enhance the oral uptake of various pharmaceuticals, such as interferon, erythropoietin or granulocyte colony-stimulating factor. Such biodegradable nanoparticles have already attracted considerable attention as potential drug- delivery devices mainly because of their ability to be reabsorbed by the body (Tao & Desai, 2003).

It is just a little step further to combine these two characteristics—attaching or encapsulating the therapeutic agent within a carrier material and providing this material with the means to home in on target cells—to create a drug-delivery vessel that will increase drug efficiency while circumventing side effects in non-targeted tissues. One possibility is to use homing peptides with high selectivity *in vivo*. Blood vessels, for instance, express molecular markers that distinguish between the vasculatures of individual organs, tissues and tumours, and unique sets of peptides with high selectivity for these markers have been identified. Akerman *et al.* (2002) showed the effective targeting of the vasculatures of normal lungs and tumours in a living mammalian organism by using peptide-coated QDs. These results opened up the possibility of using other nanomaterials with more far-reaching properties, for example a material that could target tumour cells through the folate receptor, while also having a fluorescent label for imaging and tracking of the drug. Quintana *et al.* (2002) developed dendrimer-based nanodevices optimized for the intracellular targeting of drugs, imaging agents and other materials.

Some of the most advanced cutting-edge technologies to synthesize such complex drug-delivery vessels involve the use of dendrimers and fullerenes when used in combination with peptides (Akerman *et al.*, 2002). Dendrimers are polymeric monodisperse macromolecules with a precisely defined chemical structure (Fig. 2). These branching synthetic molecules can be grown



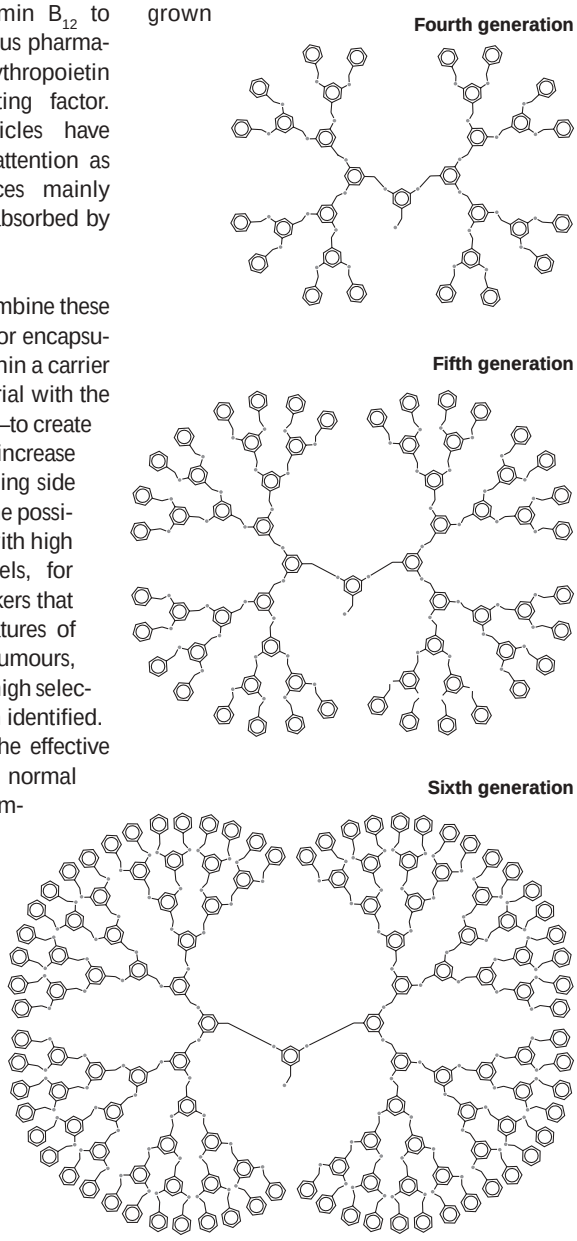


Fig. 2 | Schematic view of a dendrimer and its synthesis. A dendrimer is a polymer built up from a single monomer (3,5-dihydroxy-benzyl alcohol in this case). With each step of the dendrimer generation, new branches are added to create a three-dimensional tree-like structure.

> Particularly interesting is the process of molecular self-assembly, in which molecules—or parts of molecules—spontaneously form ordered aggregates without any human intervention

nanometre by nanometre to reach the desired size. They have a spherical shape and sufficiently large openings and cavities to carry small molecules. Dendrimers can be used as potential drug-delivery agents in at least two ways: drug molecules can be either physically trapped inside the molecule or covalently attached to the surface. In both cases, the dendrimer backbone serves just as a scaffold to arrange the functional units in space, without having any other inherent function. At present, the most active research area in dendrimer-based therapeutics is the entrapment of drugs for subsequent and controlled release, as has been shown with the cytostatic agents Adriamycin and methotrexate. Another potential application could be the use of dendrimers coated with sialic acid—crucial for the influenza virus to attach to the cell surface—to 'fool' the virus into attaching itself to these dendrimers instead of to the cell. Dendrimers have also been used commercially as non-viral vectors for DNA delivery: PolyFect (Westburg, Leusden, The Netherlands) is a commercially available *in vitro* transfection agent based on a dendritic structure (Liu & Frechet, 1999; Striba *et al.*, 2002). In addition, dendrimers are non-immunogenic and are thus uniquely suited as carrier structures for drugs or bioactive molecules without falling prey to the immune system (Freitas, 2002).

Several research groups have been testing another, completely different, class of molecules for drug delivery that has also raised huge interest among chemists and physicists: fullerenes. Fullerenes and their relatives—endohedral and metal-coated fullerenes, carbon nanotubes, carbon nanoparticles and porous carbons—are carbon-based nano-sized structures that represent the third allotropic form of elemental carbon after graphite and diamond. By far the most common is C_{60}, also called a 'buckyball'. Foley *et al.* (2002) proposed using fullerenes as drug-delivery agents because their structure mimics the clathrin scaffolds that mediate endocytosis. They showed that a fullerene derivative is able to cross the external cellular membrane and that it localizes preferentially to mitochondria. However, drug delivery by fullerenes is still confined to the research laboratory and it will take many years of research to develop the first clinical applications. Nevertheless, fullerenes have been shown to hold promise as radical scavengers for the protection of neurons, for the inhibition of HIV protease and as an antibacterial agent (Friedman *et al.*, 1993; Tsao *et al.*, 2002). Another possible application is to be found in nuclear medicine, in which fullerenes could be used as an alternative to chelating compounds that prevent the direct binding of toxic metal ions to serum components. This could increase the therapeutic potency of radiation treatments and decrease their adverse effect profile, because fullerenes are resistant to biochemical degradation within the body (Cagle *et al.*, 1999).

Gene therapy is another application that might benefit from nanotechnology, given that it faces the same problems of delivering a therapeutic agent. Although the classic use of gene therapy is to treat monogenetic diseases by supplying a correct form of the defective gene, most of the approved and tested gene therapy protocols available at present aim at treating cancer patients. Nevertheless, the main problem now in gene therapy is to find safe methods for delivering genes to human cells. Three main types of gene-delivery system have been described: viral vectors, non-viral vectors and the direct injection of corrective DNA or RNA into tissues. There are several serious questions about the safety of viral vectors, such as immunogenicity and the reversion of the engineered form to the wild type. Non-viral vectors and direct injection seem in many ways advantageous with regard to safety, but they are less effective (Davis, 1997). This is where nanotechnology could provide a solution to this problem.

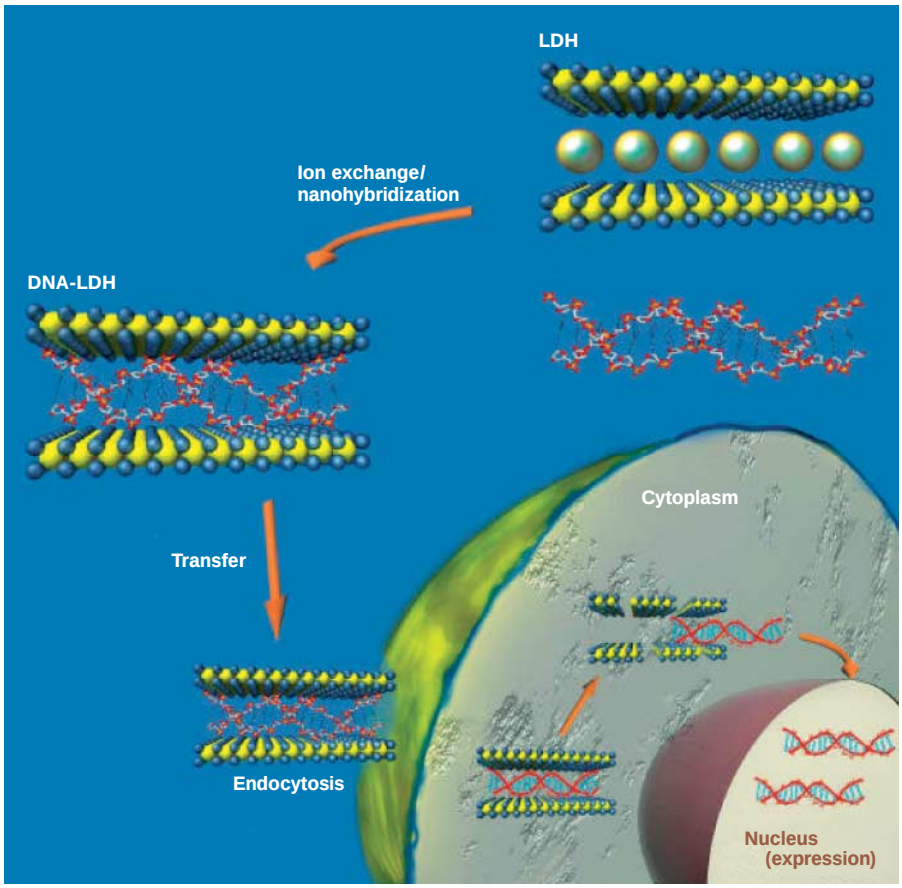


Fig. 3 | Schematic illustration of the hybridization and expected transfer mechanism of the bio-LDH nanohybrid into a cell. LDH, layered double hydroxide. (Modified from Choy *et al.*, 2000.)

Antisense DNA is a potential gene-specific therapeutic agent. These short oligodeoxynucleotide fragments are complementary in sequence to a portion of the targeted mRNA. The aim of antisense therapy is to use them to interfere with gene expression, thereby preventing the translation of mRNA into protein (Lambert *et al.*, 2001). There are some problems in antisense therapy, mainly the poor stability of oligonucleotides against nuclease activity and their low intracellular penetration, which limits their therapeutic use. To solve these problems, particulate nano-sized carriers have been proposed as a delivery method. Cationic polymerized lipid-based nanoparticles, for instance, when coupled to the $\alpha v\beta_3$-integrin-targeting ligand, can deliver genes selectively to angiogenic blood vessels in tumour-bearing mice. The $\alpha v\beta_3$-integrin receptor is strongly expressed in endothelial cells and has a key function in cell survival during angiogenesis *in vivo*. It might be particularly useful for gene-delivery strategies to endothelial cells because it potentiates the internalization of several viruses, thereby facilitating gene transfer. Hood *et al.* (2002) tested its therapeutic efficacy by generating $\alpha v\beta_3$-ligand nanoparticles conjugated to a mutant *Raf* gene, which blocks endothelial signalling and angiogenesis *in vivo*. *Raf* is a component of the signalling pathway that is important in neovascularization, and blocking this pathway suppresses angiogenesis. The delivery of a mutant *Raf* to angiogenic blood vessels blocks endothelial *Raf* activity and induces apoptosis. Systemic injection of the $\alpha v\beta_3$-nanoparticle/*Raf*$^-$ into mice resulted in the regression of established primary and metastatic tumours.

Choy *et al.* (2000) used inorganic layered double hydroxides (LDH) that consist of cationic brucite-like layers and exchangeable interlayer anions. The unique ion-exchange capability of LDHs enables these inorganic matrices to encapsulate functional biomolecules, such as antisense DNA, that are negatively charged in aqueous media, by a simple ion-exchange reaction to form bio-LDH nanohybrids. These hydroxide layers protect the intercalated antisense molecule from degradation by DNase. Once LDH-antisense hybrids have entered the cell, the hydroxide layers are removed by dissolution in the lysosome, where the pH is slightly acidic, and the encapsulated biomolecules are released inside the cell (Fig. 3).

Nanoporous materials, another class of inorganic molecules, have recently attracted interest for use in molecular medicine. Such materials, with pore sizes ranging from less than 2 nm to 50 nm, include zeolites and related molecular sieves. Zeolites are naturally occurring or synthetic crystalline aluminosilicates. The pore network of a typical zeolite, which is confined by the framework, consists of cavities and connecting windows of uniform size (Fig. 4). As a result of these unique properties, zeolites can absorb gas and water molecules, facilitate ion exchange and act as 'molecular sieves' with long-term chemical and biological stability. Zeolites have therefore already become interesting subjects in different areas of chemical research and are now widely used in industrial, agricultural, environmental and biological technology (Mumpton, 1999). There are obvious structural similarities between the cages of zeolites and the substrate-binding sites of enzymes, which have led to the development of zeolite structures that mimic enzyme functions. Another approach would be to incorporate key features of selected enzymes, such as metal complexes, in zeolites—so-called 'ship-in-a-bottle' complexes—that could be used as oxygen carriers, mimicking haemoglobin, cytochrome P450 and iron–sulphur proteins (Bedioui, 1995).

Fig. 4 | Various biomedical applications of natural and synthetic zeolites

> The fact that biological processes are in a way dependent on molecular machines and clearly defined structures shows that building new nanomachines is physically possible

Several toxicological studies of a natural zeolite named clinoptilolite proved that this compound is non-toxic and safe for use in human and veterinary medicine. Recent experiments *in vitro* and *in vivo* have suggested that clinoptilolite could be used as an adjuvant in anticancer therapy (Pavelic *et al.*, 2001). So far, zeolites have been successfully used as detoxicants and decontaminants—when added to animal diets they reduce the level of heavy metals and organopoisoning, radionuclides and ammonia—as well as antibacterial and antidiarrhoeal agents. Zeolites have been used for haemodialysis, and also in cartridges in haemoperfusions. Zeolite powder has been shown to be effective in the treatment of athlete's foot and in reducing the healing time of wounds and surgical incisions. Tissue conditioners containing silver-exchanging zeolites showed a strong antimicrobial effect. On the basis of these findings and positive double-blind clinical studies, the drug Zeomic (Sinamen-Zeomic, Nagoya, Japan) was recently approved in Japan as an antimicrobial agent for dental treatments (Fig. 4; Pavelic & Hadzija, 2003).

In the long term, nanotechnologies will further enable the construction of clearly defined two-dimensional and three-dimensional structures on inorganic, organic or biological templates. The fabrication of such nanostructures on various substrates can be achieved by exploiting a variety of procedures based on either molecular self-assembly or micropositioning technology. Particularly interesting is the process of molecular self-assembly, in which molecules—or parts of molecules—spontaneously form ordered aggregates without any human intervention. Such self-assembly is centrally important in many processes in life. The formation of molecular crystals, colloids, lipid bilayers and phase-separated polymers, the folding of nucleic acids into their functional forms, and the association of a ligand with its receptor are all examples of self-assembly (Whitesides & Boncheva, 2002; Zhang *et al.*, 2002). Consequently, the term 'self-assembly'

was, until recently, applied almost exclusively to biological structures. However, self-assembling materials are becoming a significant topic in current research and it is assumed that in the twenty-first century they might become new building blocks comparable to those of alloys, plastics and semiconductors in the twentieth century.

Numerous self-assembling systems have already been developed: block copolymers, surfactant-like materials, scaffolds for three-dimensional cell culture, DNA-based structures and models with which to study protein folding and protein conformational diseases, such as Alzheimer's disease. A research team at the Scripps Research Institute (La Jolla, CA, USA) has developed antibiotic agents based on self-assembling cyclic peptide nanotubes that attach to, and poke holes through, bacterial cell membranes, thus killing the cell. These self-assembling peptide nanotubes cleared infections of antibiotic-resistant bacteria in mice, even when injected far from the site of infection (Fernandez-Lopez *et al.*, 2001). Another promising example is a vaccine consisting of self-assembling virus-like particles for the prevention of infection of the genital tract by human papilloma virus, which can cause cervical cancer. Such particles are now being developed by MedImmune (Gaithersburg, MD, USA) and GlaxoSmithKline (Uxbridge, UK). Moreover, self-assembling biological materials are of great interest in advanced medicine because they can serve as bioactive extracellular matrices (ECMs). They provide cells and tissues with the appropriate three-dimensional architecture for normal growth and development, which is crucial for the proper understanding required for any successful clinically relevant therapies and for targeted drug delivery. PuraMatrix (3DM, Cambridge, Massachusetts, USA), for example, is one commercially available line of synthetic ECMs (Holmes, 2002).

The fact that biological processes are in a way dependent on molecular machines and clearly defined structures shows that building new nanomachines is physically possible. It also means that we will not necessarily need fundamental new technologies to make further advances in nanotechnology if we can make proper use of those found in nature—which renders the future of nanotechnology foreseeable. Although many of the ideas developed in nanomedicine might seem to be in the realm of science fiction, only a few more steps are needed to make them come true, so the 'time-to-market' of these technologies will not be as long as it seems today. Nanotechnology will soon allow many diseases to be monitored, diagnosed and treated in a minimally invasive way and it thus holds great promise of improving health and prolonging life. Whereas molecular or personalized medicine will bring better diagnosis and prevention of disease, nanomedicine might very well be the next breakthrough in the treatment of diseases.

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