REVIEW ARTICLE

Nanodevices for the immobilization of therapeutic enzymes

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Abstract

Therapeutic enzymes are one of the most promising applications of this century in the field of pharmaceutics. Biocatalyst properties can be improved by enzyme immobilization on nano-objects, thereby increasing stability and reusability and also enhancing the targeting to specific tissues and cells. Therapeutic biocatalyst–nanodevice complexes will provide new tools for the diagnosis and treatment of old and newly emerging pathologies. Among the advantages of this approach are the wide span and diverse range of possible materials and biocatalysts that promise to make the matrix–enzyme combination a unique modality for therapeutic delivery. This review focuses on the most significant techniques and nanomaterials used for enzyme immobilization such as metallic superparamagnetic, silica, and polymeric and single-enzyme nanoparticles. Finally, a review of the application of these nanodevices to different pathologies and modes of administration is presented. In short, since therapeutic enzymes constitute a highly promising alternative for treating a variety of pathologies more effectively, this review is aimed at providing the comprehensive summary needed to understand and improve this burgeoning area.

Introduction

During recent decades, a large number of proteins able to catalyze many reactions have been isolated, tested, and characterized for different biotransformation processes. In addition, the trends of industrial processes are in the forward direction toward the so-called *green-chemistry* syntheses where biocatalysts are playing a major role (Castro & Knubovets, 2003; Torres et al., 2011). Enzymes are able to perform reactions under a myriad of environmental operational conditions. From both the industrial and the therapeutic points of view, the main challenge is to convert a natural biological catalyst into a robust biocatalytic engine (Illanes et al., 2012). Nanotechnology is now providing powerful tools and devices to develop novel alternative strategies for increasing the performance, stability, and activities of biologic molecules for using outside the cell. Complex biologic man-made functional systems can be produced by combining biomolecules – such as proteins, DNA, and others – with natural or modified materials for a variety of useful applications. Among such utilizations, the powerful area of diagnosis, monitoring, and treatment of pathologies through the use of nanoscale devices is burgeoning

and has been classified as nanomedicine by the National Institutes of Health (NIH, USA). Within this area, therapeutic biocatalyst nano-object complexes will provide new tools for the diagnosis and treatment of old and emerging pathologies. The advantages of this approach are the broad compass and diverse scope of materials and biocatalysts that put the modality of the matrix–enzyme combination into a class of its own. Classical techniques for immobilizing enzymes and other biomolecules are quite similar and based mostly on traditional chemistry. Accordingly, through either application or analogy, the amount and the diversity of available methods for enzyme immobilization – either on or within a carrier, by means of nanotechnologic techniques – are increasing rapidly (Miletic et al., 2012). The selection of a given immobilization method is mostly dependent on the enzyme's final therapeutic target and is related to both the biomolecule and the choice of carrier. Moreover, the toxicological properties of the immobilized enzyme, its components, and the potential degradation subproducts must also be considered before application to clinical use. In addition, from the operational standpoint, the immobilization procedures should stabilize the required enzymatic activity in order to guarantee a minimal level of catalysis during the time required for effective therapy.

The administration of immobilized enzymes for therapeutic purposes may be divided into two main categories: (i) immobilized biocatalysts synthesized for use throughout extended circulation in the body, and (ii) enzymes that must

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be constantly present in specific tissues and/or organs as an integral part of that local physiology of the body. In the second category, the immobilized enzyme is used to act locally for the treatment of specific organs or restricted compromised areas, e.g. thrombi, tumors, skin, and heart injuries. Mainly for discrete lesions, biocompatible nanoparticles have become novel versatile devices for the development of exceptional structures for enzyme stabilization for two main reasons: the small size and the large surface area. The material characteristics such as hardness, porosity, and topology of many nanoparticles furthermore provide a strong platform for enzyme immobilization (Ansari & Husain, 2012; Costa et al., 2004; Datta et al., 2013; Hwang & Gu, 2013; Wang et al., 2013b).

This review will discuss the nanodevices currently available for enzyme therapy along with presenting relevant examples of various biomaterial supports for enzyme immobilization and a list of potential therapeutic enzymes for different specific biomedical applications.

Immobilization techniques

One of the main problems in using enzymes out of their native niche is the lack of the physicochemical environment provided by the cells for efficient catalysis. The main challenges associated with the optimization of catalytic performance are related to the complexity of the protein three-dimensional structure and its conformational changes and structural dynamics during the biocatalytic conversion of substrate to product. An alternative to solving this problem is found in the development of immobilization techniques that provide an extensive platform to produce robust biocatalysis. Immobilization technique refers to the interaction between an enzyme and a selected solid support that changes the physical state of that biocatalyst. The immobilization procedure results in protein structures that are more rigid and have different specific chemical, biocatalytic, mechanical, and kinetic properties from the respective features of the native enzyme. In general, most of the immobilization procedures are detrimental to the catalytic activity, as expressed by common kinetic parameters such as K_m, V_m, and turnover rate. The robustness of enzyme-immobilization techniques, however, is generally expressed by a high increase in enzyme stability under extreme environmental operational conditions in terms of pH, elevated temperatures, the presence of solvents, and/or widely varying ionic strengths. From the standpoint of therapeutic use, immobilized enzymes have advantageous properties, among which a greater resistance to lytic activities such as the significant autolysis occurring in some hydrolases can be cited (e.g. the serine proteases and esterases), a highly extended catalytic half-life, a resistance to shearing effects, a reusability of the biocatalyst throughout several batch preparations, and an increase in the enzyme's *in vivo* residence time (i.e. a prolonged half-life of circulation within the body).

Enzyme's primary properties, such as molecular mass, composition of amino-acid functional groups, and requirement for cofactors, are key elements in selecting the immobilization method. In addition, the catalytic and substrate-binding sites along with the enzyme's topology and dynamic tertiary protein structure are crucial

considerations in the choice of the appropriate immobilized biocatalyst. Furthermore, the intrinsic characteristics and properties of the support material and the method of immobilization have a strong influence on the resulting catalytic properties of the immobilized enzyme, with the mechanical and physicochemical properties of the support material being crucial to the *in vivo* application of the enzyme cargo. An extensive review of the different enzyme-immobilization methods and their characteristics is beyond the scope of the present article, but has recently been published in great detail (Brady & Jordaan, 2009; Elnashar, 2004; Sheldon & van Pelt, 2013). Instead, here we will provide a brief introduction to the most common approaches to affixing an enzyme to a solid support. In general, enzyme-immobilization methods can be roughly grouped into the following categories.

Enzyme immobilization through the use of matrices

(1) Interaction of the biocatalyst within the support matrix by simple adsorption methods (hydrophobic or Van-der-Waals interactions) and/or ionic interactions – either one with or without subsequent covalent attachment to the support – can also include an activation of the support with bifunctional reagents able to covalently bind the enzyme to the matrix (e.g. via amine groups). One of the characteristics of this procedure is that the biocatalyst is usually exposed to the surface of the support, although certain nanodevices contain the cargo inside the matrix. The matrices generally used are microparticles or membranes. The supports are extremely diverse, ranging from ion-exchange resins to inorganic or organic matrices and biopolymers (Breccia et al., 1999; Castro et al., 2005).

(2) Inclusion of the biocatalyst inside the matrix, i.e. encapsulation or entrapment, where the enzyme is located inside the matrix and is not exposed to the surface of the support. Many matrices acting as supports in this fashion have been reported, ranging from inorganic to organic to natural molecules, i.e. biopolymers (Costa et al., 2008; Islan et al., 2013; Martínez et al., 2013; Pierre, 2004; Zhang et al., 2011).

Enzyme self-immobilization (without supports)

Enzyme self-immobilization by cross-linking techniques has been developed in recent years (Sheldon, 2011). In this instance, the shift from a soluble to an insoluble form of the enzyme is made either by chemical cross-linking of the protein with itself to produce enzymatic crystals (CLECs) or by the formation of amorphous enzymatic aggregates referred to as CLEAs or Spherezymes (Brady et al., 2008; Illanes et al., 2012; Sheldon, 2011). The main advantages of these techniques are the absence of a carrier and the possibility of combining enzyme purification and immobilization in a single step. In addition, the simultaneous cross-linking of two or more enzymes with complementary functions – termed combi-CLEAs or CLECs – is advantageous from an operational point of view on an industrial scale (Sheldon & van Pelt, 2013).

Moreover, complementary techniques based on the tools of molecular biology such as protein fusion, enzymatic

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conjugation, and coenzyme systems are becoming more adept at improving immobilization techniques (Schoffelen & van Hest, 2012). These combination technologies involve not only molecular biology and recombinant-DNA techniques but also the acquisition of structural information on the proteins (e.g. through the use of bioinformatics) as well as specific knowledge of the enzyme's catalytic mechanism. Two main complementary approaches have been described to produce enzyme systems consisting of hybrid complexes, termed *rational redesign* and *directed evolution* (Pera et al., 2004). These technologies, however, are still too complicated, expensive, and time-consuming for scaling up to industrial applications.

Nanomaterials for enzyme immobilization in drug delivery

Recently, the development of nanotechnology has opened a new field in enzyme immobilization, which enables a myriad of novel supports with different properties and a multitude of potential applications (Table 1; Wang et al., 2013a,b,c). The effect of the nanoscale environment on enzyme stability has been investigated in various types of nanomaterials. A series of studies involving nano-objects such as carbon nanotubes and nanoparticles have established a new depth of understanding about the interaction of enzymes with such material surfaces, e.g. the nanomaterial's curvature. The effect of the nanoscale environment on enzyme stability has been found to be enzyme specific (Kim et al., 2009). In this section, we present and discuss the nanomaterials most widely employed for the immobilization of therapeutic enzymes.

Metal nanoparticles

The simplicity of assays for detecting metal nanoparticles by the optical properties of localized surface plasmon resonance (LSPR) makes these objects one of the most widely used in the nanotechnology arena (Liong et al., 2008). In fact, gold nanoparticles (AuNPs) provide attractive vehicles for the delivery of molecules in biomedical applications. AuNPs are easy to produce by several techniques and allow a control of particle surface area, shape, and size. Several approaches involving both noncovalent and covalent immobilization of many types of enzymes have been successfully developed. Noncovalent protein delivery presents a platform where the reversibility of the nanoparticle–protein interaction plays a major role that can be modulated by changing the AuNP

surface structure. A typical example is the noncovalent immobilization of β-galactosidase, an enzyme with negatively charged residues, by covering the AuNP surface with a cationic monolayer (Rana et al., 2012).

Silver nanoparticles (AgNPs) have been attracting recent interest for clinical applications because of their biologic properties, such as bactericidal activity, anti-inflammatory effects, and wound-healing efficacy, that could potentially be exploited (by enzyme immobilization, for example) in developing better dressings for wounds and ulcers (Gunasekaran et al., 2011). The toxic nature of these nanoparticles, however, and their aggregation limited their use in whole-animal systems. Nevertheless, the functionalization of the surfaces of those nanoparticles with therapeutically active molecules will probably increase the biocompatibility of AgNPs and expand their biological applications (Ravindran et al., 2013).

Superparamagnetic nanoparticles

Enzymes have been recently immobilized on magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) nanoparticles that display excellent properties, including a large surface area, high protein flexibility, and high diffusion coefficients for substrates and products. Furthermore, the application of an external magnetic field to the body enables the magnetic nanoparticles to be targeted to a restricted bodily area.

The use of magnetic nanoparticles within the body, however, requires surface modifications to not only reduce the nanoparticle toxicity (e.g. from Fenton reactions) but also maintain the enzyme activity. The choice of capping molecules and techniques depends on the type of enzyme and the particular therapeutic use (Netto et al., 2013).

Silica nanosystems

Drug delivery based on mesoporous-silica nanoparticles (MSNPs) has been widely explored because of their simplicity of synthesis, high stability under harsh environmental conditions, control of pore diameter, extensive surface area, and topology. The synthesis of MSNPs requires the polycondensation of an organosilane precursor, e.g. tetraethyl orthosilicate (TEOS), in the presence of a surfactant as a template. The main advantages of MSNPs are their high biocompatibility, biodegradability, and mechanical strength. For drug-delivery purposes, the cargo molecule can be added at the sol–gel transition during the synthesis or, alternatively, by soaking the MSNPs in a specific drug solution. The sol composition and

Table 1. Examples of biocatalysts immobilized on nanodevices.

Nanomaterial for immobilization	Enzymes	EC number*	References
Hybrid nanoparticles	α-Amylase	3.2.1.1	Wang et al. (2013a)
Metal nanoparticles	Glucose oxidase	1.1.3.4	Qin et al. (2012)
Polymer nanoparticles	Acetylcholine esterase and choline oxidase	3.1.1.7 and 1.1.3.17	Konno et al. (2004)
Supermagnetic nanoparticles	Lipase	3.1.1.3	Gardimalla et al. (2005)
Single-enzyme nanoparticles (SEN)	α-Chymotrypsin and trypsin	3.4.21.1 and 3.4.4.4	Kim & Grate (2003)
Silica nanosystems	α-Amylase and carboxylesterase	3.2.1.1 and 3.1.1.1	Bellino et al. (2010) and Edwards et al. (2011)

*From Brenda: http://www.brenda-enzymes.info/index.php4.

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the reaction conditions, e.g. pH, temperature, and reaction time as well as the nature of the precursor, define the properties of MSNPs and consequently, the drug loading, stability, and kinetic-release profiles, which are parameters that can provide a myriad of potential uses. Among the possible applications, the development of carriers and matrices based on nanohybrid amorphous silica nanoparticles provides novel alternatives for multiple and/or simultaneous applications in medicine. MSNP tailoring can be developed by the addition of single molecular annexes (e.g. proteins, drugs, dyes, and polysaccharides) and/or other nano-objects (e.g. quantum dots). Recent strategies with MSNPs focused on the development of hybrid nanoparticles with multitask functionalities such as imaging (through NMR, fluorescence, radioactivity), targeting (by specific cell markers), and/or drug delivery. Hybrid silica-magnetic nanoparticles displayed especially promising properties because of the thickness of the silica coating, which is related to the magnetic behavior of the nanoparticles (Jin et al., 2009). In essence, magnetic hybrid nanoparticles with thicker silica coatings exhibit low particle aggregation and thus exhibit the phenomenon of superparamagnetism (de la Rica et al., 2012; Nepal et al., 2008; Yin et al., 2011).

Polymeric nanoparticles

Polymer-based micro- and nanoparticles have been widely employed as carriers in drug-delivery systems (Castro et al., 2005; Jagur-Grodzinski, 2009). Among the associated properties, the polymer-based nanoparticle size plays a major role in the fluidity of these nano-objects within the body; nanoparticle size is also the key to targeting a specific tissue *in vivo*.

Natural polymers such as pectins, alginates (ALGs), carrageenans, and others would appear to be a propitious environment to interact with and protect proteins against harsh environments. Diverse mechanisms of interactions between the biopolymer and the biocatalyst, such as adsorption, entrapment, and encapsulation in different biomatrices, have been analyzed (Figure 1). Lipases, proteases, and ALG lyase upon testing have given promising results for potential applications in drug delivery and wound healing (Castro et al., 2005, Costa et al., 2008; Elnashar et al., 2014; Islan et al., 2013; Martínez et al., 2013).

Biodegradable polyesters such as polylactic acids, poly-lactic-*co*-glycolic acid, poly-L-lactide, and their mixtures; ester anhydrides; polyaminoacids made from lysine or aspartate; polyalkylcyanoacrylates; the environmentally sensitive poly(*N*-isopropylacrylamide) or PNIPAAm; interpenetrating polymeric networks; certain lipids; and several gel polymers such as polyvinyl alcohol are commonly employed for nanocarrier synthesis. The techniques used for their preparation may affect the properties and behavior of the carrier as a drug-delivery system and the enzyme as the cargo. Many methods have been developed for the synthesis of polymeric nanoparticles by means of emulsification (single or double) of monomers and/or polymeric structures down to the method of solvent evaporation. Alternatively, the emulsification of polymeric gels with simultaneous cross-linking has been extensively studied (Jagur-Grodzinski, 2009).

Single-enzyme nanoparticles

The term single-enzyme nanoparticle (SEN) implies that each biocatalyst molecule is surrounded by a layer of nanometric porous polymer matrix with or without a salt ionically linked, resulting in higher enzyme stability with no limitations in the mass transfer of substrate and product (Hegedus & Nagy, 2009; Wang et al., 2013a,b,c).

The classical chemical synthesis through the use of synthetic polymers consists mainly of three steps. The first involves covalent modification of an enzyme with a monomer of the polymer followed by solubilization of the derivative in a hydrophobic medium. The second carried out in nonpolar solvents (e.g. hexane), entails the polymerization of a vinyl group on the polymer with a subsequent hydrolysis and condensation of the trimethoxysilyl functional group (Hegedus & Nagy, 2009) as the third and final step.

In one example of the use of salt complexes, a divalent cation (Ca^{+2}) was added to a solution containing an allosteric enzyme (a calcium-dependent α-amylase) in phosphate-buffered saline (PBS). The various ratios of cross-linking agents provided different nanodevice morphologies, while the structure of the nano-object could also be associated with the enzyme activity (Wang et al., 2013a,b,c). Thus, the dual advantage of this approach was not only to have the enzyme immobilized in a nanodevice, but also to be able to tailor its activity by simply varying the concentration of the divalent ion (Ca^{+2}), the nature of the anionic buffer (PBS), and the pH (Figure 2).

Artificial enzymes

Through supramolecular interactions, enzymes are created by the self-assembly (SA) and folding of polymeric chain-like components to form molecules in the nanometer-scale with 3D structures. By using the advantages of supramolecular chemistry and the design concept of artificial enzymes, a variety of supramolecular AEs were prepared based on different supramolecular materials such as macrocycles and container molecules (e.g. cyclodextrins, crown ethers, cyclophanes, calixarenes, capsules, cavitands, molecular cages, and others), and SA nanometer-sized objects (such as, micelles, vesicles, ligand-anchoring supramolecular complexes, nanogels, nanotubes and nanoparticles) (Dong et al., 2012).

The recent development of AE designed based on supramolecular scaffolds ranging from the synthetic macrocycles to self-assembly nanometer-sized objects is useful for pharmaceutical proposes. For example, enzymes can manipulate the controlled assembly and morphological transformation of nanomaterials (Hahn & Gianneschi, 2011; Gao et al., 2011) and the controlled release of drugs in nanovalves (Liu et al., 2011).

Carbon-based nanosystems

Graphene

Graphene oxide (GO) nanodevices consist of carbon atoms bonded by SP^2 bonding in order to obtain a two-dimensional structure. This unique characteristic has persuaded scientists to design GO nanodevices for biomedical applications such as drug and gene delivery for cancer therapy, enzyme
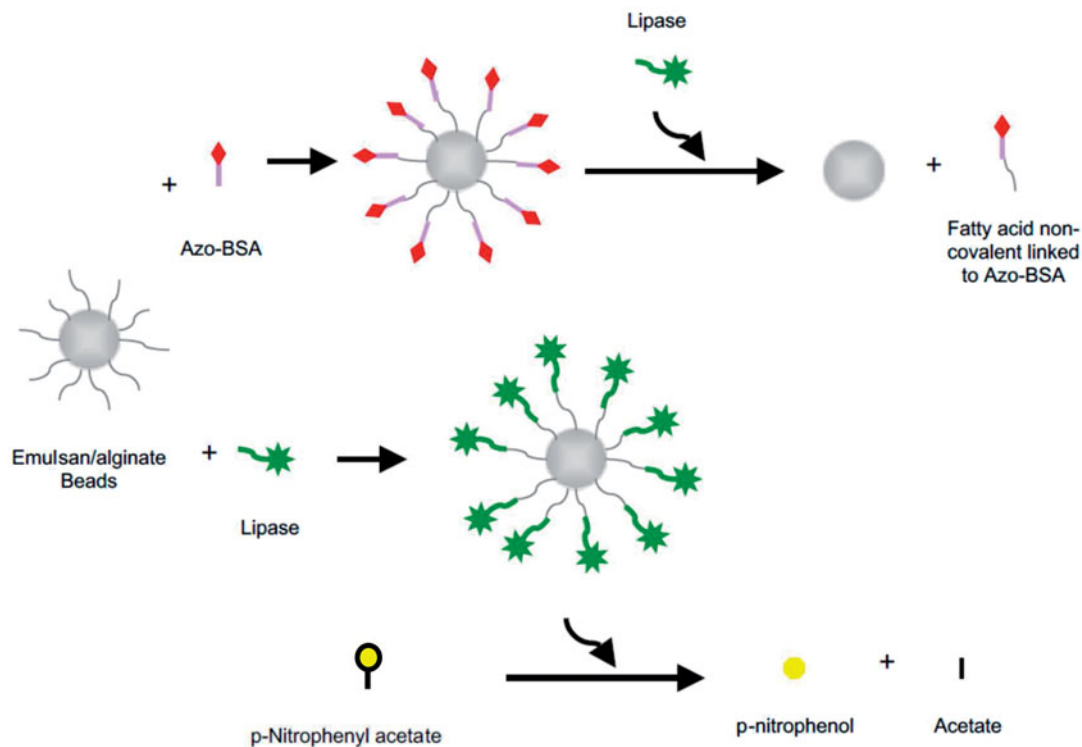
Figure 1. Cartoon of potential protein activities attached to biogel spheres. Reproduced from Castro et al. (2005) with permission of Elsevier.
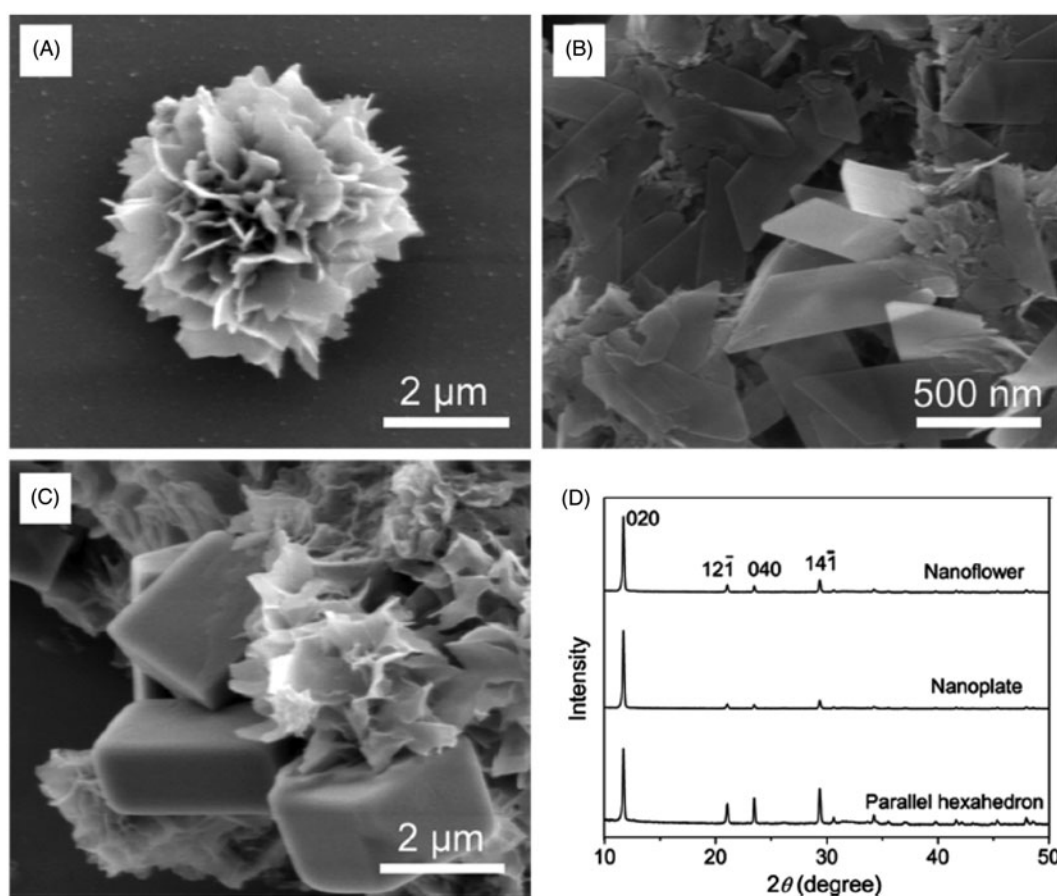

Figure 2. Scanning-electron-microscopy images of different SEN complexes of α-amylase made with ionic cross-linking of Ca^{+2} and phosphate: (A) nanoflowers, (B) nanoplates, and (C) parallel hexahedrons. (D) X-ray-diffraction patterns of the SEN complexes of nanoflowers, nanoplates, and parallel hexahedrons from top to bottom. Reproduced from Wang et al. (2013a) with permission of the American Chemical Society.

immobilization, biosensing, bioimaging, and scaffold design (Shen et al., 2012). GO toxicity, however, is the main concern due to its flat shape and charge. Also, oxidative processes may affect cellular membrane and cellular survival. In this way, functionalization of GO nanodevices is an alternative, among others, to avoid GO cytotoxicity and place them as promising devices for biomedical applications.

Carbon nanotubes

Single-walled carbon nanotubes (SWCNT) are rolled-up tubular shells of graphene sheet. Different studies revealed the interaction of CNTs with biological molecules, such as DNA, peptides, and proteins. They represent an interesting tool in enzyme immobilization, as they are stable under harsh conditions and provide a higher enzyme loading with enhanced catalytic activity (Feng & Ji, 2011; Wei & Peijun, 2011). Among them, glucose oxidase was attached to CNTs and its enzymatic activity was maintained. Enzyme adsorption generates a new device for glucose detection (Wang et al., 2003). In a similar way, the detection of extracellular glutamate levels in brain was performed by the preparation of carbon nanotubes with immobilized L-glutamate oxidase (Lee et al., 2011). However, more studies on CNT interactions with living mammalian cells are still needed. Surface modification was an alternative to make CNTs friendlier and less toxic. In this sense, functionalization with natural biopolymer is an attractive alternative to extend CNT application to living systems (Hu et al., 2004; Nepal & Balasubramanian, 2008).

Some reports on carbon nanostructures, however, indicated toxic side effects when they were administered to living organisms (Park et al., 2014). Molecular controlled release based on C-nanoforms is still under intense study by many groups because of their controversial results (Chng et al., in press; Guo & Mei, 2014).

Enzymes for disease therapies and immobilization systems

The therapeutic enzymes immobilized in nanodevices have been classified based on chemical and structural characteristics of the matrices. Table 2 lists the most significant examples of various biomaterial supports that have been used in immobilizing enzymes for different biomedical applications. The criteria for the classification of immobilized enzymes take into account the different immobilization strategies, the type of materials employed, the biomedical use and means of application, and the route of administration.

Parenteral administration of nanodevices

The most relevant examples of therapeutic enzyme administration associated with this route are the following.

Antithrombotic applications and therapy

Tissue plasminogen activator (E.C. 3.4.21.68, tPA) is a protease belonging to the serine family that is used to treat patients who have a blood clot and consequently are at risk of ischemic stroke or cardiovascular arrest.

The reaction carried out by tPA is the hydrolysis of plasminogen to plasmin. Since plasmin degrades the insoluble fibrin clot into soluble fibrin (Rouf et al., 1996), tPA is widely used in antihrombosis therapy. The short half-life, however, of free tPA is approximately from 2 to 6 min because of autolysis.

Streptokinase (E.C. 3.4.99.0, SK), a bacterial replacement for tPA, is also used for clot dissolution, but because of its nonhuman origin, it possesses undesirable side effects such as immune responses and pyrogenic reactions (Stehle & Schettler, 1986).

In addition, nattokinase (E. C. 3.4.21.62), a serine protease despite its unfortunate misnomer, is an enzyme elaborated by *Bacillus subtilis* var. natto with tPA-like activity and a similar fibrinolytic capability. Among the advantages of nattokinase is its half-life of more than 3 h and the higher fibrinogen-cleavage activity. Among the advantages of nattokinase is its half-life of more than 3 h and the higher cleavage activity for cross-linked fibrin than for plasmin cleavage (Ren et al., 2010).

Urokinase (E.C. 3.4.21.73) extracted from human urine, and another incorrectly named serine protease, is likewise an antithrombotic enzyme. Furthermore, the urokinase half-life in plasma is approximately 15 min.

All the proteases reported thus far have in common an extremely short half-life (Castro, 1999, 2000). Consequently, the administration of those enzymes for therapeutic purposes has required high and recurrent doses: for example, a 15-mg intravenous dose of SK is followed by a second bolus of 85 mg during the next 90 min. This high dose of enzymes leads to hemorrhagic complications because of the activation of circulating and fibrin-bound plasminogen (Park et al., 2001). Hence, an efficient carrier is required to extend the half-life of the enzyme and thus reduce the enzymatic dose in order to obtain the desired therapeutic effect.

Covalently immobilized tPA on magnetic nanoparticles was employed successfully for in-stent thrombosis pathology (Kempe & Kempe, 2010). The incidence of the early onset of post-stent thrombosis (before 1 month after stent insertion) is generally very low (1.0–1.5%) irrespective of the stent model, whereas the probability of thrombosis with drug-eluting stents after a prolonged time of stent insertion is higher than that with bare-metal stents. Transmission electron microscopic analysis revealed octahedral particles with a size range of 10–30 nm. In addition, magnetite nanoparticles can be injected through the guide catheter.

In vitro experiments have demonstrated that magnetic tPa nanoparticles (tPA-NPs) can be oriented successfully to a ferromagnetic spiral in the presence of a magnetic field. Nevertheless, a slight hemolysis in an induced thrombus was found in the presence of tPA-NPs. In those experiments, no short-term side effects were observed in a porcine model, suggesting that tPA-NPs would be a promising device for thrombosis treatment (Figure 3).

The plasma clearance of tPA decreases when the encapsulation carriers are liposomes coated with polyethylene glycol (PEG), the so-called *stealth* or PEGylated liposomes, the former because the PEG coating is inert with respect to the immune system. Such improvement was observed after parenteral administration of tPA encapsulated in PEGylated liposomes in rats (Kim et al., 2009). Liposomal formulations of tPA – either modified (such as with PEGylation) or unaltered – produced an extended degree of circulation

Table 2. Therapeutic enzymes on nanomaterials and common administration routes.

Enzymes (E.C.*)	Therapy	Nanomaterial	Route	References
Alcohol oxidase (E.C. 1.1.3.7) and catalase (E.C. 1.11.1.6)	Alcohol prophylaxis and antidote for intoxications	DNA-directed assembly with AuNPs	Parenteral	Liu et al. (2013)
Alkaline phosphatase (E.C. 3.1.3.1)	Increased mineralization and bone regeneration (treatment of hypophosphatasia)	microporous nanofibrous fibrin scaffolds	Local	Osathanon et al. (2009)
Asparginase (E.C. 3.5.1.1)	Acute lymphoblastic leukemia	PEGylated	Parenteral	Wang et al. (2013c)
Bilirubin oxidase (E.C. 1.3.3.5)	Treatment of neonatal jaundice	immobilized rat-serum albumin	Oral	Soltys et al. (1992)
Catalase (E.C. 1.11.1.6)	Wound treatment	Sugar–ester vesicles	Local	Thiem & Goślińska (2004), Tavano et al. (2010), and Abdelmajeed et al. (2012)
Chymotrypsin (E.C. 3.4.21.1)	Pancreatic insufficiency	SEN; polystyrene NPs; magnetic NPs; electrospinning nanofibers	Oral	Jia et al. (2003), Koneracká et al. (2002), Hegedus & Nagy (2009), and Medeiros et al. (2011)
Brachyurin (collagenase, E.C. 3.4.21.32)	Skin ulcers	Hyaluronan microparticles	Dermic	Lee et al. (2001)
β-Galactosidase (E.C. 3.2.1.23)	Lactose intolerance	Magnetic NPs	Oral	Corchero et al. (2012)
β-Galactosidase (E.C. 3.2.1.23)	Lactose intolerance	Concanavalin A layered calcium alginate–starch beads; positively charged trimethylammonium-functionalized mixed-monolayer protected clusters (MMPCs)	Oral	Haider & Husain (2008), Verma et al. (2004), and Rana et al. (2012)
Glucose-6-phosphate-dehydrogenase (E.C. 1.1.1.49)	Jaundice	Silica-based matrix	Parenteral	Cumana et al. (2013)
β-Glucosidase (E.C. 3.2.1.21)	Treatment of Gaucher's disease	Liposome	Parenteral	Braidman and Gregoriadis (1977), Gregoriadis et al. (1980)
Heparin lyase (E.C. 3.2.1.19)	Wound healing	Nanoparticles	Local	Behera et al. (2012)
Keratinase (E.C. 3.4.4.25)	Debridement of necrotic tissue	Polyvinyl-alcohol cryogels	Local	Martinez et al. (2013)
Serine endopeptidase (lumbrokinase, E.C. 3.4.21) and subtilisin (nattokinase, *E.C.* 3.4.21.62)	Antithrombotic therapy	Mnps	Parenteral	Ren et al. (2010)
Lysozyme (E.C. 3.2.1.17)	Wound and other antimicrobial therapies	Titania nanotubes, carbon nanotubes composed of biopolymers, Sba-15 mesoporous-silica rods, nanofiber mats	Local	Popat et al. (2007), Nepal et al. (2008), Ding et al. (2012), and Huang et al. (2013)
Papain (E.C. 3.4.4.10)	Wound treatment	Niosomes and nanospheres	Local	Manosroi et al. (2013)
Phenylalanine-ammonia lyase (E.C. 4.3.1.24)	Phenylketonuria treatment	CLEAs	Oral	Cui et al. (2012)
Streptokinase *(E.C.* 3.4.99.22)	Antithrombotic therapy	RGD Lips, magnetic nanoparticles (MNPs)	Parenteral	Walde & Ichikawa (2001), Torchilin et al. (2009), and Koneracka et al. (2002)
Tissue–plasminogen–activator (tPA, *E.C.* 3.4.21.68,)	Antithrombotic therapy	MNPs, PEG lips, PLGA/CS NPs, PL NPs	Parenteral	Rouf et al. (1996) and Park et al. (2001)
Trypsin (E.C. 3.4.4.4)	Wound treatment	Cotton yarn	Local	Nikolic et al. (2010)
Tyrosinase (E.C. 1.14.18.1)	Treatment of melanoma cancer skin	Polylactic-acid nanocapsules, polyethyleneglycol–polylactic-acid nanocapsules	Parental And local	Wang & Chang (2012)
u-Plasminogen activator (urokinase, E.C. 3.4.21.73)	Antithrombotic therapy	MP NPs	Potential	Piras et al. (2008)
Xanthine oxidase (E.C. 1.1.3.22)	Lesch–Nyhan treatment	Microcarrier	Oral	Palmour et al. (1989)

Abbreviations: MNPs, magnetic nanoparticles; PEG lips., PEGylated liposomes; PLGA/CS NPs, poly-(lactide-*co*-glycolide)/chitosan nanoparticles; PL NPs, polystyrene latex nanoparticles; RGD Lips, RGD-(Ar–Gly–Asp)-peptide-conjugated liposomes; MP NPs, 2-methoxyethanol hemiesters of poly(maleic anhydride-alt-butyl, vinyl ether) nanoparticles; potential: since the system has not been assayed *in vivo*, the route of administration has not yet been determined; CLEAs, amorphous enzymic aggregates.
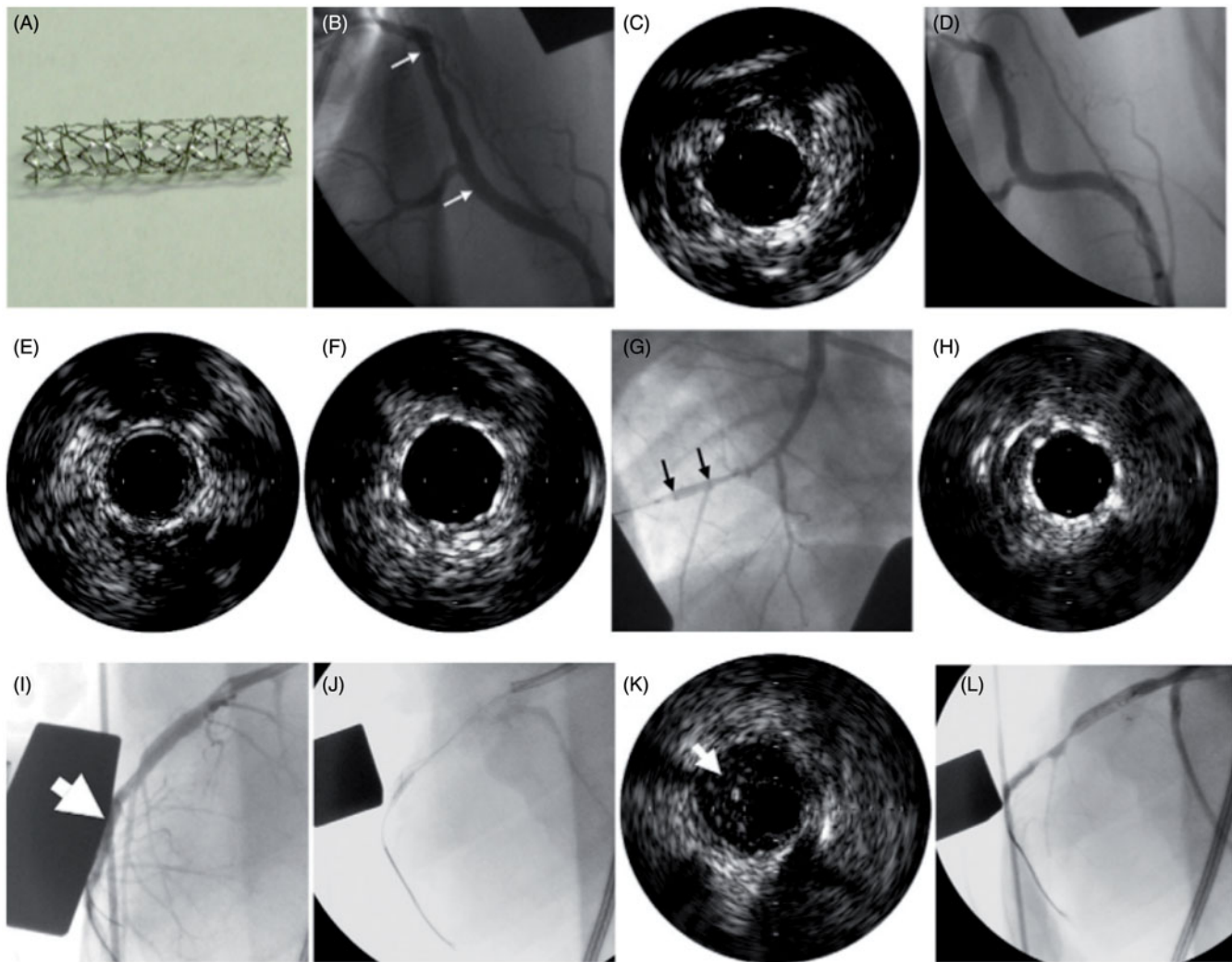
*From Brenda: http://www.brenda-enzymes.info/index.php4.

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Figure 3. *In vivo* experiments in pigs on the brachial artery (A–F) (aortoesophageal fistula) and left anterior-descending artery (LAD) (G-L). (A) An NIR Primo™ coronary stent with an interwoven Kanthal D wire in a coiled configuration. (B) Angiogram obtained directly after stent insertion in the brachial artery. Arrows indicate the stented segment. (C) Intravascular ultrasound (IVUS) obtained directly after stent insertion into the brachial artery. (D) Angiogram obtained directly after injection of nanoparticles (NPs) II. (E) IVUS obtained directly after injection of NPs II. (F) IVUS obtained 30 min after injection of NPs II. (G) Angiogram showing the position of the stent in the LAD distal to the first diagonal branch. Arrows indicate the stented segment. (H) IVUS obtained directly after stent insertion. (I) Angiogram obtained after injection of NPs II. The position of the stent is indicated by the arrow. The permanent magnet is the dark object on the left side of the figure below the arrow. (J) Angiogram obtained after thrombus formation, showing that blood flow has ceased in the LAD. (K) IVUS showing the thrombus (the dark area indicated by the arrow). (L) Angiogram illustrating the lysis of the thrombus in response to tPA-NP injection. Reproduced from Kempe & Kempe (2010) with permission of Elsevier.

in blood compared with the results with soluble tPA. Conventional and PEGylated liposomes were cleared from the plasma within 6 and 9 h, respectively; whereas the clearance of soluble tPA from the circulatory system occurred within only 1 h.

An alternative encapsulation of tPA by poly(lactide-*co*-glycolide), or PLGA, nanoparticles and derivatized with either the linear polysaccharide chitosan (PLGA-Cs) or the tripeptide Arg-Gly-Asp (RGD) that adheres to cells has been reported. The encapsulation of tPA by PLGA nanoparticles with Cs or Cs plus RGD increased the thrombolysis of a clot model system *in vitro*. The effect was attributed to the electrostatic interaction between the nanoparticles and the clotted blood (Chung et al., 2008).

Another strategy based on a site-specific nanodevice designed to accelerate the dissolution of clots was employed antifibrin antibody plus tPA conjugated to 40-nm latex nanoparticles (Yurko et al., 2009). The enzymatic activity

of the conjugated nanoparticles, however, was similar to that of the free tPA only when fibrin clots were present. In the absence of a thrombus, the enzymatic activity was reduced by two-thirds, thus decreasing the possibility of hemorrhage and improving the treatment in poststroke cases.

In vivo and *in vitro* experiments with SK encapsulated by RGD-peptide-conjugated liposomes indicated a high nanoparticle accumulation in a thrombus (Vaidya et al., 2011). A study in humans documented a higher degree of clot lyses with SK than with soluble SK in RGD-liposomes.

Urokinase was loaded into biocompatible polymeric nanoparticles composed of 2-methoxyethanol hemiesters of poly(maleic anhydride-alt-butyl vinyl ether), whose synthesis was developed by a coprecipitation technique (Piras et al., 2008) based on a form of hydrophobic bonding: the low polymer solubility in an aqueous phase and the independent hydrophobic interaction with the apolar residues of proteins form a microphase consisting of nanoparticles. Next, these

nanoparticles were PEGylated and conjugated to antifibrin antibody. After immobilization, 83% of the original enzymatic activity was retained. Cytotoxicity experiments revealed 100% cell viability compared with the controls and pointed to a potential application of this approach in future thrombolysis therapy.

Earthworm lumbrokinase (E.C. 3.4.99, LK) and the microbial nattokinases (E.C. 3.4.21.62, NK) are proteases that degrade the fibrin of thrombi. These enzymes were accordingly immobilized in magnetic FeO$_3$ nanoparticles (Ren et al., 2010). According to the report, the immobilized LK in the magnetic nanoparticles displayed over 200% of the thrombolytic activity of the soluble LK. In comparison, about 90% and 80% thrombolysis, respectively, were reported upon the use of that enzyme immobilized in magnetic nanoparticles and the free form of soluble NK. Both the lumbrokinase and nattokinase are promising enzymes for thrombosis treatment.

Treatment of Gaucher's disease

Glucocerebrosidase (or D-glucosyl-N-acylsphingosine gluco-hydrolase, E.C. 3.2.1.45), an acid β-glucosidase with glucosylceramidase activity, is a membrane-bound lysosomal hydrolase catalyzing the degradation of glucocerebroside, an intermediate in glycolipid metabolism, to glucose and ceramide through the hydrolysis of the β-glucosidic linkage. Gaucher's disease, a lipid-storage pathology, is a disorder caused by a deficiency in glucocerebrosidase (acid β-glucosidase) and characterized by the accumulation of glucocerebroside in organs such as the spleen, liver, and bones. In the first nanotechnological approach for the treatment of this disease, substitution of the deficient enzyme was carried out through the use of liposomes (Braidman & Gregoriadis, 1977). The intravenous administration of human acid β-glucosidase encapsulated in those liposomes produced a reduction in the increased liver size, indicating an improvement in the functioning of the reticuloendothelial system for a period of 13 months (Gregoriadis et al., 1980). Recent studies based on an immobilization of glucocerebrosidase on 10-nm synthetic superparamagnetic magnetite nanoparticles after silanization and aminofunctionalization with 3-aminopropyl-triethoxysilane indicated a retention of 98% enzymatic activity after immobilization (Valenzuela et al., 2012). In addition, a covalent attachment of the enzyme onto magnetic nonporous particles previously activated with cyanuric chloride and polyglutaraldehyde resulted in a high bead-immobilized enzyme activity (Alftrén & Hobley, 2013). Unfortunately, both those reports focused on that kind of immobilization for industrial applications of cellulose and lignocellulose hydrolysis, but the principles could, nevertheless, be applied to the clinical situation.

Alcohol prophylaxis and treatment

The alcohol-oxidase (E. C. 1.1.3.13), a catalase (E.C. 1.11.1.6) multienzyme system, was developed to mimic the functioning of sophisticated subcellular compartments containing enzymes able to work in tandem. Recently, a novel nanodevice for alcohol-intoxication therapy by a combined assembly of DNA and nanoencapsulation has been developed (Liu et al., 2013). Alcohol oxidase catalyzes

the conversion of ethanol to acetaldehyde and H$_2$O$_2$, while catalase decomposes the resulting H$_2$O$_2$ to prevent radical formation. These two enzymes were coencapsulated to produce a modality to prevent alcohol intoxication. A covalent linking of inhibitors such as 4-dimethylaminoanti-pyrine, glucosamine, or lactobionic acid to specific sequences of single-stranded DNA followed by self-assembly was performed to produce the scaffolds. This procedure consisted in a preconjugation of the enzymes with acryloyl groups by a classic technique involving the formation of the N-hydroxysuccinimide ester and a subsequent interaction with single-stranded DNA. Next, the DNA–enzyme complexes were encapsulated in polyacrylamide gels by means of standard protocols followed by DNA hydrolysis to produce nanonized enzymes. The gel nanodevices containing these enzymes, when tested in ethanol-fed mice, exhibited a high stability and an enhanced alcohol-oxidation rate (Liu et al., 2013).

Oral administration of nanodevices

Oral delivery is one of the most desirable approaches because the drug is absorbed in the gastrointestinal (GI) tract without the requirements of specific facilities or trained personnel. Besides, the GI tract has several protective mechanisms to prevent the uptake of potentially toxic substances that should be taken into account for the synthesis of the drug carriers in order for the payload to reach the specific drug target in adequate therapeutic concentrations. The development of nanoparticles for oral delivery could prevent the introduction of toxic levels of a drug and maintain a sustained release (Pinto et al., 2006). In this regard, the main properties of nanoparticles can be summarized as the following:

(a) Enhancement of the bioavailability and biodisponibility of drugs with poor absorption characteristics
(b) Improvement of the residence time of therapeutic molecules in the GI tract
(c) Low nanoparticle dispersion
(d) Sustained release of therapeutic agents
(e) Simultaneous delivery of certain drugs and/or molecules added in concert
(f) Drug-specific cell or tissue or organ targeting
(g) Reduction in drug toxicity and GI mucosal irritation
(h) Molecular stability along the GI tract
(i) Decrease in necessary drug concentrations by high loading/surface ratio.

The following section describes selected examples of preparations of potential therapeutic peptides and proteins for oral administration presently under investigation.

Alginate lyase (E.C. 4.2.2.3, AL): Cystic fibrosis (CF) is a congenital autosomal pathology produced by defective chloride transport in conjunction with the presence of opportunist pathogens such as *Pseudomonas aeruginosa*, a bacterium colonizing almost all CF patients. *Pseudomonas aeruginosa* infections produce large amounts of the exopolysaccharide ALG inside the lungs and intestine, thus not only drastically diminishing the respective oxygen uptake and food assimilation in such patients but also reducing the efficiency of antibiotic treatment by the presence the ALG biofilm coating the tissues. The action of AL decreases the viscosity of the biofilm by hydrolyzing the ALG chains. AL was entrapped

within ALG/pectin microspheres via ionic gelation at pH 4.0, to obtain 90% entrapment efficiency and remained inactive until arriving at a milieu with physiologic conditions (pH 7.4). AL was then released from the nanomatrix in an active form, thus becoming a promising alternative for the oral and/or nasal treatment of cystic-fibrosis patients (Islan et al., 2013). Alternatively, bovine-serum albumin (BSA) has been proposed as a type molecule for attachment to the surface of ALG-gelatin microspheres previously treated with glutaraldehyde. Azo-BSA (BSA coupled to an azo dye) bound to the gel was observed by scanning electron microscopy and fluorescence microscopy, in the latter instance along with quantification by ultraviolet absorption. The modified BSA-gel system demonstrated the ability of the gel biopolymer to support biomolecules with different biologic properties (Islan & Castro, in press).

Phenylalanine-ammonia lyase (E.C. 4.3.1.24, PAL): Phenylketonuria (PKU) is an autosomal recessive disorder that causes a metabolic disorder resulting from a deficiency in the hepatic enzyme phenylalanine 4-hydroxylase (E.C. 1.14.16.1, PHX). PHX is responsible for the oxidation of the aromatic ring of phenylalanine in the *para* position to form the amino acid tyrosine. The lack of PHX activity produces a decrease in the levels of tyrosine while markedly elevating the concentrations of phenylalanine in the blood stream. This imbalance in the levels of the circulating amino acids, if not prevented by the use of a tyrosine-supplemented diet low in phenylalanine, causes a deficiency in myelination after birth with disastrous consequences for the developing nervous system, thus producing a profound degree of mental retardation in homozygotes. Enzyme substitution therapy with PAL, which catalyzes the nonoxidative deamination of phenylalanine to *trans*-cinnamic acid and ammonia, is a possible alternative to the dietary treatment of PKU and is currently under intensive clinical investigation. The lyase, if administered without protection, would rapidly be degraded by the presence of hydrolytic enzymes in the intestinal lumen. This strategy of phenylalanine degradation by PAL has the added advantage of reducing the level of phenylalanine concentrations in the intestine, thus avoiding potential immune reactions (Sarkissian & Gámez, 2005). Several studies were performed on the immobilization of PAL in inexpensive, abundant, and safe materials, such as gelatin, that are widely used as immobilization matrices (Ates & Dogan, 2010).

Another investigation demonstrated in phenylketonuric rats that the daily oral administration of 1 unit of PAL loaded in artificial cells for 7 d reduced the circulating-phenylalanine concentration by 58%, while an increase in the dose to 5 PAL units for 6 d restored the phenylalanine levels to normal (Bourget & Chang, 1986). Similar responses were reported in PKU patients by the enteric administration of coated gelatin pills containing PAL. In addition, alternative strategies of PAL delivery entrapped by silk fibroin to the Caco-2 adenocarcinoma cell line in culture have been under study (Sarkissian & Gámez, 2005). Moreover, an enzyme-PEGylation study was performed in order to investigate protection of the enzyme from proteolysis (Sarkissian et al., 2011), while mutant PAL protein was also studied for the same purpose (Kang et al., 2010). Nevertheless, the oxidation rate of phenylalanine by the free PAL that was insensitive to proteases was extremely low,

thus requiring large doses that would be disadvantageous for long-term therapies. At the same time, efforts to devise an effective PAL oral formulation were initiated through approaches employing nanotechnology with the development of cross-linked enzymatic aggregates (CLEAs) of PAL. The PAL-CLEA exhibited enhanced enzyme stability against harsh environmental conditions such as extreme pHs/pH values and temperatures, denaturants, and organic solvents compared with the enzyme in solution (Cui et al., 2012). In addition, the process for the manufacture of multilayer-coated nanoparticles (ranging from 20 to 40 nm) and hollow-shell-based nanocomposite colloidal multilayers was developed to immobilize PAL and has recently been patented (Panzer et al., 2001).

Bilirubin oxidase (E.C. 1.3.3.5, BOX): BOX is an oxidoreductase that catalyzes the oxidation of bilirubin to biliverdin and H_2O. Neonatal jaundice is characterized by the accumulation of bilirubin in the body. Toxic levels of bilirubin can cause brain damage, as manifested in mental retardation, cerebral palsy, deafness, and kernicterus, as well as result in death. Covalent BOX immobilization on agarose beads has been proposed as a bioreactor to degrade bilirubin in an extracorporeal circuit for potential treatment of newborn blood (Sung et al., 1986). In addition, immobilized BOX administrated orally in children and adults could oxidize bilirubin in the intestine-producing products of greater solubility and thus with lower toxicity. In this regard, the concentration of bilirubin was reduced by 50% when 0.1–2.0 mg/d of immobilized BOX were administered to chronically jaundiced Gunn rats for 4 d (Soltys et al., 1992). Furthermore, new strategies to immobilize BOX in nanodevices were subsequently reported, such as a hybrid film of zirconia-coated silica nanoparticles and chitosan (Batra et al., 2013) or carbon nanotubes (Zheng et al., 2010), but those constructions were used only as biosensors. Further studies will be necessary to develop nanodevices to be used in humans.

Tyrosinase (E.C. 1.14.18.1, TYR): TYR, a member of the oxidase family, is the rate-limiting enzyme in the biosynthesis of melanin. In mammalians, tyrosinase catalyzes the conversion of only L-tyrosine or L-3,4-dihydroxyphenylalanine (DOPA) as substrates, but with the absolute requirement for the presence of DOPA as a cofactor (Hearing et al., 1980). TYR has been immobilized in biopolymers such as chitosan-based (Dinçer et al., 2012) or cellulose-based (Labus et al., 2011) carriers. The feasibility of the immobilized enzyme in an active form on different kinds of nanodevices has been described in various publications: for example, magnetic nanobeads (Sima et al., 2011; Tuncagil et al., 2009), ZnO nanorods (Gu et al., 2008), and carbon nanotubes (Mohammadi et al., 2008; Ozoner et al., 2010) have all been designed for medical applications. In a recent article, the development of two biocompatible nanocapsules containing a soluble polyhemoglobin–tyrosinase (polyHb–Tyr) complex was reported. The nanocapsules of the polyHb–Tyr complex were capable of suppressing the growth of B16F10 melanoma cells in a murine-tumor model (Wang & Chang, 2012). In the first type of nanocarrier, the circulation time of the polyHb–Tyr complex encapsulated by a PEG-poly(lactic acid) matrix (PEG-PLA) was extensive, while in the second type, the nanocapsule of polyHb–Tyr complex in PLA alone was targeted for intratumoral or local injection. The nanocapsules containing

polyHb–Tyr are able to inhibit the proliferation and attachment of murine B16F10 melanoma cell line, as demonstrated by *in vitro* assays. The biologic activity of the polyHb–Tyr complex could be attributed to a reduction of the levels of tyrosine and/or tyrosine-related metabolites. Concomitantly, the polyhemoglobin contained in the nanocapsules was associated with an exhaustion of the nitric oxide required for the replication of the melanoma cells.

Alpha-*chymotrypsin* (E.C. 3.4.21.1, α-CT): This enzyme of the exocrine pancreas is classified as a serine protease catalyzing the hydrolysis of all types of proteins in the human small intestine. The major problem for serine proteases continues to be the autolytic processes that limit the life span of these biocatalysts. In order to extend the half-life of α-CT, the enzyme was attached to magnetic nanoparticles made of Fe_3O_4 through the protein's hydroxyl groups (Koneracká et al., 2002). This procedure has also been utilized for the immobilization of many proteins, including enzymes, but only after adapting the experimental conditions to each one of them.

Another strategy for the enhancement of biocatalytic activity has been to prepare complexes between enzymes and SENs (Hegedus & Nagy, 2009; Jia et al., 2003). In one instance, the nanoparticles were synthesized in the size range of 10–40 nm in order to avoid diffusional restriction of substrates and products. Moreover, among the advantages of the SENs as nanocarriers are the enhanced half-life of the cargo under both standard and extreme operating conditions such as pH and temperature, and also autolysis in the case of most of the proteases (Illanes et al., 2012).

Recently, α-CT covalently bound to *N*-acroyloxysuccinimide was attached to magnetic particles, with latex beads of 250-nm average diameter being incorporated during the polymerization (Medeiros et al., 2011). The thermosensitive latex-containing nanoparticles sedimented six times faster under a magnetic field and retained a high α-CT activity.

β-*Galactosidase* (E.C. 3.2.1.23, β-gal): This enzyme is involved in the normal pathway for the oxidation of lactose. Different devices to immobilize β-gal were studied in search of satisfactory profiles for activity and stability during drug-delivery therapies in order to enhance the assimilation of lactose in lactose-intolerant Caucasians (Elnashar et al., 2014).

A promising strategy for the delivery of this enzyme into cells was recently developed through the use of ionic interactions between β-gal and an ionic nanodevice. In this approach, the anionic form of human β-gal ($M_W = 465$ kDa, $pI = 4.6$) interacted with colloidal AuNPs previously coated with different cationic alkane ligands of C_7 to C_{12} chain length. The β-gal–AuNP complex exhibited no activity in the absence of glutathione, but in the presence of that reducing agent, the enzyme was released from the complex. This β-gal nanodevice, upon detection in the cytosol, had retained significant enzymatic activity (Rana et al., 2012).

Intracarotid injections (Wada test) or internal-carotid-artery injection

The payload of intracarotid injection proceeds straight to the brain, avoiding broad biodistribution and unwanted toxicity in healthy tissues.

Superoxide dismutase (E.C. 1.15.1.1, SOD): SOD has been encapsulated in liposomes and chemical polymers, such as poly(butyl-cyanoacrylate) or PLGA, in order to treat cerebral ischemia and reperfusion injury in a murine model. Intracarotid injections of the nanodevices containing SOD after injury produced a 50–60% a drop in inflammatory markers and the infarct volume, thus reducing the chances of stroke in the treated animals (Yun et al., 2013).

Local administration

The following is a description of the most relevant aspects associated with this administration route.

Bone-regeneration enzymes

Alkaline phosphatase (E.C. 3.1.3.1, ALP): ALP hydrolyzes pyrophosphate (PPi)-releasing inorganic monophosphate (Pi). Since PPi is an inhibitor and Pi a promoter of hydroxyapatite synthesis, the role of ALP is crucial in the regulation of bone development (Osathanon et al., 2009); thus, this enzyme is of great relevance to medical interests. For example, an application has recently been developed for hypophosphatasia, a rare metabolic bone disease that causes a profound skeletal hypomineralization.

The use of a hydrophilic chemical polymer, poly (2-hydroxyethyl methacrylate), to immobilize ALP-produced promising results with respect to mineral deposition *in vitro* in the presence of β-glycerophosphate (Filmon et al., 2000). More recently, the covalent immobilization of ALP on a nanomatrix composed of microporous fibrin scaffolds (FSs) was carried out to produce the nanodevice ALP/FSs with the objective of effecting bone repair. This approach was successful in a model system in culture since calvarial cells seeded on ALP/FSs exhibited a high expression of an osteoblast gene marker compared with the control cultures without ALP (Osathanon et al., 2009).

In addition, ALP has been immobilized in different nanodevices such as magnetic nanoparticles (Saiyed et al., 2007), poly(styrene-acrylic-acid) nanospheres (Yin et al., 2011), or nanoporous nickel-titanium films (Zhang & Cass, 2001) for biosensing applications.

Wound-treatment enzymes

Trypsin (E.C. 3.4.21.4) is a serine protease elaborated by the exocrine pancreas. Trypsin is a highly active protease on proteins and peptides that cleaves the peptide bond on the carboxyl side of arginine and lysine residues (Brown & Wold, 1973). Autolysis, however, was found to strongly affect trypsin activity and stability (Castro, 1999). Immobilized trypsin has potential applications in wound healing. For such uses, the support must be biocompatible, biodegradable, and have the properties of softness and an affinity for the skin. In a recent study, trypsin was covalently immobilized onto sodium-periodate-oxidized cotton yarn (Nikolic et al., 2010). The immobilized trypsin displayed 1.22 U/g of the support, equivalent to only 14% of the initial catalytic activity. Nevertheless, the activity of the immobilized trypsin that remained after 60 d at 4 °C and 25 °C was 90% and 7%/12.5% of the original value, respectively. In contrast, unbound
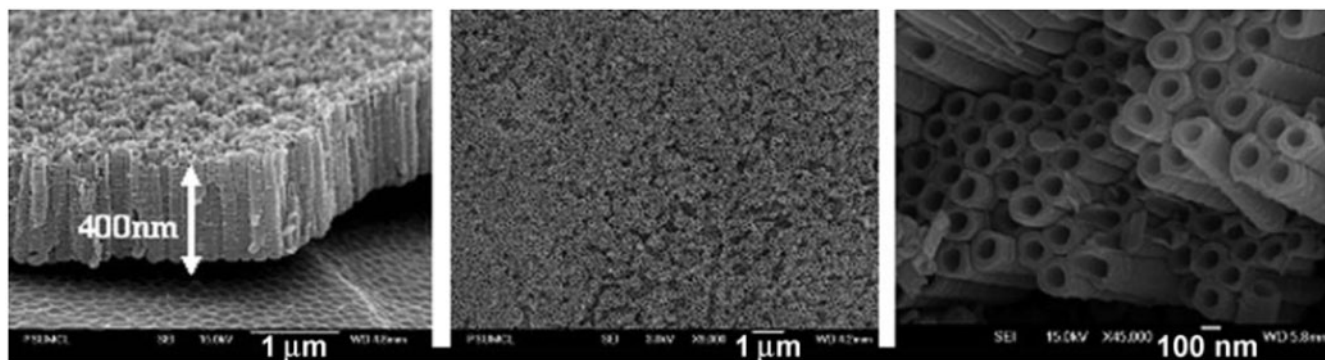
Figure 4. Scanning-electron-microscopy images of titania nanotubular surfaces. (Left) Cross-sectional view of a mechanically fractured sample demonstrating that the length of the tubes is approximately 400 nm. (Center) Top view of nanotubular surface. (Right) High-magnification top view of nanotubular surface showing the tube diameter of approximately 80 nm. Reproduced from Popat et al. (2007) with permission of Elsevier.

trypsin retained only 14.5% of the initial activity at 4 °C after 60 d because of its ongoing autolysis. Based on these results, and in view of the antimicrobial activity that is a component of the ALD system, this immobilization device shows potential for medical applications.

Keratinase (E.C. 3.4.4.25) is another member of the protease family with potential applications for debridement and wound healing through the promotion of spontaneous skin epithelialization (Martínez et al., 2013). Keratinase entrapped in gels composed of polyvinyl alcohol and pectin (PVA-pectin) retained 100% enzyme activity. The kinetic release of keratinase from coacervate gels can be tailored by changing the amount of pectin and its degree of methoxylation. Co-immobilization of keratinase and the antibiotic enrofloxacin in this PVA-pectin cryogel led to a controlled retarded release of enrofloxacin compared with the dissociation from the gel of the antibiotic alone ($15.4 \pm 0.6\%$ versus $6.9 \pm 0.7\%$ remaining at 37 °C, pH = 5.5, after a 5-h incubation). Studies involving small-angle-X-ray scattering demonstrated that the enzyme and the antibiotic were entrapped among the crystals of the PVA-pectin matrix (Martínez et al., 2013). This system has been proposed for use in the form of a dermal patch as a first stage in the treatment of wounds and scars in order to affect an enzymatic debridement of necrotic tissue, thus giving the antibiotic, upon its subsequent release, a greater access to the bacterial niche usually located underneath.

Lysozyme (E.C. 3.2.1.17, LSZ): LSZ is an enzyme typically used for the hydrolysis of the thick cell wall of Gram-positive bacteria. LSZ acts by cleaving the 1,4-β-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in the murein peptidoglycan. Unlike many antimicrobials, lysozyme has enzymatic as well as nonenzymatic activities that can be beneficial even when heat treatment is needed. LSZ, for use as an antimicrobial enzyme, was immobilized (alone or with trypsin) in many materials (e.g. cellulose, dialdehyde cellulose and polycaproamide fibers, PEG methacrylate, silica devices, carbon and titanium nanotubes, hybrid devices, nanodiamonds, and PVA films) for different antimicrobial therapies, e.g. those requiring surgery or implantable devices or involving suppurative wounds.

Anodized surfaces of titania nanotubes were tested as a drug-delivery device with BSA and lysozyme as cargos (Popat et al., 2007). The protein loading in the nanotubes was

between 60% and 80% regardless of the starting protein concentration, and the release occurred in the 25–110 min range depending on the initial amount of protein bound. In addition, positively charged proteins, such as lysozyme, displayed a higher binding to the nanotubes than negatively charged or neutral proteins. A further advantage of the titania nanotubes is the positive interaction with the tissue (Figure 4). Another strategy consisted in depositing LSZ on the surface of single-wall carbon nanotubes (SWNTs; Nepal et al., 2008). The thickness of the SWNTs was only 1.6 nm, while the orientation of the nanotube made by an air stream enabled a combination of the two principal strategies used in nanotechnology: SA and a bottom-up approach in a hybrid device (Figure 5). The authors reported long-term antibacterial activity that could be extended to Gram-negative bacteria (with thinner peptidoglycan-containing cell walls between an inner and an outer membrane than the walls of the Gram-positives) just by the addition of chelators such as ethylene diamine tetraacetic acid (Nepal et al., 2008).

Highly ordered mesoporous silica rods (SBA-15), when also tested as vehicles of LSZ, displayed a capability for rapid and high enzyme adsorption. The main advantage of the SBA-15 silica nanodevice was the capacity for varying the pore size, and consequently the enzyme adsorption, by changing the acidity (Ding et al., 2012). The LSZ-adsorption rate by SBA-15 rods and the enzyme's loading capacity in them increased inversely to the pore size, reaching equilibrium at up to $835 \, \mathrm{mg \, g^{-1}}$ of protein in less than 10 min (Figure 6).

Cellulose–acetate nanofibrous mats synthesized by electrospinning were used in an SA strategy for LSZ entrapment (Huang et al., 2013). In the SA system, a nanocomposite made of a positively charged complex – LSZ–[*N*-(2-hydroxy-3-trimethyl-ammonium)-propyl-chitosan chloride] (LSZ-HTCC) and negatively charged sodium ALG – was deposited on alternate cellulose pads. The topology and LSZ activity of the mats were regulated by the amount of bilayers. Field-emission scanning electron microscopy revealed an increase in the fiber diameter that correlated with the number of bilayers, while the LSZ activity depended on the enzyme's access to the environment. The best antimicrobial activity was found in 10.5 LSZ–HTCC layers (Figure 7).

Catalase (E.C. 1.11.1.6, CAT): CAT is a H_2O_2 oxidoreductase present mainly in the peroxisomes of mammalian

Figure 5. Schematic diagram of layer-by-layer assembly of LSZ-SWNT and DNA-SWNT. LSZ: lysozyme and scanning-electron-microscopy images of layer-by-layer assembly of LSZ-SWNT–DNA-SWNT of the (a) 8th layer and (b) 68th layer. Reproduced from Nepal et al. (2008) with permission of Elsevier.
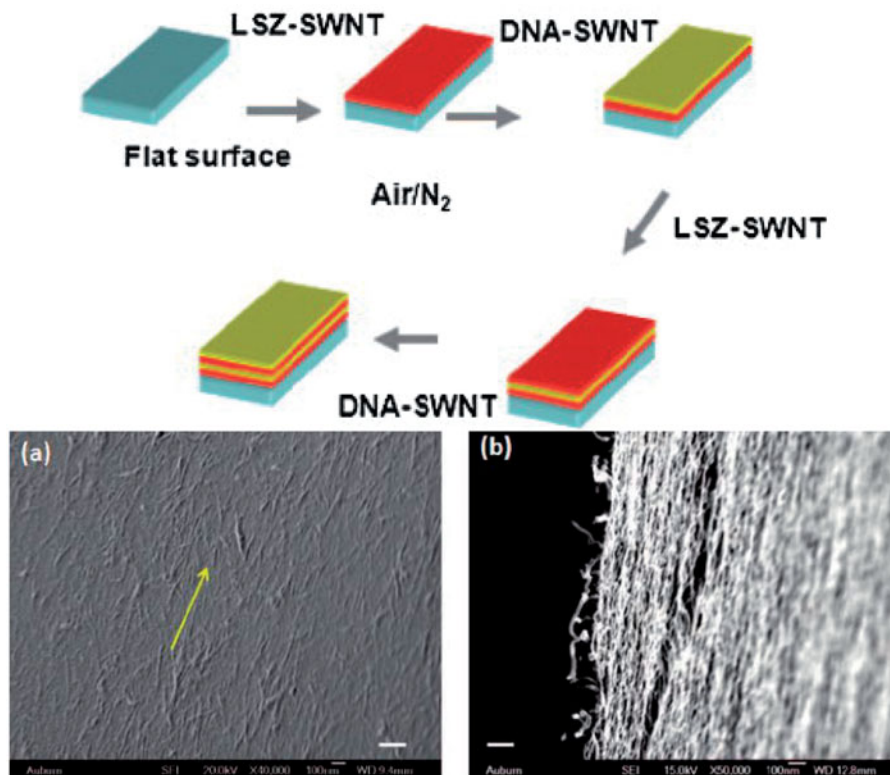

Figure 6. Scanning-electron-microscopy images of different samples showing the changes of length and width of the rods at different acidities produced by varying the HCl concentration: (a) 0.5 M HCl: 3.0 μm length by 0.26 μm width, (b) 1.0 M HCl: 2.1 μm length and 0.28 μm width, (c) 2.0 M HCl: 1.2 μm length, and 0.30 μm width, and (d) 2.5 M HCl: 0.6 μm length and 0.40 μm width. Reproduced from Ding et al. (2012) with permission of Elsevier.
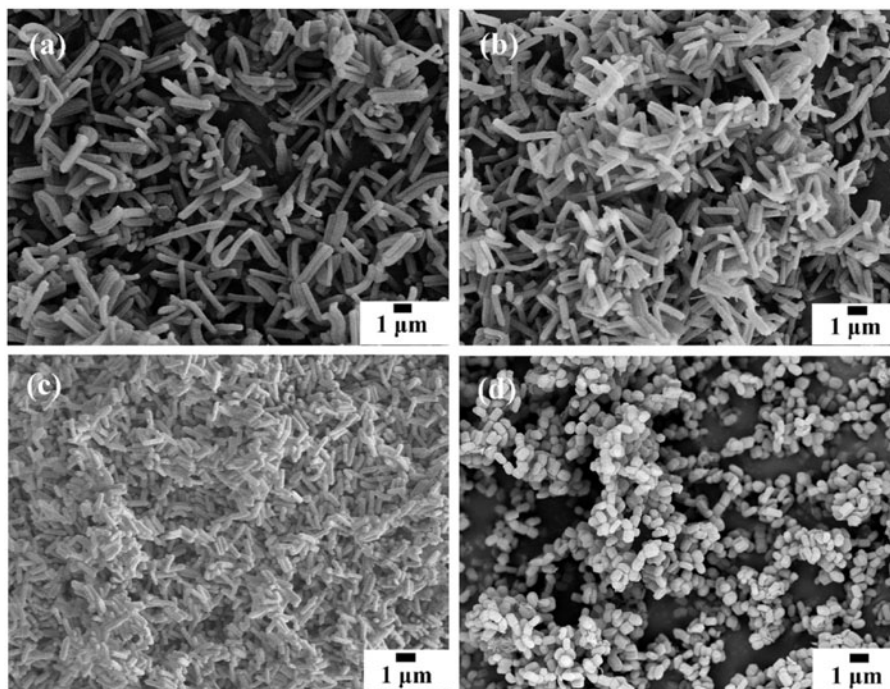
cells, where the enzyme catalyzes the conversion of hydrogen peroxide to water and molecular oxygen with no free-radical production. The presence of free radicals in cells is commonly associated with several pathologies including cancer and aging. Enzymes scavenging free radicals have potential biomedical applications in the prevention of oxidative damage and in wound healing (Castro et al., 2009; Thiem & Goślińska, 2004). An example of topical enzyme delivery is catalase encapsulated in flexible sugar–ester vesicles (SEVs).

The main advantages of SEVs are the properties of biodegradability and biocompatibility with respect to low toxicity (Abdelmajeed et al., 2012; Tavano et al., 2010). CAT showed a much higher stability upon encapsulation, where the enzyme remained stable for 90 d at a 95% residual activity. In contrast, free CAT retained only 7% of its initial activity after that same time period. *In vivo* studies have indicated a significant effect of CAT-nanovesicle preparations on wound healing.

Figure 7. Field-emission-scanning-electron-microscopy images of (a) cellulose-acetate fibrous mats and layer-by-layer structured mats coated with: (b) LSZ–HTCC-ALG, 5 bilayers; (c) LSZ–HTCC-ALG, 5.5 bilayers; (d) LSZ–HTCC-ALG, 10 bilayers; and (e) LSZ–HTCC-ALG, 10.5 bilayers. The average diameter (nm) of the layer-by-layer film-coated cellulose–acetate nanofibers is indicated in each image. The *inset*s represent the same fields but at different magnifications. Reproduced from Huang et al. (2013) with permission of Elsevier.
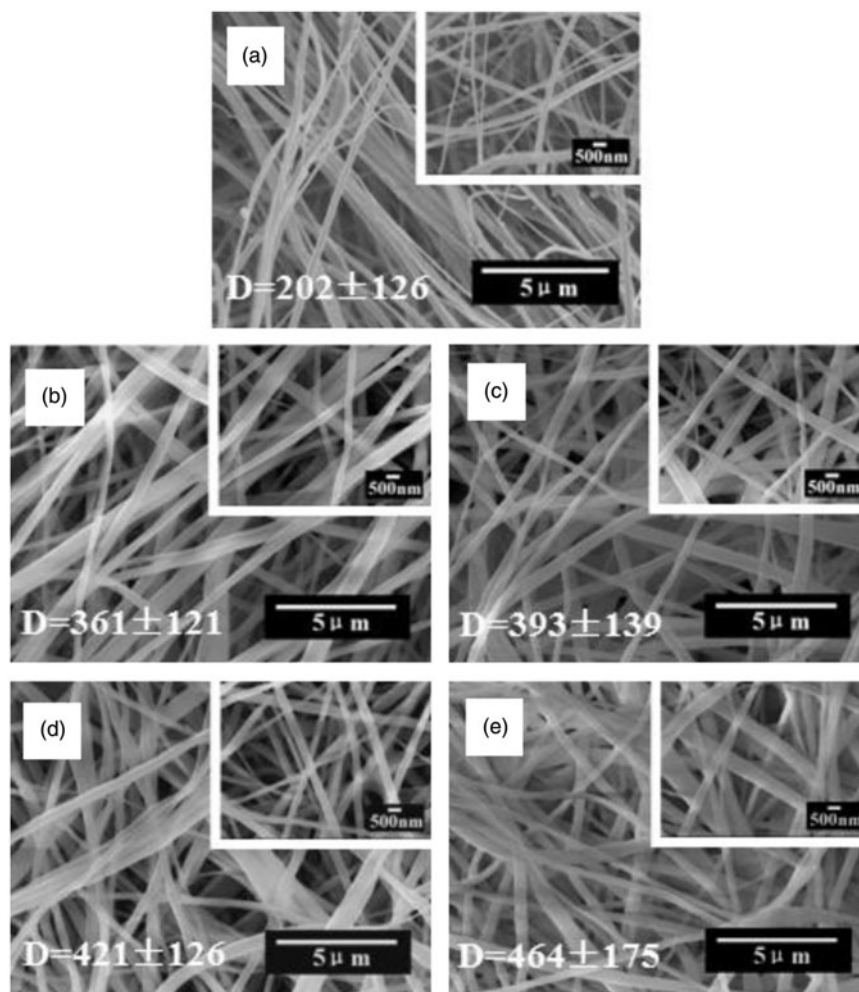
Papain (E.C. 3.4.22.2): This enzyme is a cysteine protease present in *Carica papaya* plants. The enzyme's medical application includes the debridement of necrotic tissue as an alternative to the surgical removal of the necrotic areas. Papain encapsulated in nanospheres and two types of niosomes included in gels were formulated within a diameter range of 2.21–5.20 μm (Manosroi et al., 2013). Scars induced in rabbits showed decreases in hypertrophy by factors of 10.2-, 2.73-, and 2.31-fold after applications of the gels containing the elastic niosomes, free papain, and nonelastic niosomes, respectively, for 28 d.

Finally, Table 3 lists enzymes having potential biomedical applications but not yet immobilized in nanodevices. These enzymes, which show a potential to be tested *in vitro* and *in vivo*, or to be modified and/or adapted for some therapies involving nanodevices, are discussed below.

β-N-Acetyl-D-hexosaminidase (E.C. 3.2.1.35): Tay–Sachs or infantile disease is an autosomal-recessive genetic disorder characterized by the absence of the lysosomal enzyme β-*N*-acetylhexosaminidase resulting in a progressive deterioration of nerve cells correlated with a decrease in mental and physical capabilities. The mechanism is based on the accumulation of large amounts of gangliosides in the cerebral neurons leading to cellular death. Up to the present, no treatment or cure has been reported. Nanobiotechnology, however, could conceivably present new possibilities for the treatment of Tay–Sachs disease through the use of nanodevices to provide the target cells with the deficient in the enzymatic activity.

In a previous report, the immobilization of β-*N*-acetyl-D-hexosaminidase on nanodevices was described. Concanavalin A, a carbohydrate-binding protein, was used to obtain a high immobilization yield of the enzyme (Farooqui & Srivastava, 1981). In addition, the enzyme was co-immobilized with BSA and glutaraldehyde and then cast as membranes (Yeung et al., 1979). No further research and/or pharmaceutical reports, however, have appeared in the literature.

Arginase (E.C. 3.5.3.1): This enzyme, which catalyzes the final reaction in the urea cycle, converts L-arginine to L-ornithine and urea. Recently, arginase has been considered as an alternative treatment for cancer. Certain studies demonstrated that the growth of particular tumors was dependent on a plentiful supply of arginine and that withdrawal of that amino acid would have a devastating effect on many types of cancer (Hsueh et al., 2012). An immobilized enzyme that removes amino acids from the bloodstream is an alternative approach to the use of a free enzyme, especially one that would not have an optimum stability of activity when maintained in the circulation (Schmer & Chandler, 1988). In one study, rat-liver arginase was covalently trapped in a fibrin clot and the physicochemical properties of the enzyme were characterized.

Table 3. Some examples of immobilized enzymes with potential therapeutic use.

Enzyme	E.C. Number*	Device	References
β-*N*-acetyl-D-hexosaminidase	3.2.1.35	Concanavalin A	Farooqui & Srivastava (1981)
alginate lyase	4.2.2.3	Alginate-pectin	Islan et al. (2013)
alkaline phosphatase (ALP)	3.1.3.1	Modified silica coatings	Ehlert et al. (2010)
α-Amylase (food intolerance; improvement of fat digestibility and enhancement of growth performance)	3.2.1.1	Halloysite nanotubes, zeolites, carboxymethyl-tamarind-gum-silica nanohybrids	Zhai et al. (2010), Maedeh et al. (2011), and Singh & Kumar (2011)
Arginase	3.5.3.1	Fibrin clot, Au NPs	Diez et al. (1990), Stasyuk et al. (2011), and Kuen-Chaná Lee et al. (2012)
Catalase	1.11.1.6	Bentonite and sepiolite nanoparticles, starch-based polymers, metal-chelated affinity cryogels	Cengiz et al. (2012), Costa & Reis (2004), and Tüzmen et al. (2012)
Lysozyme	3.2.1.17	Au nanorods; silica-coated magnetic NPs	Yang et al. (2007)
Monooxygenase	1.14.14.1	Mesoporous materials	Weber et al. (2010)
Papain	3.4.4.10	Carbon nanotubes, CLEA, porous-CLEAs (cross-linked enzyme aggregates)	Wang et al. (2010, 2011a,b)
Pepsin	3.4.4.1	Polyurethane microsphere/Au NP	Phadtare et al. (2003)

*From Brenda: http://www.brenda-enzymes.info/index.php4.

The immobilized arginase displayed the same substrate affinity as the soluble enzyme, at room temperature was more stable and more resistant to denaturation, and even exhibited a higher catalytic activity at physiologic pH. The properties examined thus far may point to the use of immobilized arginase in cancer therapy (Diez et al., 1990). Nanodevices were later developed for arginase immobilization. An engineered form of arginase with a single protruding cysteine site, which was deliberately placed away from its active center by site-directed mutagenesis, was found to facilitate the enzyme's attachment to a AuNP surface with an exquisitely high degree of precision, resulting in no apparent loss in catalytic activity (Stasyuk et al., 2011). In addition, metal-containing nanoparticles functionalized through the use of β-mercaptohexadecanoic acid were utilized as a support for the immobilization of human recombinant arginase type I expressed in *Hansenula polymorpha* and derivatized by the carbodiimide-pentafluorophenol method. The remaining type-I arginase activity was 40% and 25% in AuNPs and AgNPs, respectively, compared with the residual activity of the free enzyme after storage at 4 °C for 25 d (Stasyuk et al., 2011). Immobilized arginase is being used as a biosensor in the biomedical and food industry (Kuen-Chaná Lee et al., 2012). These systems, however, are at the early stage of development and require preclinical and clinical testing.

Concluding remarks

In comparison with the traditional strategies of drug delivery, the addition of enzymes associated with nanomaterials alone or combined with other molecules (e.g. antibiotics, peptides, or metals) may enhance the therapeutic power of medication on and/or inside the body in terms of activity, life span, and stability. The chiral stereospecificity and the enantioselectivity of enzymes are discriminative properties that can be used for the specific targeting of substrates involved in pathologic states.

Based on the prodigious number of enzymes presently or potentially available, a new class of nanodevices containing enzymes on their surfaces or inside their matrices can be developed for a wide range of medical applications. Hence, considering the current strategies and available tools of enzyme technology, such as directed evolution through genetic engineering and artificial protein synthesis, new approaches can be developed to find a cure for or an amelioration of both old and novel pathologies through nanotechnology.

The future development of nanodevice–enzyme complexes will likely be focused on several multiple and functional areas of medicine to solve practical problems wherein the nanomaterial can constitute the link between the advanced biocatalyst functions used in therapeutics and diagnostics – in the form of sensors and the controlled release of molecules – and a more efficient mode of therapy that will be capable of treating all aspects of a number of pathologies.

References

Abdelmajeed NA, Khelil OA, Danial EN. (2012). Immobilization technology for enhancing bio-products industry. Afr J Biotechnol, 11, 13528–39.

Alftrén J, Hobley TJ. (2013). Covalent immobilization of glucosidase on magnetic particles for lignocellulose hydrolysis. Appl Biochem Biotechnol, 169, 2076–87.

Ansari SA, Husain Q. (2012). Potential applications of enzymes immobilized on/in nano materials: a review. Biotechnol Adv, 30, 512–23.

Ates S, Dogan NS. (2010). Properties of immobilized phenylalanine ammonia lyase and investigation of its use for the prediagnosis of phenylketonuria. Turk J Biochem, 35, 58–62.

Batra B, Lata S, Rana J, Pundir CS. (2013). Construction of an amperometric bilirubin biosensor based on covalent immobilization of bilirubin oxidase onto zirconia coated silica nanoparticles/chitosan hybrid film. Biosens Bioelectron, 44, 64–9.

Behera BK, Sahoo SK, Mohapatra R, et al. (2012). Biodegradable and bioinspired polymers for pharmaceutical formulations and drug delivery: a brief review. World J Pharm Res, 1, 591–625.

Bellino MG, Regazzoni AE, Soler-Illia GJAA. (2010). Amylase-functionalized mesoporous silica thin films as robust biocatalyst platforms. ACS Appl Mater Interfaces, 2, 360–5.

Bourget L, Chang T. (1986). Phenylalanine ammonia-lyase immobilized in microcapsules for the depletion of phenylalanine in plasma in phenylketonuric rat model. Biochim Biophys Acta, 883, 432–8.

Brady D, Jordaan J. (2009). Advances in enzyme immobilisation. Biotechnol Lett, 31, 1639–50.

Brady D, Jordaan J, Simpson C, et al. (2008). Spherezymes: a novel structured self-immobilisation enzyme technology. BMC Biotechnol, 8, 8.

Braidman IP, Gregoriadis G. (1977). Rapid partial purification of placental glucocerebroside beta-glucosidase and its entrapment in liposomes. Biochem J, 164, 439–45.

Breccia JD, Hatti-Kaul R, Castro GR, Siñeriz F. (1999). Isolation of β-xylanase from whole broth of *Bacillus amyloliquefaciens* by adsorption on a matrix in fluidized bed with low degree of expansion. Bioseparation, 8, 273–80.

Brown WE, Wold F. (1973). Alkyl isocyanates as active-site-specific reagents for serine proteases. Reaction properties. Biochemistry (USA), 12, 828–34.

Castro GR. (1999). Enzymatic activities of proteases dissolved in organic solvents. Enzyme Microb Technol, 25, 689–94.

Castro GR. (2000). Properties of soluble alpha-chymotrypsin in neat glycerol and water. Enzyme Microbial Technol, 27, 143–50.

Castro GR, Kamdar RR, Panilaitis B, Kaplan DL. (2005). Triggered release of proteins from emulsan-alginate beads. J Control Release, 109, 149–57.

Castro GR, Knubovets T. (2003). Homogeneous biocatalysis in organic solvents and water-organic mixtures. Crit Rev Biotechnol, 23, 195–231.

Castro GR, Zuluaga N, Panilaitis B, Kaplan DL. (2009). Evaluation of antioxidant activity of emulsan in vitro. Chapter 3. In: Pandey A, Larroche C, Soccol CR, Dussap CG, eds. New horizons in Biotechnology 2007. New Delhi, India: Asiatech Publishers Inc, 13–22.

Cengiz S, Çava L, Yurdakoç K. (2012). Bentonite and sepiolite as supporting media: immobilization of catalase. Appl Clay Sci, 65–66, 114–20.

Chng ELK, Sofer Z, Pumera M. (2014). Cytotoxicity profile of highly hydrogenated grapheme. Chemistry – A European Journal 20, 6366–73.

Chung T-W, Wang S-S, Tsai W-J. (2008). Accelerating thrombolysis with chitosan-coated plasminogen activators encapsulated in PLGA nanoparticles. Biomaterials, 29, 228–37.

Corchero JL, Mendoza R, Ferrer-Miralles N, et al. (2012). Enzymatic characterization of highly stable human alpha-galactosidase A displayed on magnetic particles. Biochem Eng J, 67, 20–7.

Costa L, Bosio VE, Pandey A, Castro GR. (2008). Effects of organic solvents on immobilized lipase in pectin microspheres. Appl Biochem Biotechnol, 151, 578–86.

Costa SA, Azevedo HS, Reis RL. (2004). Enzyme immobilization in biodegradable polymers for biomedical applications. In: Reis RL, San Roman J, eds. Biodegradable systems in tissue engineering and regenerative medicine. Boca Raton, USA: CRC Press, 301–24.

Costa S, Reis R. (2004). Immobilisation of catalase on the surface of biodegradable starch-based polymers as a way to change its surface characteristics. J Mater Sci Mater Med, 15, 335–42.

Cui J-D, Zhang S, Sun L-M. (2012). Cross-linked enzyme aggregates of phenylalanine ammonia lyase: novel biocatalysts for synthesis of L-phenylalanine. Appl Biochem Biotech, 167, 835–44.

Cumana S, Simonsen J, Liese A, et al. (2013). Immobilization of glucose 6-phosphate dehydrogenase in silica-based hydrogels: a comparative study. J Mol Catal B Enzym, 85–86, 220–8.

Datta S, Christen RL, Rani Y, Rajaram S. (2013). Enzyme immobilization: an overview on techniques and support materials. 3-Biotechnology, 3, 1–9.

Diez A, Campo M, Soler G. (1990). Properties of arginase immobilized in a fibrin clot. Biotechnol Appl Biochem, 12, 237–44.

De la Rica R, Aili D, Stevens MM. (2012). Enzyme-responsive nanoparticles for drug release and diagnostics. Adv Drug Deliv Rev, 64, 967–78.

Dinçer A, Becerik S, Aydemir T. (2012). Immobilization of tyrosinase on chitosan–clay composite beads. Int J Biol Macromol, 50, 815–20.

Ding Y, Yin G, Liao X, et al. (2012). A convenient route to synthesize SBA-15 rods with tunable pore length for lysozyme adsorption. Microporous Mesoporous Mater, 170, 45–51.

Dong Z, Luo Q, Liu J. (2012). Artificial enzymes based on supra-molecular scaffolds. Chem Soc Rev, 41, 7890–908.

Edwards JS, Kumbhar A, Roberts A, et al. (2011). Immobilization of active human carboxylesterase 1 in biomimetic silica nanoparticles. Biotechnol Prog, 27, 863–9.

Ehlert N, Müller PP, Stieve M, Behrens P. (2010). Immobilization of alkaline phosphatase on modified silica coatings. Microporous Mesoporous Mater, 131, 51–7.

Elnashar MMM. (2011). The art of immobilization using biopolymers, biomaterials and nanobiotechnology. In: Elnashar MMM, ed. Biotechnology of Biopolymers. Rijeka: Intech, ISBN: 978-953-307-179-4, InTech, DOI: 10.5772/23696. Available from: http://www.in-techopen.com/books/biotechnology-of-biopolymers/the-art-of-immo-bilization-using-biopolymers-biomaterials-and-nanobiotechnology.

Elnashar MM, Awad GE, Hassan ME, et al. (2014). Immobilization of β-galactosidase onto biomaterial based on κ-carrageenan gel beads and its optimization using response surface methodology. Scientific World J. Article ID 571682, 7 pages. http://dx.doi.org/10.1155/2014/571682.

Farooqui AA, Srivastava PN. (1981). Changes in the kinetic properties of _-N-acetylhexosaminidase and arylsulfatase a upon immobilization on concanavalin A. Int J Biochem, 13, 893–7.

Feng W, Ji P. (2011). Enzymes immobilized on carbon nanotubes. Biotechnol Adv, 29, 889–95.

Filmon R, Basle M, Barbier A, Chappard D. (2000). Poly (2-hydroxy ethyl methacrylate)-alkaline phosphatase: a composite biomaterial allowing in vitro studies of bisphosphonates on the mineralization process. J Biomater Sci Polym Ed, 11, 849–68.

Gao L, Wu J, Gao D. (2011). Enzyme-controlled self-assembly and transformation of nanostructures in a tetramethylbenzidine/horserad-ish peroxidase/H_2O_2 system. ACS Nano, 8, 6736–42.

Gardimalla HM, Mandal D, Stevens PD, et al. (2005). Superparamagnetic nanoparticle-supported enzymatic resolution of racemic carboxylates. Chem Commun, 21, 4432–4.

Gregoriadis G, Neerunjun D, Meade T, et al. (1980). Experiences after long-term treatment of a type I Gaucher disease patient with liposome-entrapped glucocerebroside: beta-glucosidase. Birth Defects Orig Artic Ser, 16, 383–92.

Gu B, Xu C, Zhu G, et al. (2008). Tyrosinase immobilization on ZnO nanorods for phenol detection. J Phys Chem B, 113, 377–81.

Gunasekaran T, Nigusse T, Dhabaraju MD. (2011). Silver nanoparticles as real topical bullets for wound healing. J Am Coll Clin Wound Spec, 3, 82–96.

Guo X, Mei N. (2014). Assessment of the toxic potential of graphene family nanomaterials. J Food Drug Anal, 22, 105–15.

Hahn ME, Gianneschi NC. (2011). Enzyme-directed assembly and manipulation of organic nanomaterials. Chem Commun, 47, 1814–21.

Haider T, Husain Q. (2008). Concanavalin A layered calcium alginate–starch beads immobilized _galactosidase as a therapeutic agent for lactose intolerant patients. Int J Pharm, 359, 1–6.

Hearing VJ, Ekel TM, Montague PM, Nicholson JM. (1980). Mammalin tyrosinase. Stoichiometry and measurement of reaction products. Biochim Biophys Acta-Enzymol, 611, 251–68.

Hegedus I, Nagy E. (2009). Improvement of chymotrypsin enzyme stability as single enzyme nanoparticles. Chem Eng Sci, 64, 1053–60.

Hsueh EC, Knebel SM, Lo W-H, et al. (2012). Deprivation of arginine by recombinant human arginase in prostate cancer cells. J Hematol Oncol, 5, 1–6.

Hu H, Ni Y, Montana V, et al. (2004). Chemically functionalized carbon nanotubes as substrates for neuronal growth. Nano Lett, 4, 507–11.

Huang W, Li X, Xue Y, et al. (2013). Antibacterial multilayer films fabricated by LBL immobilizing lysozyme and HTCC on nanofibrous mats. Int J Biomacromol, 53, 26–31.

Hwang ET, Gu MB. (2013). Enzyme stabilization by nano/microsized hybrid materials. Eng Life Sci, 13, 49–61.

Illanes A, Cauerhff AA, Wilson L, Castro GR. (2012). Recent trends in biocatalysis engineering. Bioresour Technol, 115, 48–57.

Islan GA, Bosio VE, Castro GR. (2013). Alginate lyase and ciprofloxacin co-immobilization on biopolymeric microspheres for cystic fibrosis treatment. Macromol Biosci, 13, 1238–48.

Islan GA, Castro GR. (2014). Tailoring of alginate–gelatin microsphere properties for oral Ciprofloxacin controlled release against *Pseudomonas aeruginosa.* Drug Delivery, 21, 615–26..

Jagur-Grodzinski J. (2009). Polymers for targeted and/or sustained drug delivery. Polymer conjugates as anticancer nanomedicines. Polym Adv Technol, 20, 595–606.

Jia H, Zhu G, Wang P. (2003). Catalytic behaviors of enzymes attached to nanoparticles: the effect of particle mobility. Biotech Bioeng, 84, 406–14.

Jin Y, Li A, Hazelton SG, et al. (2009). Amorphous silica nanohybrids: synthesis, properties and applications. Coord Chem Rev, 253, 2998–3014.

Kang TS, Wang L, Sarkissian CN, et al. (2010). Converting an injectable protein therapeutic into an oral form: phenylalanine ammonia lyase for phenylketonuria. Mol Gen Metab, 99, 4–9.

Kempe H, Kempe M. (2010). The use of magnetite nanoparticles for implant-assisted magnetic drug targeting in thrombolytic therapy. Biomaterials, 31, 9499–510.

Kim J, Grate JW. (2003). Single-enzyme nanoparticles armored by a nanometer-scale organic/inorganic network. Nano Lett, 3, 1219–922.

Kim J-Y, Kim J-K, Park J-S, et al. (2009). The use of PEGylated liposomes to prolong circulation lifetimes of tissue plasminogen activator. Biomaterials, 30, 5751–6.

Koneracká M, Kopcansk P, Timko M, et al. (2002). Direct binding procedure of proteins and enzymes to fine magnetic particles. J Mol Catal B Enzym, 18, 13–8.

Konno T, Watanabe J, Ishihara K. (2004). Conjugation of enzymes on polymer nanoparticles covered with phosphorylcholine groups. Biomacromolecules, 5, 342–7.

Kuen-Chaná Lee G, HaoáYu C, EdmanáTsang SC. (2012). Immobilization of engineered arginase on gold-carbon nanotubes. Chem Commun, 48, 7693–5.

Labus K, Turek A, Liesiene J, Bryjak J. (2011). Efficient *Agaricus bisporus* tyrosinase immobilization on cellulose-based carriers. Biochem Eng J, 56, 232–40.

Lee GJ, Choi SK, Choi S, et al. (2011). Enzyme-immobilized CNT network probe for in vivo neurotransmitter detection. Meth Mol Biol, 743, 65–75.

Lee JE, Park JC, Kim JG, Suh H. (2001). Preparation of collagen modified hyaluronan microparticles as antibiotics carriers. Yonsei Med J, 42, 291–8.

Liong M, Lu J, Kovochich M, et al. (2008). Multifunctional inorganic nanoparticles for imaging, targeting, and drug delivery. ACS Nano, 2, 889–96.

Liu JS, Du XZ, Zhang XF. (2011). Enzyme-inspired controlled release of cucurbituril nanovalves by using magnetic mesoporous silica. Chem Eur J, 17, 810–15.

Liu Y, Du J, Yan M, et al. (2013). Biomimetic enzyme nanocomplexes and their use as antidotes and preventive measures for alcohol intoxication. Nat Nanotech, 8, 187–92.

Maedeh T, Fazilati M, Kamali M, et al. (2011). Investigation of stability and activity of alpha-amylase immobilized on nano pore zeolite. Clin Biochem, 44, S21–2.

Manosroi A, Chankhampan C, Manosroi W, Manosroi J. (2013). Transdermal absorption enhancement of papain loaded in elastic niosomes incorporated in gel for scar treatment. Eur J Pharm Sci, 48, 474–83.

Martínez YN, Cavello I, Hours R, et al. (2013). Immobilized keratinase and enrofloxacin loaded on pectin PVA cryogel patches for anti-microbial treatment. Bioresour Technol, 145, 280–4.

Medeiros S, Santos A, Fessi H, Elaissari A. (2011). Stimuli-responsive magnetic particles for biomedical applications. Int J Pharm, 403, 139–61.

Miletić N, Nastasović A, Loos K. (2012). Immobilization of biocatalysts for enzymatic polymerizations: possibilities, advantages, applications. Bioresour Technol, 115, 126–35.

Mohammadi A, Moghaddam AB, Dinarvand R, et al. (2008). Bioelectrocatalysis of methyldopa by adsorbed tyrosinase on the surface of modified glassy carbon with carbon nanotubes. Int J Electrochem Sci, 3, 1248–57.

Netto CGCM, Toma HE, Andrade LH. (2013). Superparamagnetic nanoparticles as versatile carriers and supporting materials for enzymes. J Mol Catal B: Enzym, 85–86, 71–92.

Nepal D, Balasubramanian S, Simonian AL, Davis VA. (2008). Strong antimicrobial coatings: single-walled carbon nanotubes armored with biopolymers. Nano Lett, 8, 1896–901.

Nikolic T, Kostic M, Praskalo J, et al. (2010). Sodium periodate oxidized cotton yarn as carrier for immobilization of trypsin. Carbohydr Polym, 82, 976–81.

Osathanon T, Giachelli CM, Somerman MJ. (2009). Immobilization of alkaline phosphatase on microporous nanofibrous fibrin scaffolds for bone tissue engineering. Biomaterials, 30, 4513–21.

Ozoner SK, Yalvac M, Erhan E. (2010). Flow injection determination of catechol based on polypyrrole–carbon nanotube–tyrosinase biocomposite detector. Curr Appl Phys, 10, 323–8.

Palmour R, Goodyer P, Reade T, Chang TS. (1989). Microencapsulated xanthine oxidase as experimental therapy in Lesch–Nyhan disease. Lancet, 334, 687–8.

Panzer S, Endert G, Essler F, et al. (2001). Nanocapsules having a polyelectrolyte envelope. WO Patent 2,001,064,330; 2001.

Park E-J, Zahari NEM, Lee E-W, et al. (2014). SWCNTs induced autophagic cell death in human bronchial epithelial cells. Toxicol In Vitro, 28, 442–50.

Park Y-J, Liang J, Yang Z, Yang VC. (2001). Controlled release of clot-dissolving tissue-type plasminogen activator from a poly (L-glutamic acid) semi-interpenetrating polymer network hydrogel. J Control Release, 75, 37–44.

Pera LM, Baigori MD, Castro GR. (2004). Hybrid enzymes. In: Pandey A, Webb C, Soccol CR, Larroche C, eds. Enzyme technology. Berlin: Springer-Verlag, 652–71.

Phadtare S, Kumar A, Vinod V, et al. (2003). Direct assembly of gold nanoparticle ''shells'' on polyurethane microsphere ''cores'' and their application as enzyme immobilization templates. Chem Mater, 15, 1944–9.

Pierre AC. (2004). The sol-gel encapsulation of enzymes. Biocat Biotransform, 22, 145–70.

Pinto Reis C, Neufeld RJ, Ribeiro AJ, Veiga F. (2006). Nanoencapsulation II. Biomedical applications and current status of peptide and protein nanoparticulate delivery systems. Nanomedicine, 2, 53–65.

Piras AM, Chiellini F, Fiumi C, et al. (2008). A new biocompatible nanoparticle delivery system for the release of fibrinolytic drugs. Int J Pharm, 357, 260–71.

Popat KC, Eltgroth M, LaTempa TJ, et al. (2007). Titania nanotubes: a novel platform for drug-eluting coatings for medical implants? Small, 3, 1878–81.

Qin Y, Kong Y, Xu Y, et al. (2012). *In situ* synthesis of highly loaded and ultrafine Pd nanoparticles-decorated graphene oxide for glucose biosensor application. J Mater Chem, 22, 24821–6.

Rana S, Bajaj A, Mout R, Rotello VM. (2012). Monolayer coated gold nanoparticles for delivery applications. Adv Drug Deliv Rev, 64, 200–16.

Ravindran A, Chandran P, Khan SS. (2013). Biofunctionalized silver nanoparticles: advances and prospects. Colloids Surf B Biointerfaces, 104, 342–52.

Ren L, Wang X, Wu H, et al. (2010). Conjugation of nattokinase and lumbrukinase with magnetic nanoparticles for the assay of their thrombolytic activities. J Mol Catal B: Enzym, 62, 190–6.

Rouf SA, Moo-Young M, Chisti Y. (1996). Tissue-type plasminogen activator: characteristics, applications and production technology. Biotechnol Adv, 14, 239–66.

Saiyed Z, Sharma S, Godawat R, et al. (2007). Activity and stability of alkaline phosphatase (ALP) immobilized onto magnetic nanoparticles (Fe3O4). J Biotechnol, 131, 240–4.

Sarkissian CN, Gámez A. (2005). Phenylalanine ammonia lyase, enzyme substitution therapy for phenylketonuria, where are we now? Mol Gen Metab, 86, 22–6.

Sarkissian CN, Kang TS, Gámez A, et al. (2011). Evaluation of orally administered PEGylated phenylalanine ammonia lyase in mice for the treatment of Phenylketonuria. Mol Gen Metab, 104, 249–54.

Schmer G, Chandler WL. (1988). Enzyme reactors for the removal of amino acids from plasma. Method Enzymol, 137, 479–91.

Schoffelen S, van Hest JCM. (2012). Multi-enzyme systems: bringing enzymes together in vitro. Soft Matter, 8, 1736–46.

Sheldon RA. (2011). Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs). Appl Microbiol Biotechnol, 92, 467–77.

Sheldon RA, van Pelt S. (2013). Enzyme immobilization in biocatalysis: why, what and how. Chem Soc Rev, 42, 6223–35.

Shen H, Zhang L, Liu M, Zhang Z. (2012). Biomedical applications of graphene. Theranostics, 2, 283–94.

Sima VH, Patris S, Aydogmus Z, et al. (2011). Tyrosinase immobilized magnetic nanobeads for the amperometric assay of enzyme inhibitors: application to the skin whitening agents. Talanta, 83, 980–5.

Singh V, Kumar P. (2011). Carboxymethyl tamarind gum–silica nanohybrids for effective immobilization of amylase. J Mol Catal B Enzym, 70, 67–73.

Soltys PJ, Mullon C, Langer R. (1992). Oral treatment for jaundice using immobilized bilirubin oxidase. Artif Organs, 16, 331–5.

Stasyuk N, Serkiz R, Mudry S, et al. (2011). Recombinant human arginase I immobilized on gold and silver nanoparticles: preparation and properties. Nanotechnol Dev, 1, 11–15.

Stehle G, Schettler G. (1986). Review and current status of thrombolytic therapy with streptokinase. Tokai J Exp Clin Med, 11, 51–66.

Sung C, Lavin A, Klibanov AM, Langer R. (1986). An immobilized enzyme reactor for the detoxification of bilirubin. Biotechnol Bioeng, 28, 1531–9.

Tavano L, Muzzalupo R, Cassano R, et al. (2010). New sucrose cocoate based vesicles: preparation characterization and skin permeation studies. Colloids Surf B Biointerfaces, 75, 319–22.

Thiem B, Goślińska O. (2004). Antimicrobial activity of Rubus chamaemorus leaves. Fitoterapia, 75, 93–5.

Torres S, Pandey A, Castro GR. (2011). Organic solvent adaptation of Gram positive bacteria: applications and biotechnological potentials. Biotechnol Adv, 29, 442–52.

Torchilin V, Papisov M, Orekhova N, et al. (2009). Magnetically driven thrombolytic preparation containing immobilized streptokinase-targeted transport and action. Pathophysiol Haemost Thromb, 18, 113–16.

Tuncagil S, Kayahan SK, Bayramoglu G, et al. (2009). L-Dopa synthesis using tyrosinase immobilized on magnetic beads. J Mol Catal B Enzym, 58, 187–93.

Tüzmen N, Kalburcu T, Denizli A. (2012). Immobilization of catalase via adsorption onto metal-chelated affinity cryogels. Process Biochem, 47, 26–33.

Vaidya B, Nayak MK, Dash D, et al. (2011). Development and characterization of site specific target sensitive liposomes for the delivery of thrombolytic agents. Int J Pharm, 403, 254–61.

Valenzuela R, Castro JF, Parra C, et al. (2012). β-Glucosidase immobilisation on synthetic superparamagnetic magnetite nanoparticles and their application in saccharification of wheat straw and *Eucalyptus globulus* pulps. J Exp Nanosci, 9, 177–185.

Verma A, Simard JM, Worrall JW, Rotello VM. (2004). Tunable reactivation of nanoparticle-inhibited β-galactosidase by glutathione at intracellular concentrations. J Am Chem Soc, 126, 13987–91.

Walde P, Ichikawa S. (2001). Enzymes inside lipid vesicles: preparation, reactivity and applications. Biomol Eng, 18, 143–77.

Wang L-B, Wang Y-C, He R, et al. (2013a). A new nanobiocatalytic system based on allosteric effect with dramatically enhanced enzymatic performance. J Am Chem Soc, 135, 1272–5.

Wang M, Jia C, Qi W, et al. (2011a). Porous-CLEAs of papain: application to enzymatic hydrolysis of macromolecules. Bioresour Technol, 102, 3541–5.

Wang M, Qi W, Yu Q, et al. (2010). Cross-linking enzyme aggregates in the macropores of silica gel: a practical and efficient method for enzyme stabilization. Biochem Eng J, 52, 168–74.

Wang Q, Zhou L, Jiang Y, Gao J. (2011b). Improved stability of the carbon nanotubes–enzyme bioconjugates by biomimetic silicification. Enzyme Microb Technol, 49, 11–6.

Wang SG, Zhang Q, Wang R, et al. (2003). Multi-walled carbon nanotubes for the immobilization of enzyme in glucose biosensors. Electrochem Commun, 5, 800–3.

Wang R, Billone PS, Mullett WM. (2013b). Nanomedicine in action: an overview of cancer nanomedicine on the market and in clinical trials. J Nanomat, Article ID 629681, 12 pages. DOI:org/10.1155/2013/629681.

Wang R, Zhang Y, Lu D, et al. (2013c). Functional protein-organic/inorganic hybrid nanomaterials. Wiley Interdiscip. Rev Nanomed Nanobiotechnol, 5, 320–8.

Wang Y, Chang TMS. (2012). Nanobiotechnological nanocapsules containing polyhemoglobin-tyrosinase: effects on murine B16F10 melanoma cell proliferation and attachment. J Skin Cancer, Article ID 673291, 9 pages. doi:10.1155/2012/673291.

Weber E, Sirim D, Schreiber T, et al. (2010). Immobilization of P450 BM-3 monooxygenase on mesoporous molecular sieves with different pore diameters. J Mol Catal B Enzym, 64, 29–37.

Wei F, Peijun J. (2011). Enzymes immobilized on carbon nanotubes. Biotechnol Adv, 29, 889–95.

Yang T, Li Z, Wang L, et al. (2007). Synthesis, characterization, and self-assembly of protein lysozyme monolayer-stabilized gold nanoparticles. Langmuir, 23, 10533–8.

Yeung K-K, Owen AJ, Dain JA. (1979). Effect of immobilization on stability and properties of N-acetyl-β-D-hexosaminidase from *Turbo cornutus*. Carbohydr Res, 75, 295–304.

Yin Z, Liu Y, Jiang L-P, Zhu J-J. (2011). Electrochemical immunosensor of tumor necrosis factor _ based on alkaline phosphatase functionalized nanospheres. Biosens Bioelectron, 26, 1890–4.

Yun X, Maximov VD, Yu J, et al. (2013). Nanoparticles for targeted delivery of antioxidant enzymes to the brain after cerebral ischemia and reperfusion injury. J Cereb Blood Flow Metab, 33, 583–92.

Yurko Y, Maximov V, Andreozzi E, et al. (2009). Design of biomedical nanodevices for dissolution of blood clots. Mater Sci Eng C, 29, 737–41.

Zhai R, Zhang B, Liu L, et al. (2010). Immobilization of enzyme biocatalyst on natural halloysite nanotubes. Catal Commun, 12, 259–63.

Zhang JK, Cass A. (2001). A study of His-tagged alkaline phosphatase immobilization on a nanoporous nickel–titanium dioxide film. Anal Biochem, 292, 307–10.

Zhang Y, Xu J, Yuan Z, et al. (2010). Artificial neural network-genetic algorithm based optimization for the immobilization of cellulase on the smart polymer Eudragit L-100. Bioresour Technol, 101, 3153–8.

Zheng W, Zhao H, Zhou H, et al. (2010). Electrochemistry of bilirubin oxidase at carbon nanotubes. J Solid State Electrochem, 14, 249–54.

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