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Review article

Opportunities for nanomaterials in enzyme therapy

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ABSTRACT

In recent years, enzyme therapy strategies have rapidly evolved to catalyze essential biochemical reactions with therapeutic potential. These approaches hold particular promise in addressing rare genetic disorders, cancer treatment, neurodegenerative conditions, wound healing, inflammation management, and infectious disease control, among others. There are several primary reasons for the utilization of enzymes as therapeutics: their substrate specificity, their biological compatibility, and their ability to generate a high number of product molecules per enzyme unit. These features have encouraged their application in enzyme replacement therapy where the enzyme serves as the therapeutic agent to rectify abnormal metabolic and physiological processes, enzyme prodrug therapy where the enzyme initiates a clinical effect by activating prodrugs, and enzyme dynamic or starving therapy where the enzyme acts upon host substrate molecules. Currently, there are >20 commercialized products based on therapeutic enzymes, but approval rates are considerably lower than other biologicals. This has stimulated nanobiotechnology in the last years to develop nanoparticle-based solutions that integrate therapeutic enzymes. This approach aims to enhance stability, prevent rapid clearance, reduce immunogenicity, and even enable spatio-temporal activation of the therapeutic catalyst. This comprehensive review delves into emerging trends in the application of therapeutic enzymes, with a particular emphasis on the synergistic opportunities presented by incorporating enzymes into nanomaterials. Such integration holds the promise of enhancing existing therapies or even paving the way for innovative nanotherapeutic approaches.

1. Background

Enzymes are extremely effective tools for *ex vivo* utilization as they catalyze biotransformations with exquisite selectivity and specificity. They are environmentally friendly, biologically compatible, and represent an attractive green alternative to chemical catalysts. Enzyme-directed evolution and immobilization techniques have improved enzyme stability, reusability, and storage, making it more feasible to use enzymes for reactions under no physiological conditions [1]. This has led to a rapidly expanding number of enzymes' applications beyond their vital role in living animals. Indeed, they are currently extensively used not only for manufacturing active pharmaceutical ingredients (APIs), health supplements, agrochemicals, and biofuels; but also, for environmental monitoring, food processing, and the diagnosis or treatment of several diseases [2].

Although enzyme utilization for industrial applications has been prevalent for centuries, their potential as therapeutics remained practically unexplored until the early 1960s, when the administration of enzymes for the therapy of lysosomal storage diseases was proposed for the first time.[3] Since then, different enzyme therapy strategies have been rapidly developed to catalyze critical biochemical reactions that have a remedial effect on the body. These therapies are especially relevant in treating rare genetic diseases caused by a deficiency or dysfunction of a crucial metabolic enzyme. While these treatments provide a temporary solution and necessitate lifelong administration, various clinical studies have demonstrated that enzymes are highly effective and safe for treating most of these rare inherited diseases [2]. In turn, the implementation of enzyme therapy has been extended beyond the use in genetic disorders, being currently explored in the treatment of a myriad of other diseases such as cancer therapy [4,5]

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neurodegenerative diseases such as Alzheimer's disease [6,7], wound healing, inflammation diseases [8,9], infectious diseases [10], and gene editing based therapies [11,12]. However, a wide number of these new enzyme-based therapies are still technologically immature and will require a few more years before human experimentation [5].

Therapeutic enzymes can fulfil two discernible roles: i) the enzyme itself constitutes the therapeutic agent correcting abnormal metabolic and physiological processes (enzyme replacement treatment) (ERT), ii) the enzyme indirectly triggers a clinical effect by either activating prodrugs (enzyme prodrug therapy) (EPT) or dealing with host substrate molecules (enzyme dynamic or starving therapy) (EDT and EST respectively). But regardless of the role, enzymes used as therapeutics have differential features that make them specific and on occasions more effective compared to traditional drugs.

Enzymes have properties that are key for their therapeutic success: they have high specificity, high selectivity, high turnover number, and biodegradability. However, they also have several drawbacks for this application. Indeed, challenges remain in extending the half-life of the enzymes for longer periods inside the body, developing tools that enable them to successfully cross biological barriers (*e.g.,* blood-brain barrier, cell membranes, *etc.*), decreasing their production costs, or avoiding side effects that arise from immunogenicity of the enzyme and/or its OFF-target accumulation [2]. To address these limitations, the implementation of new advances in protein engineering added to the integration of novel nanomaterials could reveal entirely new opportunities in the field. In this sense, protein engineering has helped to modify and optimize enzymes in terms of their stability and even their specificity/bioconversion efficiency towards non-natural substrates [13–15]. Techniques such as *de novo* design, rational design, and directed evolution have been employed to overcome issues like instability under physiological conditions, low substrate affinity, susceptibility to proteolytic cleavage, and promiscuous activity, which can lead to side effects during treatment. As a result, these approaches have effectively improved the pharmacokinetic and pharmacodynamic properties of biocatalysts applied in therapy.

For instance, rational design was successful in arginine deiminase (ADI) from *Pseudomonas furukawaii* (PfADI), an enzyme in Phase III clinical trials for treating arginine auxotrophic tumors [16]. The sequence-structure-based immunogenic properties of PfADI were compared with ADI currently in clinical trials (MhADI) to evaluate its suitability for ADI-based therapeutics. B-cell epitope density analysis was conducted to predict the intensity of the humoral immune response against the enzyme, and the relative frequency was calculated to estimate epitope density. Following *in silico* predictions, experiments were performed to assess the biochemical properties of the enzyme in comparison to MhADI, demonstrating its good potential for therapeutic applications. In another successful example of the impact of genetic engineering in therapeutic enzymes, directed evolution was applied to β-glucuronidase (BGus). This enzyme was approved by FDA to treat MPS VII, a lysosomal storage disorder caused by deficiency of this enzyme, which causes an abnormal buildup of toxic materials in the body's cells. The authors were able to find nine variants that showed up to 130-fold improved kcat/KM value towards a synthetic substrate and up to 108-fold shifted specificity towards an alternative substrate over the wild-type enzyme [17]. This demonstrated the enzyme variant's potential to accept a broader range of substrates, thereby enhancing its therapeutic impact. More recently, following an elegant approach, Giessel et al.,[18] used generative deep learning to engineer an improved ornithine transcarbamylase (OTC), an enzyme whose deficiency generates strong metabolic disorders that are treated *via* replacement approaches with exogenous enzymes or mRNAs. The approach trained a neural network on a large alignment of OTC sequences to generate novel, near-human variants that retain functional correlations. These variants exhibited improved stability and specific activity, outperforming a consensus library that lacked residue-residue correlations. The results demonstrated that generative modelling, by capturing the diversity of natural sequences and higher-order co-evolutionary relationships, holds significant potential for engineering therapeutic enzymes.

For a more comprehensive understanding and additional information on genetic engineering, readers are encouraged to consult recent reviews that have thoroughly revised the impact of different strategies for the genetic modification of therapeutic enzymes to improve desired properties for their application [19–21].

Regarding the integration of therapeutic enzymes in materials, increased attention has been centered on developing novel carriers, which can enable improvements for enzyme therapy application in terms of enzyme stability, biodistribution, pharmacokinetics, or even diminish their immunogenicity. Several advantages arise from applying nanomaterials as carriers of therapeutic enzymes.

One major advantage is the possibility of tailoring the design of a material (*e.g.,* size, shape, crystal structure, surface functionalization, *etc.*), so that it perfectly suits any therapeutic outcome. For instance, it is possible to create a large surface area for high-density loading of enzyme molecules in a small volume or allow a favorable microenvironment for efficient conversion of reactant to products, which in many cases improves enzyme stability. This is the case of the use of different nanomaterials that have been employed to co-deliver cancer-starving enzymes increasing their stability and blood circulation half-life [22]. Moreover, to achieve better biodistribution results, the surface of the nanoparticles can be functionalized with different targeting molecules (*e.g.*, antibodies) to facilitate the crossing of biological barriers and/or to bind overexpressed specific ligands in the surface of the target cells *(e.g.,* a tumorigenic cells) to improve therapy selectivity and cellular internalization. Nanodevices also display a high cargo capacity to enable the encapsulation of several functional molecules, allowing synergistic therapeutic approaches combining conventional drugs and enzymes [23,24].

Besides, the development of enzyme-nanoparticles hybrids also allows the integration of the specific recognition and unique catalytic properties of enzymes with the size-dependent unique properties of nanoparticles. In this sense, nanomaterials have electronic, optical, magnetic, and even catalytic properties [25] that provide these enzyme-nanomaterials hybrids with advanced unique features that enzymes do not possess by themselves. Examples of this synergy have been demonstrated: i) in nanomaterial-mediated direct electron transfer to redox-enzymes for improving oxidation/reduction mediated catalysis [26–28]; ii) in the combination of enzymes with nanomaterials with intrinsic catalytic activity for the development of catalytic ensembles with synergic and complementary functions [29–31], and even iii) in the remote control of enzyme activity triggered by nanomaterials acting as nanoactuators that respond to external stimuli (*e.g.*, light [32,33], alternating magnetic fields [34–36] or NIR irradiation [31,37]).

For all the aforementioned, integrating nanomaterials into the design of enzyme therapeutics has the potential to become a forerunner to catalyze a healthy society as Zayed et al. have coined [2]. Therefore, the main aim of this review is to provide an overview of current biomedical applications of enzyme technology and to show how nanomaterials can be used to modify and enhance enzyme properties for therapeutic applications. To the best of our knowledge, the overlap of enzymes and nanotechnology and their synergy for their application in therapy has not been revised to date. Our work includes a description of the types of enzyme therapies, their pros and cons as well as the treatments currently approved. Furthermore, we outline the advantages and challenges derived from the integration of enzymes and nanomaterials in medical applications, and finally, we provide a detailed description of the main nanomaterials reported so far for the improvement of enzyme-based therapeutic schemes.

2. Enzyme therapy: hallmarks, advantages, and limitations

Enzyme-based therapies depend on the administration of an

externally produced enzyme for triggering the targeted therapeutic action. Thus, as in any enzyme technology application, enzyme availability is crucial for knowledge advancement and the development of new application protocols. Enzyme therapies sometimes constitute the only treatment option for certain pathologies. Pompe disease, which causes progressive weakness in the heart and skeletal muscles, lacked a specific treatment until 2006 when enzyme replacement therapy (ERT) with alglucosidase alfa (Myozyme, Genzyme) was approved [38]. For hypophosphatasia, which affects the development of bones and teeth, ERT with asfotase alfa (Strensiq®Alexion, Pharmaceuticals, Inc) was approved in 2015 [39]. Another example is Niemann-Pick disease, a lethal disorder that disrupts the cellular metabolism of cholesterol and lipids. Presently, the available treatment options are limited to supportive care. However, a promising avenue of investigation involves the study of ERT utilizing Olipudase alfa® [40].

The progress in recombinant technologies to produce new and improved biocatalysts has undoubtedly facilitated enzyme therapy, which thus benefits from the constant availability of its main technological tool [41,42]. The advantages of producing therapeutic enzymes using recombinant expression systems include facile genetic modification for the preparation of alternate versions of the enzymes, easier production scale-up, downstream purification and cost-effective preparations of highly homogeneous materials. A recent case of the benefits of recombinant technology for enzyme therapy is exemplified by the development of human cystathionine β-synthase (hCBS) variants for the treatment of cystathionine beta-synthase (CBS)-deficient homocystinuria (HCU), the most common inborn error of sulfur amino acid metabolism [42]. In a series of genetic modifications reviewed by Bublil & Mjtan [43], hCBSe was truncated eliminating an autoinhibitory domain and modifying its catalytic site to increase its efficiency. Indeed, the complex functional and structural properties of this CBS that once impaired its use despite its potential for enzyme therapy, were eased thanks to recombinant and genetic technologies. The new version of hCBS is commercialized as pegtibatinase, and it is included in a product called OT-58 developed by Kraus and Majtan group at the University of Colorado Anschutz Medical Campus with Orphan Technologies and can be administered subcutaneously to metabolize homocysteine to cystathionine in the bloodstream.

Despite their readiness, enzymes need appropriate catalytic properties for an adequate performance in enzyme therapy. Low Michaelis constants and high turnover numbers are desired as the enzyme is expected to rapidly convert low concentrations of substrate into the desired therapeutic product [44]. This would lead to the ideal scenario where a single enzyme molecule generates many drug molecules leading to higher drug concentrations on a specific site, compared to the systemic use of the drug alone. Consequently, these properties would allow for smaller quantities of the prodrug to be administered. The relevance of catalytic properties in therapeutic enzymes is demonstrated by the current interest in improving enzymes that are already approved for their application in therapy (*e.g.,* Glucarpidase [45] and Oncaspar®/ Erwinase® [46]).

The drug specificity of enzymes advantageously minimizes the risk of interfering with normal biological processes [47]. A clear example is an enzymatic therapy that removes or lyses blood clots or thrombi restoring the perfusion of the affected tissue during thrombosis. Using fibrinolytic enzymes for thrombolytic therapy has advantages over conventional drugs (anticoagulants and antiplatelets) as they can act upon the existing clot by directly degrading the fibrin [48]. It has been shown that fibrinolytic serine metalloproteases can be more effective in eliminating thrombi than traditional anti-coagulant drugs such as heparin, whose mechanism of action is based on slowing down the clot formation process. Besides, they have the advantage of not showing proteolytic activity towards blood plasma proteins like haemoglobin, γ-globulins, and transferrin [49].

Enzyme therapy also provides the possibility to reduce the secondary effects derived from conventional drugs using exogenous enzymes. This is a clear characteristic of pro-drug enzyme therapy, which is based on the *in situ* bioconversion of non-toxic or less-toxic pro-drugs into the therapeutic agent of interest. For example, 5-fluorouracil (5-FU) is a chemotherapeutic used in aerodigestive tract, colorectal, breast, head and neck cancer treatment [50]; but its usage leads to secondary effects such as mucositis, myelosuppression, dermatitis, and cardiac toxicity. However, the enzyme-prodrug system composed of the prodrug 5- fluorocytosine (5-FC) and the enzyme cytosine deaminase (CD) proved to be a more efficient therapy. The advantage of this system lies in the fact that CD is not expressed in mammalian cells which reduces off-target effects, and that 5-FC, a less toxic precursor of 5-fluorouracil, is converted into 5-FU by CD allowing the benefit of the cytotoxic effect of 5-FU without suffering from its side effects. Indeed, Mitchell et al. [51] reported a reduction in leukopenia, a 5-FU-derived side effect, by using the 5-FC/CD system.

Despite the advantages that enzymes provide over conventional therapeutics, only a few have been effective in clinical phases, and even fewer have been approved by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) (Table 1).

The limited number of commercially available therapeutic enzymes might respond to global and specific limitations depending on the enzyme for a specific therapeutical use [48–50]. In this sense, despite their differences, all enzyme therapy strategies share the following general limitations:

2.1. Poor biodistribution

A major challenge in the field of enzyme therapy is the necessity of site-specific delivery of the therapeutic enzymes to achieve a sustained therapeutic action at the targeted site and to reduce unwanted side reactions**.** Therefore, enzymes sequentially encounter different biological barriers (BBs) before reaching their site of action (Fig. 1). The challenge to overcome these BBs is strictly related to the administration route. Intravenous injection (IV) is a commonly chosen and effective route for many enzyme therapies due to its rapid and widespread delivery capabilities. However, it is important to note that IV administration may not be suitable for all therapeutic scenarios and/or pathologies. Other common administration routes are oral and nasal [52,53]. Throughout the following section, we will discuss the modifications that enzymes may suffer and that may alter their biodistribution and pharmacokinetics.

Organ level BBs: Right after the IV administration, exogenous/recombinant enzymes are recognized and removed by the immune cells of the reticuloendothelial system (RES) and filtrated in the liver or the spleen. Briefly, the RES is composed of phagocytic cells (macrophages and monocytes) distributed in the blood, liver, spleen, lymph nodes, and bone marrow, among other organs. Thus, we can assume that both the RES and the liver and spleen are major BBs after IV administration. Consequently, the pharmacokinetics of the therapeutic enzyme is reduced.

Eventually, therapeutics must extravasate from the bloodstream to reach the target organ or tissue. To accomplish this, enzymes need to face a new BB, known as the endothelial layer. This layer is distinguished by fenestrations of approximately 50-60 nm in width in gastrointestinal mucosa and renal capillaries, while in the spleen, bone marrow, and liver, the fenestrations are characterized by a larger size, ranging from 100 to 175 nm in width [54].

However, for addressing some pathologies other BBs are involved after IV administration. This is the case of the Blood-Retinal-Barrier (BRB,) a specialized barrier in the eye that separates the bloodstream from the retina and other ocular tissues, and the Blood-Brain-Barrier (BBB) that protects the central nervous system (CNS) allowing only certain molecules to pass through [55,56]. In this sense, addressing neurological disorders constitutes a major challenge for enzyme therapy since the BBB is not permeable to enzymes administered intravenously [57]. As a consequence, based on pre-clinical data obtained in animal

Table 1
Therapeutic enzymes approved by FDA and EMA.

Enzyme	Mechanism of action	Source	Commercial name	Approved indications	Approved year	Enzyme therapy
Adenosine deaminase	Mediates conversion of adenosine into inosine, and of deoxyadenosine into deoxyinosine.	Recombinant adenosine deaminase (rADA) based on bovine amino acid sequence	Revcovi (peg-ademase)	Adenosine deaminase severe combined immune deficiency (ADA-SCID)	2018 (FDA)	ERT
		Recombinant adenosine deaminase from bovine	Adagen (peg-ademase)		1990 (FDA)	ERT
alfa-Galactosidase A	Catalyze the removal of terminal α-galactose groups from substrates such as glycoproteins and glycolipids.	Recombinant DNA technology in a Chinese hamster ovary cell line	Fabrazyme	Fabry disease (α-galactosidasa A deficiency)	2003 (FDA)	ERT
			Replagal		2001 (EMA)	
Alglucosidase alfa	Breaks down sugar stored as glycogen into glucose	Recombinant DNA technology in a Chinese hamster ovary cell line	Myocime	Pompe disease (α -glucosidase deficiency)	2006 (FDA) 2001 (EMA)	ERT
			Lumizyme		2010 (FDA)	
			Nexviazyme/ Nexviadyme (avalglucosidase alfa-ngpt)		2021 (FDA) 2022 (EMA)	
alfa -L-Iduronidase	Catalyzes the hydrolysis of terminal α-L-iduronic acid residues of dermatan sulfate and heparan sulfate.	Recombinant DNA technology in a Chinese hamster ovary cell line	Aldurazyme	Hurler and Hurler-Scheie forms of Mucopolysaccharidosis I	2003 (FDA) 2003 (EMA)	ERT
alpha-mannosidase	Degradation of mannose–rich oligosaccharides	Recombinant DNA technology in a Chinese hamster ovary cell line	Lamzede (velmanase alfa)	alpha-Mannosidosis	2018 (EMA) 2023 (FDA)	ERT
Asfotase alfa	Cleaves inorganic pyrophosphate, releasing inorganic phosphate to combine with calcium to form hydroxyapatite crystals that mineralise bone and so restore skeletal integrity. Enzyme activity also permits pyridoxal to enter cells to act as a cofactor for many enzymatic reactions.	Recombinant DNA technology in a Chinese hamster ovary cell line	Strensiq	Hypophosphatasia	2015 (FDA) 2015 (EMA)	ERT
Asparaginase	Breaking up amino acid asparagine	*Escherichia coli*	Elspar	Acute Lymphocytic Leukemia.	1978 (FDA)	EDT
		Escherichia coli	Oncaspar (peg-asparaginase)		1994 (FDA) 2016 (EMA)	
		Erwinia chrysanthemi	Erwinaze		2011 (FDA)	
		Escherichia coli	Spectrila		2016 (EMA)	
		Escherichia coli	Asparlas (calaspargase pegol-mknl)		2018 (FDA)	
beta-Glucuronidase	Catabolism of glycosaminoglycans	Recombinant DNA technology in a Chinese hamster ovary cell line	Mepsevii	Sly Syndrome mucopolysaccharidosis VII	2017 (FDA) 2018 (EMA)	ERT
Carboxy-peptidase	Hydrolyzes the carboxyl-terminal glutamate residue from folic acid and classical antifolates such as methotrexate	Recombinant bacteria	Voraxaze	Treatment of toxic plasma methotrexate concentrations	2012 (FDA)	EDT
Cerliponase alfa	Serine protease, cleaving N-terminal tripeptides from a broad range of protein substrates.	Recombinant DNA technology in a Chinese hamster ovary cell line	Brineura	Batten disease (tripeptidyl-peptidase-1 deficiency)	2017 (FDA) 2017 (EMA)	ERT
Collagenase	Metalloproteinase that breaks peptide bonds in collagen	*Clostridium histolyticum*	Santyl (Collagenase Santyl Ointment (CSO)	Debriding chronic dermal ulcers and severely burned areas	1965 (FDA)	EDT
			Xiaflex	Treatment of Dupuytren's contracture/ Peyronie's disease	2010 (FDA)	
			Xiapex		2011 (EMA)	
Dornase alfa (desoxirribo-nuclease I recombinant human)	Cleaves extracellular DNA to 5′-phosphodinucleotide and 5′-phosphooligonucleotide end	Recombinant DNA technology in a Chinese hamster ovary cell line	Pulmozyme	Cystic fibrosis	1993 (FDA)	EDT

(*continued on next page*)

Table 1 (*continued*)

Enzyme	Mechanism of action	Source	Commercial name	Approved indications	Approved year	Enzyme therapy
	products without affecting intracellular DNA.					
		Recombinant DNA technology in a Chinese hamster ovary cell line	Cerezyme		1994 (FDA) 1997 (EMA)	ERT
Glucocerebrosidase	Catalyzes the hydrolysis of the glycolipid glucocerebroside to glucose and ceramide	Plant-cell expressed recombinant form of glucocerebrosidase	Elelyso (taliglucerase alfa)	Gaucher disease	2012 (FDA)	ERT
		Produced in an HT-1080 human fibroblast cell line by recombinant DNA technology.	VPRIV (velaglucerase alfa)		2010 (FDA) 2010 (EMA)	ERT
Glycosaminoglycan N–acetylgalactosamine 4-sulfatase	Catabolism of glycosaminoglycans	Recombinant DNA technology in a Chinese hamster ovary cell line	Naglazyme	Maroteaux-Lamy syndrome Mucopolysaccharidosis VI	2005 (FDA) 2006 (EMA)	ERT
Iduronate-2-sulfatase	hydrolyzes the 2-sulfate esters of terminal iduronate sulfate residues in the lysosomes	Recombinant DNA technology in a human cell line	Elaprase	Hunter syndrome mucopolysaccharidosis II	2006 (FDA) 2007 (EMA)	ERT
Lysosomal acid lipase	Hydrolysis of cholesteryl esters and triglycerides to free cholesterol, glycerol and free fatty acids	Recombinant DNA technology in a Chinese hamster ovary cell line	Kanuma	Lysosomal Acid Lipase deficiency	2015 (FDA) 2015 (EMA)	ERT
N-Acetylgalactosamine-6-sulfatase	Catabolism of glycosaminoglycans	Recombinant DNA technology in a Chinese hamster ovary cell line	Vimizim	Morquio A syndrome Mucopolysaccharidosis type IVA	2014 (FDA) 2014 (EMA)	ERT
Ocriplasmin	Degradation of proteins between the vitreous humour and the retina	*Pichia pastoris*	Jetrea	Vitreomacular Adhesion.	2012 (FDA) 2013 (EMA)	EDT
Phenylalanine ammonia lyase	Degradation of phenylalanine	Phenylalanine ammonia lyase from the Cyanobacterium *Anabaena variabilis* expressed in *E coli.*	Palynziq (peg-valise)	Phenylketonuria	2018 (FDA) 2019 (EMA)	ERT
Tissue plasminogen activator (tPA)	Serin protease that catalyzes the conversion of plasminogen to plasmin	Recombinant DNA technology in a Chinese hamster ovary cell line.	Alteplase	Ischemic stroke, ST-elevation (STEMI), acute massive pulmonary embolism, and those with central venous access devices	1996 (FDA)	EDT
			Reteplase		1998 (FDA) 1996 (EMA)	
			Tenecteplase		2000 (FDA) 2001 (EMA)	
Urate-oxidase	Oxidation of uric acid to allantoin	Aspergillus flavus-derived urate oxidase expressed in *Saccharomyces cerevisiae*	Elitek	Hyperuricemia in children with leukemia, lymphoma, and solid tumors.	2009 (FDA)	EDT
Uricase	Oxidation of uric acid to allantoin	Mammalian uricase enzyme derived from a genetically modified strain of *E. coli.*	Krystexxa (peg-uricase)	Chronic gout	2010 (FDA)	EDT

studies [58–60], approaches based on invasive surgical methods for their administration are currently being tested in clinical trials mostly focused on gaining access to the CNS. Additional BBs are encountered when the administration route is other than IV. *i)* the nasal or lung epithelia (encountered following nasal or pulmonary administration); *ii)* the skin (after dermal/transdermal administration); *iii)* the mucosal epithelia of the mouth and the gastrointestinal (GI) tract (after oral administration). The main property that hinders these BBs' penetration is the size of the enzyme, which results in low membrane permeability and, therefore, low absorption. Indeed, the cut-off for penetration of epithelium through passive diffusion is <500 Da in the gastrointestinal (GI) tract and skin, < 1 kDa for the nasal mucosa, < 76 kDa for macromolecules diffusion to inner and outer plexiform layers of the retina while <150 kDa to reach the inner retina [61].

2.1.1. Suborgan level BBs

When enzymes pass the endothelial barrier, they have to penetrate the 3D structure of the extracellular matrix (ECM). The ECM is located within the interstitial space that lies between blood vessels and cells. This ECM provides the fluid and structural environment surrounding those cells, conveying both mechanical and chemical signals to them. It consists of a combination of structural proteins and glycosaminoglycans intricately intertwined. Thus, ECM is a physical barrier for enzymes that need to penetrate its complex 3D structure. Indeed, the mechanical rigidity of ECM hinders the therapeutics penetration in pathologies such as cancer or fibrosis. Specifically, when dealing with Pancreatic Ductal

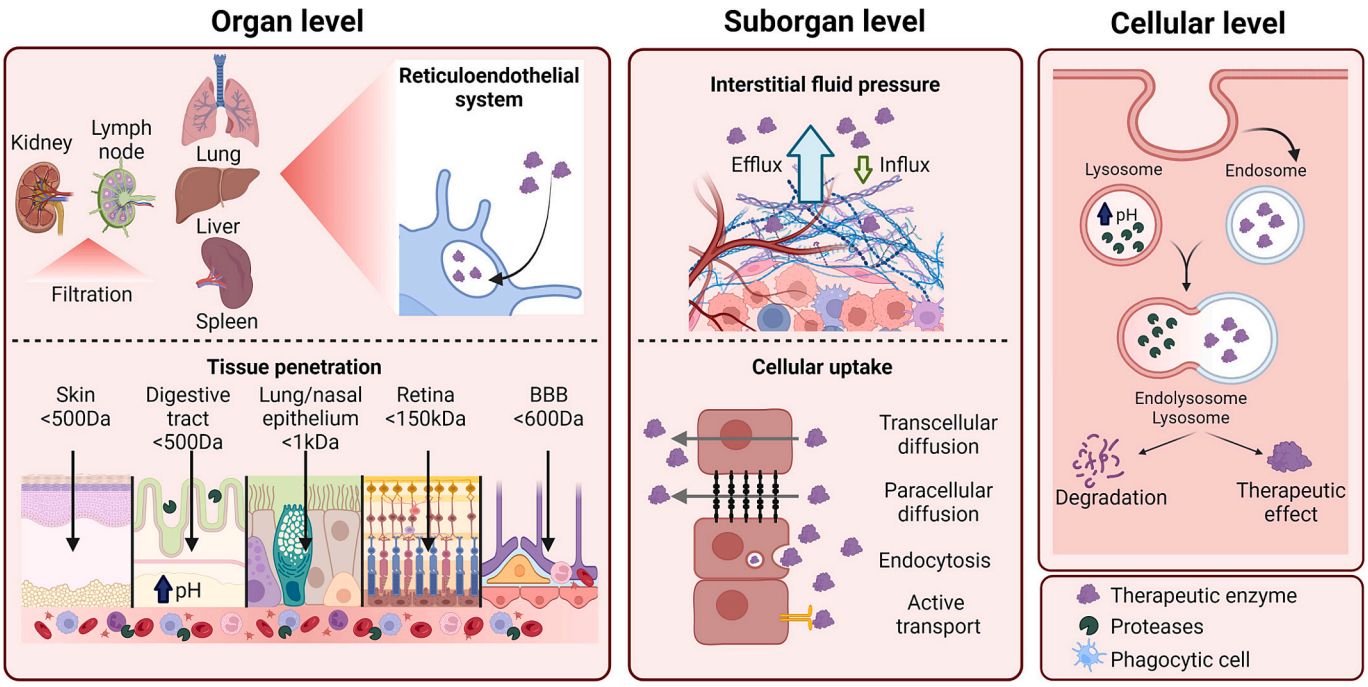

Fig. 1. Biological barriers faced by therapeutic enzymes in the body. *Organ level*. Once in systemic circulation, enzymes encounter barriers such as filtration by the liver, the spleen and lymph nodes, as well as the reticuloendothelial system (RES). If they can reach their target tissue/organ, additional barriers must be overcome. As it is depicted in the figure, each tissue has a limiting size for penetration. *Suborgan level*. Once in the target organ, the enzyme must cope with interstitial flow pressure (IFP), usually caused by a matrix (*e.g.*, tumor matrix). The influx (green arrow) or movement into the cells is lower than the efflux (blue arrow) or the outward movement. On the other hand, to reach the interior of the cell, it encounters both passive (transcellular and paracellular diffusion and endocytosis) and active (receptor-mediated) uptake mechanisms. *Cellular level*. Most of the cellular uptake mechanisms end up in endosomes, and consequently, in the lysosome. Lysosomal release is essential for avoiding the degradation of the therapeutic enzyme to carry out its function in the cytosol if the therapy is not to fight lysosomal storage diseases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Adenocarcinoma (PDAC), the delivery of therapeutics including enzymes becomes even more difficult due to excessive ECM production, resulting in the development of an extremely dense fibrotic stroma. Furthermore, under normal physiological conditions, fluid from the vascular compartment consistently undergoes filtration from microvessels into the interstitial space. Subsequently, this fluid is cleared through lymphatic drainage, ultimately re-entering the venous circulation [62,63]. Tumors are characterized not only by aberrant blood vessels but also by inefficient lymphatic vessels leading to elevated interstitial fluid pressure. Importantly, the elevated interstitial fluid pressure in tumor tissue eliminates pressure gradients and hampers the transit of molecules into and out of vessels. This results in low concentrations of therapeutic enzymes and other therapeutics at tumor sites compromising their efficacy [64].

2.1.1.1. Cellular level BBs. The uptake of therapeutic enzymes by cells is mediated by different mechanisms: *i) transcellular diffusion*, the enzyme passes through the lipid membrane of the target cell; *ii) paracellular diffusion*, the enzyme crosses an epithelium by passing through the intercellular space between cells; *iii) endocytosis*, the enzyme is entrapped in a cellular membrane invagination which will lead to the formation of endosomes and eventually will evolve into lysosomes. Considering that most of the therapeutic enzymes are mainly uptake through endocytosis ending in lysosomes, proteolytic degradation is a major concern. As a result, the overall therapeutic efficacy is limited because only a very small fraction of those successfully delivered enzyme molecules can interact with their cytosolic targets (<10 %) [65]. The last mechanism is *iv) active transport*, in which the enzyme is recognized by a specific cell receptor (*e.g.*, mannose-6-phosphate receptor) and is of great importance for lysosomal disorders. Generally, these therapies take advantage of the mannose-6-phosphorylation (M6P) modification, which controls the trafficking of endogenous lysosomal enzymes. Generally, ERT takes advantage of introducing mannose-6-phosphorylation (M6P) modification to the therapeutic enzyme. However, this does not ensure selective enzyme delivery at the targeted cells due to: *i)* the abundance of the M6P receptor in the liver and on RES cells and *ii)* incomplete and unpredictable M6P modification of recombinant enzymes hampers. In addition, >70 % of all patients affected by lysosomal storage disorders have varying degrees of central nervous system involvement, so ERT must cross the BBB. However, as already explained, the molecular weight of the enzymes makes paracellular or transcellular diffusion of therapeutic enzymes through the BBB almost nonexistent [66,67].

2.1.1.2. Instability. Another significant challenge in enzyme therapy is the instability of enzyme preparations [68]. Each particular treatment or administration route may have its requirements in terms of enzyme stability for an effective therapy. For instance, of all the administration routes, the oral one is the most challenging regarding enzyme stability. It offers the advantage of self-administration with high patient acceptability and compliance. However, the gastrointestinal (GI) tract is a hurdle in the stability of therapeutic enzymes as its major function is protein degradation mainly by the digestive enzymes: trypsin and chymotrypsin. Moreover, therapeutic enzymes are potentially subject to denaturation by intestinal bile salts and are generally poorly absorbed across the intestinal epithelium into the bloodstream [69]. For example, the enzyme phenylalanine ammonia-lyase (PAL) used in the treatment of phenylketonuria is rapidly inactivated in the GI tract by the acidic gastric pH and the GI enzymes [70]. Indeed, PEGylated PAL was developed (Palynziq®) as an enzyme substitution therapy for the parenteral treatment of this genetic inborn error in metabolism that causes intellectual disability, often severe, and other neurologic features that can include autistic behavior, seizures, tremors, and ataxia [71].

2.1.1.3. Clearance. Other obstacles to the development of enzyme therapy are related to the short half-life times resulting from fast renal clearance and enzymatic degradation in the systemic circulation, which is caused by enzymes present in the blood, liver, and kidneys [72]. Consequently, higher concentrations are required for achieving therapeutic doses [73]. While factors such as isoelectric point, state of aggregation, glycosylation pattern, and size have been demonstrated to influence the plasma clearance rate of specific proteins, there are no general properties that can reliably predict the clearance rate of foreign proteins [74,75]. This makes it difficult to select a recombinant enzyme variant with the highest therapeutic outcome.

Although very promising strategies have been developed *in vitro*, the limited half-life of therapeutic enzymes poses challenges for *in vivo* applications. Clear examples illustrating these limitations include human hyaluronidase, which can effectively remove hyaluronic acid-dependent tumor cell extracellular matrices *in vitro* but has a short half-life in serum ($t1/2 < 3$ min) [76], as well as commercial agalsidase alfa, a therapeutic enzyme for Fabry disease, with a half-life of only 13 min [77]. Another striking example is *N*-acetylgalactosamine-6-sulfate sulfatase, which is rapidly cleared from the blood circulation of Morquio A mice, boasting a plasma half-life of a mere 2.9 min [78]. Even if polymers or fusion-tags have been explored for stabilizing the enzyme in the bloodstream and avoiding proteolytic degradation, high costs and low yield are limiting their therapeutic application [79,80]. A remarking example of it is the case of urate oxidase (UOx) treatment for chemotherapy-induced hyperuricemia. Najjari and colleagues increased the half-life of the UOx by integrating a small amino acids proline, alanine, and serine sequence (PASylation) to the native structure, but the synthetic process needs to be improved to obtain a higher yield of production of the recombinant enzyme [80,81].

2.1.1.4. Immunogenicity. The administration of therapeutic enzymes, whether recombinant or not [82], typically results in the induction of anti-drug antibodies (ADAs) and, in particular, neutralizing antibodies which are able to impair the activity of the enzyme. As a result, the generation of neutralizing antibodies leads to short plasma half-lives of enzymes.

Abs recognizing the therapeutic enzyme can affect the therapy outcome due to several reasons: *i)* abs can recognize the epitopes involved both in the recognition of the receptor for the enzyme uptake by the target cell and/or in the substrate recognition region (enzyme active site), *ii)* abs can recognize domains in the catalytic site increasing or decreasing enzyme turnover, *iii*) the ab binding to an exogenous enzyme could be a signal for the phagocytic cells to ingest and process it (process known as opsonization) [83].

Indeed, antibodies against therapeutic enzymes can be found in the serum of patients enrolled in clinical trials. The percentage of patients that generate ADAs is wide and broad depending on the therapeutic enzyme: 47 % for Idursulfase [84], 67 % for Hunterase® [85], 72 % for alglucosidase alpha [86], 79 % for cerliponase alfa [87], 100 % for elosulfase alfa [88]. The production of Abs can be induced by a single treatment cycle, as for the carboxypeptidase G2 (CPG2), an EPT enzyme developed for cancer treatment [89], or by repeated administration, such as for the agalsidase-α and agalsidase-β for the treatment of Fabry disease, a rare X-linked inherited lysosomal storage disease [86,90]. While the impact of ADAs on the sustained effectiveness of treatment remains a subject of ongoing debate with varying perspectives [91,92], increasing evidence supports their role in interfering with or neutralizing enzyme therapeutic efficacy [87,88]. For example, infantile Pompe disease patients with high sustained antibody titer have attenuated therapeutic response to enzyme replacement therapy (ERT) with Myozyme® [93]. In acute lymphoblastic leukemia, it has been demonstrated that the higher the titer of anti-asparaginase antibodies is, the lower the enzyme's therapeutic efficiency. Besides, a correlation of the antibody titer was found both with allergic reactions as well as with a decrease in the event-free and overall survival [94]. Indeed, to perform an effective EPT therapy patients needed to be treated with immunosuppressive drugs.

Although the above-mentioned are global limitations shared among the different enzyme therapy strategies, each of them has significant differences and, thus, specific advantages and limitations. Therefore, we will now discuss the pros and cons of the different types of enzyme therapies.

3. Types of enzyme therapy

As previously stated, enzyme therapies are classified according to the role played by the enzyme, which can be exerted both intracellular or extracellular. Enzyme therapies are classified according to if the enzyme itself constitutes the therapeutic agent (*enzyme replacement treatment*) or the enzyme indirectly triggers a clinical effect by activating prodrugs (*enzyme prodrug therapy*) or by manipulating host substrate molecules (*enzyme dynamic and starving therapy*) (Fig. 2).

3.1. Enzyme replacement therapy

Enzyme replacement therapy (ERT) was the first reported use of enzymes for therapeutic purposes, and it was first applied in 1964 to treat lysosomal storage diseases [95]. A highly important rationale for the development of ERT was that only 1 %–5 % of normal intracellular enzyme activity is required to correct the metabolic defects in those enzyme-deficient cells [96]. Today, it is employed in multiple enzyme deficiency disorders like Gaucher disease, Fabry disease, and Pompe disease among others [2,38,97]. Nowadays, in addition to these lysosomal storage diseases, pancreatic disorders [98–100] benefit from ERT. The indications, mechanism and adverse effects of the enzymes used in ERT have been thoroughly discussed by Baldo et al. [101]. All these disorders are associated with mutations that impair the activity of essential enzymes that take part in various biochemical pathways in the body, leading to the accumulation of harmful substances. ERT consists of replacing the mutated and defective enzyme with periodic administration of a recombinant and functional enzyme presenting mannose-6-phosphate (M6P) residues on its oligosaccharide chains. This allows specific binding of the enzyme to M6P receptors on the cell surface, thus triggering its internalization and lysosomal targeting as already explained [102].

Various enzymes have been approved, tested or are in clinical trials for this type of therapy [103] as is the case of β-glucocerebrosidase to treat Gaucher disease a rare disorder caused by inherited deficiency of the lysosomal enzyme glucocerebrosidase. This defect leads to the accumulation of glucocerebrosides, which causes multiple complications such as enlargement of the spleen and liver, anaemia with subsequent fatigue, discomfort, infection, and bleeding as well as bone pain [2]. Upon approval by the FDA and EMA, results from clinical trials and benefits in patients proved not only the efficacy but also the safety of ERT in the treatment of the above-mentioned multisystem, progressive disorder. Besides, reduced toxicity over traditional therapeutic alternatives has been confirmed. Indeed, the ERT strategy used for this disorder consists of the intravenous supplementation of the deficient protein glucocerebrosidase (Taliglucerase alpha®, Imiglucerase®, or Velaglucerase alpha®) [104–106]. The traditional drug-based treatment available, however, involves the oral administration of inhibitors of the enzyme glucosylceramide synthase (GCS) to slow down the production of glucocerebrosides (substrate reduction therapy or SRT)[107,108]. SRT efficacy against Gaucher disease depends on several factors such as the genotype and renal function and has a higher incidence of adverse effects than ERT (up to 80 % gastrointestinal complaints and tremors) [108]. Indeed, Smid et al. [107] compared the effect on biochemical markers reflecting disease burden between SRT and ERT treatment modalities, finding less response to the treatment when using the GCS inhibitor Miglustat® (Actelion Pharmaceuticals) than when using ERT.

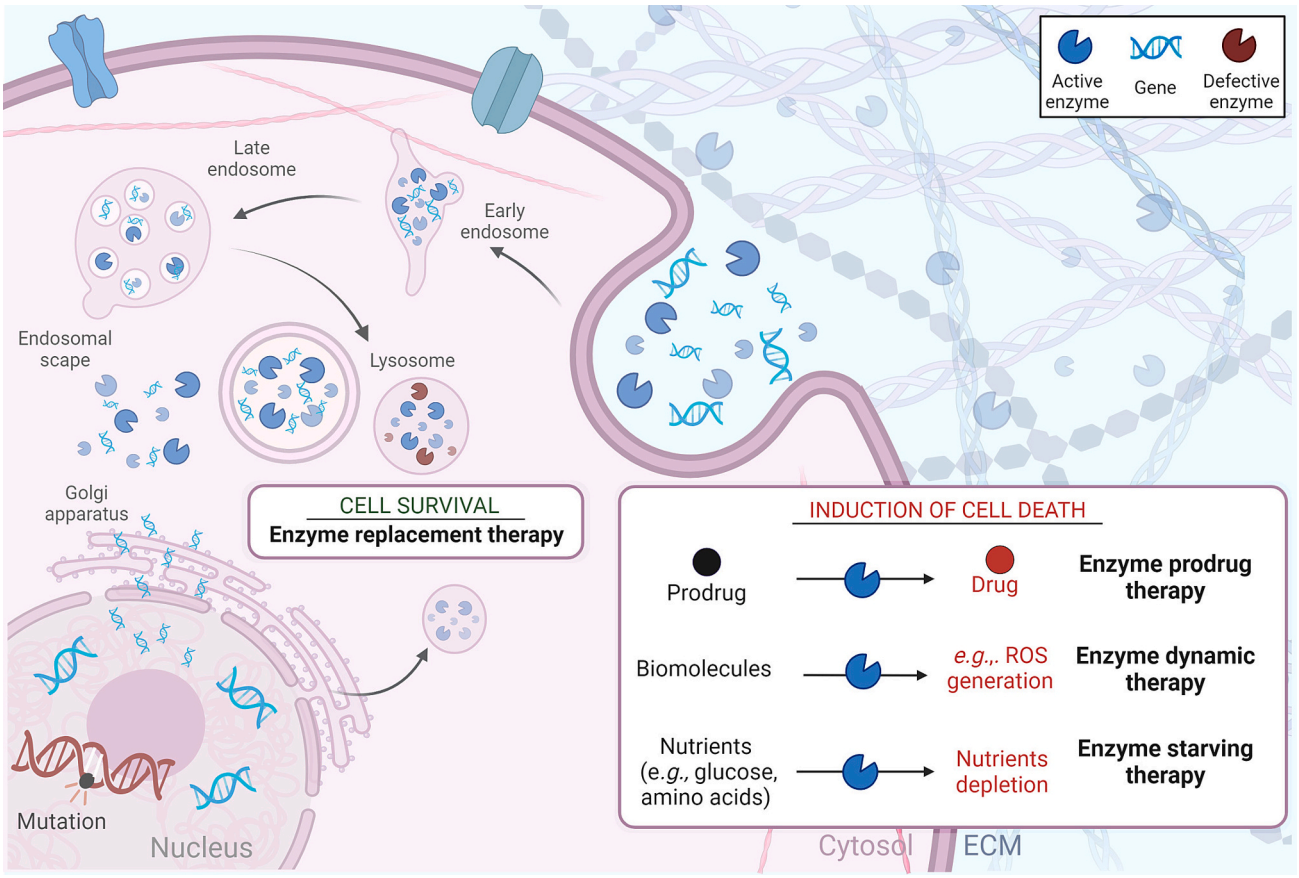

Fig. 2. Representation of the four types of enzyme therapies. There are two primary modalities for therapy approaches with enzymes: the introduction of a therapeutic enzyme-encoding gene or the direct administration of the enzyme itself into a recipient cell. Following internalization, both the enzymes and genes progress through a series of cellular compartments, moving from the early endosome to the late endosome. At this stage, the enzymes and genes face a decision point, either being directed towards the lysosome or escape from the endosomal compartment. If the gene gets into the nucleus, it is transcribed and transduced into a protein and released in vesicles. One common therapeutic application is Enzyme Replacement Therapy (ERT), which typically involves directing the enzyme to lysosomes. Here, the enzyme can serve to replace or supplement deficient enzymes within the cellular environment. Alternatively, the therapeutic enzyme may be released into the cytoplasm, and from there, it can venture into the extracellular matrix to partake in various therapeutic strategies. These strategies include Enzyme Prodrug Therapy (EPT), where the enzyme activates prodrugs, transforming them into active forms that can selectively target specific diseases or conditions. Enzyme Starving Therapy (EST) involves the degradation of nutrients, resulting in the deprivation of essential substrates. On the other hand, Enzyme Dynamic Therapy (EDT) relies on the active engagement of the enzyme in metabolic processes, consequently influencing and modifying cellular functions. Figure created with Biorender.com.

Having demonstrated remarkable clinical responses, ERT is the standard treatment of several genetic disorders, nonetheless, it also presents important limitations. In many cases, the recombinant enzyme options are expensive and present low activity due to changes in its quaternary structure or the absence of key post-translational modifications [109,110]. Besides, the infused recombinant enzymes have a short half-life (*e.g.*, in circulation) due to rapid uptake into visceral organs (liver and spleen). Thus, cartilaginous organs such as the trachea and bronchi, bones and eyes are poorly impacted by ERT probably due to limited penetration in these specific tissues. Besides, due to the inefficacy of recombinant enzymes to cross the BBB, there are no benefits of ERT for central nervous system (CNS) involvement. Virus vectors have been explored for enhancing biodistribution, even in the CNS, maximizing functional enzyme expression at the target location, and, thus, therapy efficacy. Despite the numerous clinical trials involving adenovirus (NCT01395641, NCT03952637, NCT03612869), lentivirus (NCT038524,98), and retrovirus (NCT00794508, NCT00001234, NCT00004454), there are still concerns regarding the safety of using these current vector systems [111,112].

In addition to this, as ERT is a lifelong therapy, it requires weekly intravenous infusions of the recombinant enzymes, and each infusion could take 3 to 4 h. Thus, most infusions must be given in hospital settings to avoid severe reactions. Another limitation is that most ERTs treatments produce an anti-drug antibody (ADA) response which, as already explained, can potentially reduce efficacy or lead to hypersensitivity reactions [113]. Hence, there is a clear need for technological developments to overcome these limitations. As will be elaborated later, the integration of nanomaterials for the development of novel strategies for ERT may enhance the BBB permeability and mitigate the long-term impact of immune responses against the exogenous/recombinant enzyme. This will improve the effectiveness of ERT. Furthermore, enzyme integration on nanomaterials may lead to the development of patient-friendly administration methods.

3.2. *Enzyme prodrug therapy*

Enzyme prodrug therapy (EPT) involves the delivery of therapeutic enzymes to specifically convert prodrugs into potent cytotoxic effector agents. Prodrugs are modified medications that are designed to have low toxicity until they are metabolized into an active form. It was conceived as a tool not only to increase the efficacy of conventional anti-tumoral chemotherapy whilst diminishing its toxicity but above all to achieve an *in situ* site-specific drug production [39,114,115]. This *in situ* bioconversion has an amplifying effect since a single enzyme can activate many pro-drug molecules, which enables drug accumulation in the targeted tissue, generating a more selective and efficient therapy

[115,116]. There are two strategies for the administration of the therapeutic enzyme: the exogenous enzyme can be administered using an antibody, a polymer or implantable biomaterials to facilitate enzyme localization at the desired site [89,117,118]. Alternatively, the gene encoding the enzyme can also be delivered using viruses, stem cells, and even bacteria as vectors [119–122].The mechanism of action of all these different enzyme-prodrug therapeutic strategies has been previously reviewed, being mostly focused on the treatment of tumors [123]. A summary of the different ways used to accomplish the localization of the enzyme at the desired site is described in Table 2.

>50 enzyme-prodrug combinations have been developed over the last 20 years, having to meet both the enzyme and the prodrug requirements for EPT to be efficient [89,125]. For the enzyme: *i)* it should be of non-human origin, or if it is human, it should be absent or expressed at very low concentration in healthy tissue; *ii)* it must have high catalytic activity and must achieve a sufficient concentration at the target site. For the prodrug: *i)* it should be a good substrate for the administered enzyme but not for endogenous enzymes present in non-targeted tissues; *ii)* the difference in cytotoxicity between the prodrug and the active drug must be as high as possible; *iii)* it should be able to cross the targeted cell membrane if the therapeutic enzyme localization is intracellular; *iv)* the activated drug should be highly diffusible and with enough half-life to kill multiple neighboring targeted cells by utilizing the so-called bystander effect. Indeed, a clear advantage of EPT is that it not only triggers a "local effect" caused by the drug diffusion through the cell membrane, *via* intercellular gap-junctions, or endocytosis of apoptotic bodies released by dying cells. In addition, it also triggers a "distant effect" in which the tumoral cells that are dying elicit a systemic immune response by releasing damage associated molecular patterns (DAMPs) that result in the activation of cytotoxic T-lymphocytes. This triggering of immune response has also the advantage that could lead to the destruction of disseminated metastases originating from the primary tumor [126]. The synergy between both effects greatly increases the therapy efficiency allowing to achieve the total eradication of the tumor even if low levels of expression/accumulation of the therapeutic enzyme are achieved. In fact, in the case of gene enzyme prodrug therapy (GEPT), total tumor eradication was reported with transfections efficiencies of tumoral cells between 2 and 10 % [121].

While EPT provides several therapeutic advantages, it also faces various constraints. Similar to ERT, GEPT-based therapies require a clear need for enhancing the design and safety of delivery vectors to introduce the genes encoding therapeutic enzymes into tumor cells. Because of these risks, those strategies based on the delivery of active enzymes rather than genes into tumors, are of great relevance for clinical applications. In this sense, antibody directed enzyme prodrug therapy (ADEPT) is the most extended strategy used for this aim which is based on targeting the enzyme to tumors by attaching it to an antibody directed to a tumor associated antigen. Unlike GEPT, internalization of the enzyme-antibody conjugate is not required as the drug could be generated in the extracellular areas of the tumor triggering a therapeutic effect due to a bystander killing of tumoral cells [89]. But despite these advantages over GEPT, ADEPT also has significant limitations: *i)* the need for high affinity monoclonal antibodies to be linked to the therapeutic enzyme, *ii)* the need for enzymes with optimal pH values close to that of the tumor environment, *iii)* the immunogenicity raised by the antibody-enzyme conjugate that limits multiple application cycles, *iv)* the need of a lag time between the enzyme and the prodrug application to avoid system toxicity as a measure to ensure that the antibody-enzyme conjugate is only accumulated in the tumor rather than in blood or normal tissues. The duration of this interval has to be optimized and results from clinical phases have shown that it could be needed extended periods (*e.g.*, up to seven days) [125]. Indeed, this last limitation could greatly reduce ADEPT efficiency due to stability issues of the antibody-enzyme conjugate caused by the acidic pH and high content of proteases at tumors microenvironments [127]. Thus, the use of polymers as carriers for therapeutic enzymes emerged as an alternative to overcome some of these limitations including: *i)* improving the stability of the enzymes against proteases and acidic pHs, *ii)* improving

Table 2
Classification and description of the main modalities for enzyme therapy.

Classification	Definition	Main features	Disadvantages	References
		Passive carrier		
Virus directed enzyme therapy	The enzyme is expressed by targeted cells upon infection	Gene expression may be controlled by tumor cell-specific promoters. Prodrug is activated intracellularly Highly dependent on bystander effect.	Vector transfection efficiency	[121]
Mesenchymal stromal cells (MSC)	Mesenchymal stroma cells (MSC) are transduced and used as vehicles for targeted vectorization.	MSC migrates and reaches the targeted tissue. Cytotoxic drugs are produced extracellularly and diffuse inside the tumor. Highly dependent on bystander effect.	Vector transfection efficiency	
Bacteria directed enzyme therapy	Bacteria can be transfected with a plasmid containing the gene of the enzyme of interest under a bacterial promoter Endogenous gene expression of the enzyme of interest. Bactofection of plasmid into tumoral cells	Motility and ability to swim against pressure or diffusion gradients Bystander effect as bacteria growth is in colony and they adhere to or invade tumor cells Huge genome allows to accommodate a variety of exogenous therapeutic genes Bacteria can be killed with antibiotics if complications arise	Plasmid transfection efficiency Loss of the plasmid encoding the enzyme Low prodrug/drug accessibility due to bacteria cell wall	[119]
		Active enzyme targeting		
Antibody -directed enzyme therapy	The enzyme is conjugated to an antibody that targets the enzyme at the desired place by interaction with antigens expressed onto the target cell.	Antigens should be expressed only at the targeted site or expressed only at low concentrations in normal tissues. Drugs are generated extracellularly and diffuse inside the cell.	Immunogenicity reaction	[89,115]
Polymer directed enzyme therapy	The enzyme and the prodrug are conjugated to a polymer	Reduced plasma residence time No immunogenicity. Low systemic toxicity	Reduced activity due to polymer conjugation	[118]
Substrate mediated enzyme prodrug therapy	The enzyme is immobilized on a biocompatible material (*i.e.* hydrogel)	Flexibility of delivering multiple drugs Changeable dosage Site-specific	Critically dependent on the substrate, the enzyme, and the prodrug	[123,124]

their circulation half-life by decreasing renal and/or immune system clearance, *iii)* improving their retention and accumulation within the targeted tumor taking advantage of the enhanced permeability and retention (EPR) effect related to the anatomical and pathophysiological differences from normal tissues, and *iv)* providing a cover shell that serves as a surface for anchoring active targeting agents improving selectivity and/or internalization of the therapeutic enzyme [118]. However, off-target activation is a limitation when using polymers or other nanomaterials as enzyme carriers. Indeed, liver and/or spleen accumulation is a predominant problem of therapeutic nanocarriers due to fenestration in endothelial cells and high blood flow of these organs which is part of the natural mechanism used by our body to remove foreign material in a way similar to how the EPR effect works [128,129].

From all that has been previously said regarding EPT, further development in the technology is necessary to achieve better specificity in the accumulation of the enzyme at the site of interest, or in turn the spatiotemporal control of the pro-drug biotransformation. The latter strategy can be achieved by programming a response by a nanocarrier or the enzyme itself triggered either by conditions naturally present at the area of interest (internal stimulus) or by an external stimulus applied remotely [36,118,130–133]. As elaborated in a subsequent section (Section 4.2 Targeted Delivery), the incorporation of nanomaterials may serve to address these challenges effectively.

3.3. Enzyme starving therapy

Enzyme Starving Therapy (EST) is emerging as a promising strategy for the development of new therapeutic agents against cancer. This therapy is based on differences in the metabolism of normal and cancer cells. Indeed, the latter exhibit an altered metabolism rate to ensure increased biomass and energy production and to maintain redox homeostasis [134,135]. EST is therefore based on the use of enzymes capable of metabolizing nutrients such as glucose, amino acids and oxygen, depriving their supply to the tumor and inducing cell death by activating apoptotic pathways.

Over the past 50 years, several enzymes have been selected for the development of this therapy (Table 3). Their activity act on well-known metabolic pathways of cancer, such as glucose consumption, protein synthesis and oxygen deprivation. In this sense, a hallmark of cancer cells is the so-called Warburg effect, defined as an increased glucose uptake and anaerobic fermentation production of energy rather than *via* respiratory metabolism [136]. Glucose concentration is therefore key to the energy supply and metabolism of the tumor. Thus, this molecule is considered an excellent target for cancer cell starvation and subsequent tumor reduction. Glucose oxidase has been studied for the starvation of tumor cells by intratumoral glucose consumption. It catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide (H_2O_2) in the presence of O_2. On one hand, it reduces the glucose concentration and on the other hand, induces ROS production that can cause cancer cell apoptosis if produced at high concentrations [137,138].

Amino acid metabolism is dysregulated in many tumor tissues, in particular, some of these show a lack of gene expression of proteins required for the synthesis of asparagine, arginine, and methionine. In this context, EST has focused on the depletion of these exogenous amino acids. In the late 1970s, the FDA approved the use of asparaginase for the treatment of lymphoblastic leukemia. This tumor shows a deficit in the synthesis of asparagine due to reduced or no expression of asparagine synthetase, which converts aspartate to asparagine [134,139], being the exogenous one the only source of asparagine for tumoral cells. In this case, treatment with bacterial asparaginase is effective, as the complete depletion of exogenous arginine prevents the tumor survival. The use of this enzyme for cancer therapy has been established for >40 years, with considerable progress being made in the formulation of the enzyme's administration [140]. Other amino acid related enzyme therapies are already in clinical trials such as arginine deiminase formulations and methioninase, which also lead to cell death by modification in DNA methylation, protein homeostasis, and apoptosis [141].

Although very promising, ESTs are often associated with acute toxicities, and in rare cases with severe pancreatitis, thrombosis, or allergic reactions. Furthermore, under this metabolizing pressure of starvation, tumoral cells enter in autophagy. It has been described that autophagy has a cytoprotective role as it provides metabolic plasticity. This allows the tumor to benefit from additional nutrients from the catabolic metabolism leading to tumor growth. Another limitation is the insurgence of drug resistance caused by the overexpression of enzymes involved in the synthetic route of amino acids [142–144]. Thus, the therapeutic effect of EST alone is limited, needing the combination of other therapies such as autophagy regulators or chemotherapy for

Table 3
Enzymes, mode of action, and clinical stage of developed enzyme starvation (EST) therapies.

Target	Enzyme	Mode of action	Pathology	Stage	Reference
Arginine	Arginine deiminase (EC 3.5.3.6)	Catabolizes the guanidino deamination of L-arginine (L-arg) to L-citrulline (L-cit) and ammonia. Starvation of cancer cells that lack of activity of urea-cycle enzymes.	Hepatocellular carcinoma, melanomas, pleural mesothelioma, non-Hodgkin's lymphoma, high-grade gliomas, breast cancer, lung cancer.	ADI-PEG20 in Clinical trials stage I and II	[134,135,142,149,150]
Arginine	Arginase (EC 3.5.3.1)	Catalyzes the conversion of L-arginine to l-ornithine and urea leading to cancer cells autophagy, cell cycle arrest and apoptosis.	Melanoma, Prostate Adenocarcinoma, Sarcomas, Pediatric Solid Tumor, Pediatric AML, Pediatric ALL, Hepatocellular Carcinoma	phase I and II clinical trials	
Asparagine	L-Asparaginase (EC 3.5.1.1)	Starvation of cancer cell that lack of L-asparagine synthetase by conversion of exogenous L-asparagine	Acute lymphocytic leukemia (ALL), Pancreatic cancer, ovarian cancer, adenocarcinome	Approved for ALL.	[134,139,142,151]
Glucose	Glucose oxidase (EC 1.1.3.4)	Catalyze glucose oxidation and produce gluconic acid and H2O2 in the presence of oxygen inducing hypoxia, acidity, and ROS in tumor environment.	Breast cancer, colon cancer	N.A.	[152–154]
Methionine	Methionase (EC 4.4.1.11)	L-methionine has role in DNA methylation, polyamine synthesis, methylation reactions, cytoprotection, mammalian protein synthesis, antioxidative stress defense, synthesis of vitamins and antioxidants controlled gene expression. Converts methionine into ammonia, α-ketobutyrate, and methanethiol. It is depleting the methionine in methionine-dependent cancer.	Colon carcinoma, neuroblastoma, glioblastoma, lymphoma, breast, lung and renal cancer	phase I clinical trials	[134,142,151,155]

effectiveness [138–140]. These various therapeutic elements can be effectively combined within a nanomaterial serving as a carrier for therapeutic enzymes, cytotoxic drugs, and/or autophagy inhibitors. Furthermore, this nanomaterial can be further functionalized with antibodies or other biomolecules to enable targeted therapy towards specific cells. This approach not only safeguards the enzymes from degradation but also enhances efficiency by integrating various therapies into a single nanotherapeutic [145–148].

3.4. Enzyme dynamic therapy

Enzyme dynamic therapy (EDT) relies on the administration of endogenous-like enzymes (*e.g.,* catalase [156], collagenase [157], caspase [158], uricase [159], ribonuclease [160]) to break down or transform pathology associated-molecules.

The first EDT strategy developed was based on the modulation of reactive oxygen species (ROS) in tumoral regions. ROS encompass a broad class of reactive ions and radicals, including hydrogen peroxide (H_2O_2), superoxide radicals (*e.g.,* superoxide anion •O2–), hydroxyl radicals (•OH), and singlet oxygen ($^1O2$), that play roles in various physiological processes. At low levels, ROS function as intracellular messengers, while at high levels, they regulate the fate of the cell, inducing apoptosis or necrosis *via* oxidation of several targets within the cells (proteins, lipids, DNA). Tumoral cells generally exhibit higher sensitivity to ROS compared to normal cells and rely on antioxidants to downregulate the high ROS concentration [161]. As for photodynamic therapy (PDT) and sonodynamic therapy (SDT), enzyme dynamic therapy uses O_2 to induce cell death. While the PDT and SDT rely on an external stimuli application, EDT produces ROS *via* biochemical enzymatic oxidation of different molecules. Induction of cytotoxic stress by biochemical ROS production has been evaluated for cancer treatment since the early 1980's, when Nathan and Cohn developed a therapy using glucose oxidase (GOX) as generator of H_2O_2 *in situ* using glucose as substrate. The concept was further extended to the use of other enzymes in 1995 by Yoshikawa and colleagues who studied the antitumoral effect of xanthine oxidase (XO) *via* the generation of ROS (O_2^- and H_2O_2) by oxidation of its substrate (hypoxanthine). Indeed, they observed a reduction in VX2 liver rabbit carcinoma tumoral mass when hypoxanthine was exogenously administered [162]. However, XO also induces systemic vascular damage and hypertension due to its high affinity to blood vessels. Different strategies have been explored to reduce XO's off-target activation, such as the PEGylation when it is exogenously administered [163] or the specific activation of endogenous XO present in the tumor cells [164]. Other researchers have explored instead the use of other oxidases for anti-tumoral therapeutics *via* ROS-generation. Indeed, inspired by the ROS-responsive cytotoxicity of neutrophils, which induce microbial cell death by the oxidation of halide ions (Cl^-) catalyzed by myeloperoxidase (MPO) in the presence of H_2O_2, the use of chloroperoxidase (CPO) from *Caldariomyces fumago* has been explored. As MPO, CPO catalyzes the production of hypochlorous acid (HOCl) which subsequently decompose to produce cytotoxic singlet oxygen ($^1O2$) from H_2O_2 but with higher resistance to oxidative inactivation compared to its neutrophil counterpart [165,166]. However, all these strategies for enhancing tumoral cell death by inducing increased ROS production in tumor tissue have limitations. First, they rely on natural substrates that are ubiquitous and present in the human body, leading to systemic toxicity and unwanted side reactions. Besides, their therapeutic efficiency is compromised due to the low stability of these enzymes in biological fluids and tumoral environment, and the presence of natural ROS scavenger agents (*e.g.,* reductants such as glutathione) [167]. Specifically, in the case of therapies relying on CPO, their efficacy is constrained by the utilization of endogenous H_2O_2 as a substrate, which is only available in limited concentrations of approximately 100 μM within tumors.

EDT extends beyond its role in promoting tumor cell death by boosting ROS levels. Its ability to interact with a wide range of endogenous biomolecules makes it a versatile therapeutic approach for combating a broad spectrum of diseases. For instance, cystic fibrosis (CF) is among the conditions that can benefit from EDT [164,165]. In *CF*, the high viscoelasticity of mucus is linked to the release of DNA from neutrophils responding to chronic infections typical of this disease. DNA-rich mucus blocks the action of aminoglycoside antibiotics commonly used to treat lung infections, reducing their effectiveness. In 1993, the FDA approved the enzymatic drug dornase alfa (Pulmozyme) for its treatment. This enzyme selectively degrades the DNA present in the sputum and bronchial mucus of cystic fibrosis patients and reduces its viscosity in the lung [168]. Although Pulmozyme ameliorated the outcome of the treated patients, it still induced antibody production in 5% of them. Furthermore, protein aggregation occurs after inhalation [169,170].

Another example of EDT involves ocriplasmin, a modified recombinant variant of plasmin. It received approval from the FDA and EMA in 2012 and 2013, respectively, for treating vitreomacular adhesion. Ocriplasmin catalyzes the breakdown of the extracellular matrix resulting from abnormal cell proliferation within the epiretinal membrane [171,172]. Although this therapy resolved vitreomacular traction and closed macular holes, the treatment presents side effects like eye pain, photopsia and retinopathy [173]. Besides, ocriplasmin suffers of unfolding and concentration-dependent autolysis that reduce its pharmacological effects [174].

A similar approach has been approved in chronic wounds and burns with the degradation of the devitalized tissue by collagenase from Clostridium. Commercialized as Collagenase Santyl Ointment, it acts by selectively digesting collagen debris [175], promoting cell migration of fibroblast and keratinocytes and thus promoting regenerative adult wound healing [176]. Another enzyme approved for burn healing treatment is bromelain, commercialized as NexoBrid and Debrase [177]. It is a plant-derived mixture of enzymes capable of hydrolyzing the fibrin clot and degrade extracellular matrix components. As for collagenase, the debris production induces a cytokine cascade and the release of angiogenic and growth factors, which promote cellular proliferation.

In addition to degrading endogenous biomolecules, EDT can be used to metabolize drugs that can cause unwanted side reactions or toxicity [178]. For example, glucarpidase (also known as carboxypeptidase-G2), commercialized as Voraxaze, is used to reduce plasma levels of methotrexate (MTX), a drug used as an anticancer and anti-inflammatory in various malignancies and autoimmune diseases. High levels of methotrexate can induce encephalopathy and nephrotoxicity thus this therapeutic enzyme is currently approved for reversing MTX toxicity in patients with delayed MTX clearance resulting from renal dysfunction [179].

The significant variations in the 3D complexity and localization of target biomolecules give rise to distinct limitations in different applications of EDT within specific therapies. Nevertheless, it can be summarized from the preceding discussion that there exist common limitations also shared with other enzymatic therapies besides EDT, such as: i) off-target activation, leading to systemic toxicity; ii) rapid enzyme clearance, resulting in a low percentage of accumulation at the intended target sites; iii) diminished enzyme stability at the site of action, due to unfolding, aggregation, or protease cleavage; iv) initiation of immunological responses upon repeated drug administration; v) the need to achieve synergistic effects in combination with other therapies. In this context, nanomaterials offer a myriad of advantages for improving the therapeutic efficacy of EDT. For instance, it has been recently showed that the co-entrapment of CPO enzyme in a self-assembled hybrid nanogel allowed its protection and the possibility to increase the peroxide concentration in the tumor by co-immobilization with the superoxide dismutase (SOD) enzyme [180]. The obtained nanogels mimicked the membrane-bound lysosomes of neutrophils, enabling them to overcome the limitations already explained for the use of CPO as antitumor therapy. In fact, it has been observed that the co-encapsulation of SOD and COP enabled to increase of the local

concentration of the H_2O_2 over the threshold of the vulnerable tumor while same time allow to stabilize the protein [177].

4. Role of nanotechnology in enzyme therapy

Nanomaterials are a class of materials that fulfil the requirement of involving at least one of its dimensions in the size range between 1 and 100 nm [181]. The increasing awareness of nanomaterials in biomedicine is mostly due to their remarkable optical, magnetic, electrical, mechanical and catalytic properties, which are substantially different from their bulk counterparts. For example, nanomaterials exhibit lower melting points, unique surface plasmon resonance (SPR), and superparamagnetism [182]. These unique characteristics arise because of the large surface area and the confinement (quantum) effect due to their nanoscale size [183]. A major advantage of these materials is that these properties can be tuned on demand to suit any therapeutic outcome *via* the control of their size, shape, synthesis conditions and functionalization (Fig. 3).

In this context, researchers have engineered various nano-enzymatic systems, capitalizing on the unique attributes that nanoscale materials offer. Table 4 provides a comprehensive overview of the most frequently used nanomaterials in conjunction with enzymes, offering insights into the advantages and disadvantages of each nanomaterial. While metal nanomaterials have typically been explored to achieve precise spatio-temporal control over enzymatic activity, silica nanoparticles have been preferred for their ease of synthesis, scalability, and good biocompatibility. Conversely, polymeric and lipid-based nanostructures facilitate controlled release triggered by endogenous stimuli while also ensuring enzyme protection.

Although each specific material presents advantages and disadvantages, as summarized in Table 4, they could be used for the development of versatile and multifunctional platforms for transporting enzymes. Even some examples of enzyme therapy applications are based on combining these materials to further enhance the versatility and multifunctionality of these platforms. They accomplish this by simultaneously safeguarding them from degradation while enabling precise spatio-temporal control of their activity, thereby mitigating systemic toxicity. Besides, they can be engineered to accommodate the co-delivery of other therapeutics, multiple enzymes, or even imaging agents, thus amplifying treatment precision and efficiency. In sum, the integration of nanomaterials into enzyme therapy not only tackles existing limitations but also opens new possibilities for the development of innovative and highly effective therapeutic strategies. All this translates not only into a greater efficiency of the therapy, but also into a weakening of side effects, and better patient compliance. Fig. 4 summarizes the advantages offered by integrating nanomaterials into the development of enzyme therapies. Subsequently, a more detailed description of each of these advantages is provided.

4.1. Nanomaterials for enzyme stabilization

The three-dimensional structure of enzymes is what endows them with biological activity but also makes them sensitive to the action of proteases and external physical factors such as heat, pH, ionic strength, and solvent polarity. These factors have a detrimental effect, often irreversible, on the enzyme activity as they trigger extensive conformal changes in its structure. In this sense, the use of nanocarriers for enzyme vectorization encompasses their enhanced stability not only against detrimental physical factors present at the target site (*e.g.,* low pH at the tumoral stroma) but also against proteases present in blood, tissues, or cell compartments (*e.g.,* lysosomes). Hence, for any enzyme-based therapy, the enzyme must maintain its activity for the entire duration of the treatment. This presents an important challenge, as discussed in Section 2 *(2_Instability* and *3_Clearance* challenges*)*, when using free enzymes as therapeutics.

Although, the stabilization of enzymes by binding or encapsulation in nanomaterials is a well-reported fact for their use in biotechnological applications of industrial interest [221], it has also been reported for their use in therapeutic applications. For instance, a significant improvement on the use of entrapped over soluble collagenase for the treatment of fibrosis has been recently reported. Fibrosis is a common lesion in different pathologic diseases (*e.g.,* palmar fibromatosis, Dupuyten disease, or Peyronie disease among others) characterized by the excessive accumulation of collagen. The use of collagenase has been approved for its treatment by FDA and EMA. It has been shown that the entrapment of collagenase within polymeric nanocapsules resulted in a gradual release of the collagenase up to ten days after its injection with a retention of >50 % of the catalytic capacity. This represents a substantial enhancement when contrasted with the use of the unbound (free) enzyme, which experienced a decline of over 80 % in its activity within just one day following injection. [157].

Another example to be pointed out for stabilization of a therapeutic enzyme through encapsulation is the enzyme β-glucuronidase, which is a lysosomal enzyme that catalyzes the decomposition of β-D-glucoronides. The deficiency of this enzyme leads to Mucopolysaccharidosis type VII and is related, among others, to inflammatory diseases and neoplasms. This is due to a reduced breakdown of three glucuronic acid-containing glycosaminoglycans (GAGs) - dermatan sulfate, chondroitin sulfate, and heparan sulfate. As these GAGs progressively accumulate in various tissues, they cause systemic tissue and organ dysfunction, affecting the heart, airways, lungs, liver, spleen, brain, and bones. Thus, its exogenous administration is approved for ERT to catalyze the stepwise degradation of these GAGs [222]. Moreover, β-glucuronidase can be used for EPT as it is able to metabolize the innocuous SN-38-

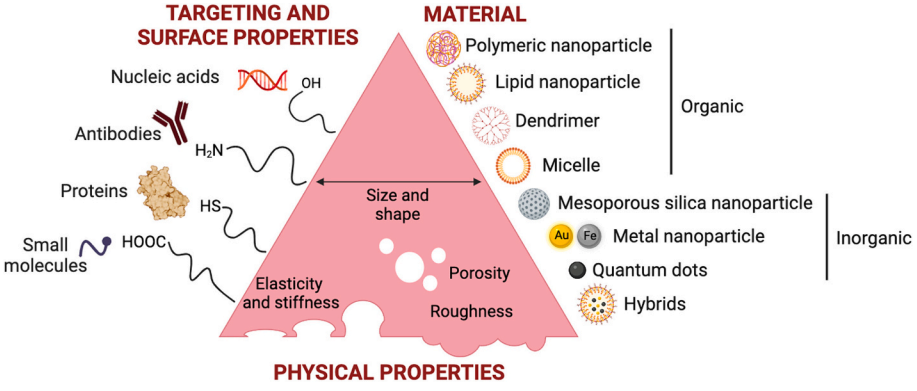

Fig. 3. Schematic representation of the physical and chemical properties of nanoparticles. Nanoparticles exhibit tunable characteristics which may be designed based on a desired application. The therapeutic outcome is strongly affected by the chemical composition, the physical properties, the functionalization, and its derived surface characteristics. Figure created with Biorender.com.

Table 4
Main nanomaterials used for enzyme therapy.

Nanomaterial		Therapeutic enzyme	Advantages	Disadvantages	References
Metal Nanoparticles	Gold nanoparticles (AuNPs)	Lipase	Colloidal stability; Easy surface functionalization; Remote activation through laser irradiation; Biocompatibility; Contrast agents for computed tomography, nuclear and photoacoustic imaging	Poor laser body penetration Non-biodegradability	[133,184–191]
	Silver nanoparticles (AgNPs)	D-amino acid oxidase (DAAO)	Antibacterial properties	Difficult synthesis Reduced stability over time Possible toxicity Non-biodegradability	
	Magnetic nanoparticles (MNPs) Fe_3O_4 and γ-Fe_2O_3, $CoPt_3$, FeP, $MgFe_2O_4$, $MnFe_2O_4$, and $CoFe_2O_4$	β-Galactosidase	High biocompatibility; Low toxicity; Remote activation by external magnetic field; Ability to generate localized heat under AMF; Contrast agents for Magnetic Resonance Imaging	Difficult surface functionalization Reduced colloidal stability	
Silica nanoparticles	Non-porous	β-Galactosidase	Ease of synthesis; Structurally tunable properties, size &shape	Diffusional issues; Long-term exposure effects need to be defined; Large-scale manufacturing of surfactant-free NPs need to be developed	[192–196]
	Porous (micro/ mesoporous)	Glucose oxidase (GOX) Horseradish peroxidase (HRP) Superoxide dismutase (SOD) Glutathione peroxidase (GPx)	Good biosafety profile Biodegradability Good hydrothermal stability High capacity for therapeutic/diagnostic agent loading. Ease of surface functionalization		
Polymeric nanomaterials	Micelles, nanocapsules, nanogels, nanofibers, dendrimers and nanocomposites	β-galactosidase Alginate lyase Catalase Galactosylceramidase	Controlled release High biocompatibility Prolonged blood circulation Inherent targeting ability with some polymers as constituents	Possible toxicity depending on constituents	[197–201]
Lipid based nanomaterials	Liposomes	Papain	Biocompatibility	Destabilization by blood lipoproteins Complex functionalization Possible synthesis-dependent cytotoxicity Cargo degradation by lysosomes Potential immune system activation Possible hepato and pulmonary toxicity	[202–207]
	Solid lipid nanoparticles (SLNs)	Catalase D-amino acid oxidase	High physical stability, Controlled release; Good biocompatibility Good elastic moduli		
Carbon-based nanosystems	Carbon nanotubes (CNT)	Catalase (CAT) Apyrase	High stability Enlarged surface area	Low colloidal stability in biological fluids Possibility of macrophage activation and inflammatory response; Low biodegradability	[208–215]
	Graphene	5'-nucleotidase α-L-iduronidase	Ease of surface functionalization; Extremely large surface area Versatility in hydrophobic and hydrophilic encapsulation; Thermal stability		
	Carbon quantum dots (CQD)	α-L-iduronidase	Ease of surface functionalization; Extremely large surface area; Biodegradability; Thermal stability; Photoluminescence	Complex synthesis; Possible toxicity; Low colloidal stability in biological fluids	
Nanoclays	Halloysite clay nanotubes	Lipase Laccase	High specific surface Tunable surface chemistry	High variable structures	[216,217]
	Kaolinite	Glucose oxidase (GOX)	Thermal resistance; Biocompatibility; High specific surface; Non-toxic nature	Slow release of the cargo	
Nanosized organic frameworks	Hidrogen bonded organic frameworks	Catalase	Preferential localization, Synergistic catalysis; Photocatalytic Activity; Neuroprotective effect	Desing complexity; Weak bonds in HOFs limit isostructural framework formation	[218–220]
	Metal bonded organic frameworks	Horseradish peroxidase (HRP)	Smart enzyme -based platform	Moderate e toxicity or side effects	
		Glucose oxidase (GOX)	Enhanced activity; Operational stability; Allow cascade catalytic reactions; Good pharmacokinetics		

glucuronide into the antiproliferative drug SN-38. Similarly, β-glucuronidase loaded in liposomes within electrospun fibers of poly(vinyl alcohol) displayed a sustained pro-drug bioconverting activity over at least seven weeks, thus showing excellent potential as implantable material for *in situ* drug generation. The activity of the soluble enzyme was, otherwise, lost due to deactivation within 48 h of incubation in cell culture media [223].

A similar effect on the stability of L-asparaginase upon immobilization was also recently reported. This enzyme is a keystone enzyme in starving therapy (EST) as mentioned in the previous section and is widely used in the treatment of lymphoblastic leukemias and lymphosarcomas. Tumor cells require high amounts of L-asparagine for their malignant progression but do not have L-asparagine synthetase for its synthesis. As a consequence, they depend on the plasma levels of L-asparagine. L-asparaginase immobilized in both aluminium oxide nanoparticles and titanium oxide nanoparticles have recently displayed a broader working pH and temperature range for its anti-neoplastic activity against leukemia cell lines along with enhanced reusability and storage stability [224].

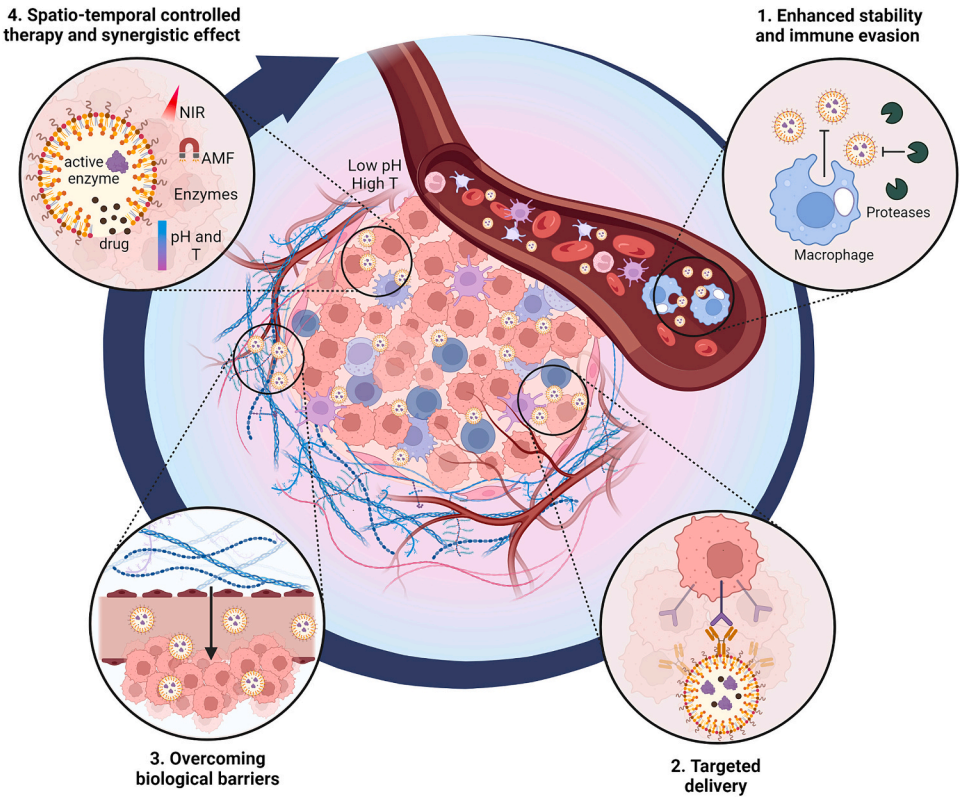

Fig. 4. Schematic representation of the advantages derived from the use of nanomaterials in enzyme therapy. *Enhanced stability and immune evasion*. The encapsulation of the exogenous enzyme and the functionalization of its nanocarrier can prevent it from becoming an immunogenic neoantigen and thus triggering an immune response. Moreover, the encapsulation leads to enhanced stability against degradation by proteases during delivery. *Target delivery and overcoming biological barriers.* Nanocarriers offer advantages for overcoming biological barriers. Their improved accumulation in the target site can be achieved by passive *(e.g.,* EPR effect) and/or active (*e.g.,* by their functionalization with targeting molecules/biomolecules) targeting. *Spatio-temporal controlled therapy*. Nanomaterials can be tailored to respond to endogenous and/or exogenous stimuli (*e.g.,* pH, temperature, proteases, magnetic fields, light…) in order to trigger activation or release of the enzyme. *Synergistic therapy.* Nanocarriers display a high cargo capacity allowing not only to bind the enzyme of interest but also other molecules, such as drugs isolated or in combination, to look for synergistic effects in the development of combination therapies. Figure created with Biorender.com.

4.2. Nanomaterials for immune evasión

As also mentioned in Section 2 (*4_Immunogenicity* challenge), the use of recombinant enzymes in enzyme therapy has limited therapeutic benefit when repeated therapy cycles are needed. In this sense, encapsulation of the enzyme of interest within nanocarriers could not only shield the enzyme from being enzymatically degraded or denatured by external factors but also avoids an immune reaction caused by its recombinant origin [225,226]. However, it is important to note that nanocarriers could elicit an immune reaction by itself (*e.g.,* recognition by the mononuclear phagocyte system). Therefore, their surface must be engineered to prevent intrinsic immunogenicity and, thus, enhance their biocompatibility. Indeed, there are several strategies to achieve this. One example is the use of poly(ethylene glycol) (PEG), which introduces hydrophilicity on the NPs surface. This has resulted in an effective strategy for shielding nanoparticles from plasma proteins, thereby hindering them from immune recognition which results in extended blood circulation half-lives [227]. As an example, it has been shown that PEG-grafted chitosan NPs reduce the clinical limitations of the enzyme streptokinase (a thrombolytic agent) by increasing its blood circulation time from 15 to 30 min up to 120 min while also delaying an immune response for 20 days *in vivo* [228]. Likewise, PEG, polymers, natural polysaccharides, antibodies, lipids mimicking the cell membranes and peptides can also be used with the same aim [229].

4.3. Nanomaterials for targeted delivery

One of the biggest limitations of therapeutic enzymes relies on reaching a high concentration at the site of action (See Section 2, Poor distribution challenge). Despite the development of antibodies or virus as vectoring agents, there is still a need of more efficient systems that can deliver the therapeutic enzyme at the targeted organs and at the targeted cells. In this sense, binding or integrating enzymes into NPs could benefits from different passive and/or active targeting schemes.

Passive targeting schemes promote the accumulation of enzyme-nanocarriers without functionalizing them with targeting moieties. Nanomaterials tend to passively accumulate at sites of enhanced vascular permeability. This effect is commonly known as Enhanced Permeability and Retention (EPR) effect, and it is exploited in cancer for solid tumor treatment. The unique characteristics of tumor vasculature, fenestrated endothelium and defective lymphatic drainage allows nanoparticle accumulation. In this sense, Chen et al. [230] developed a nanoreactor to reduce hypoxia in tumor. The nanoreactor was synthetized *via* glutaraldehyde-mediated crosslinking of albumin molecules and catalase. The nanosystem, after being IV injected, was able to accumulate in the tumor and efficiently decomposed H_2O_2 to generate oxygen. Even though EPR is considered as a main mechanism to enhance nanoparticle accumulation in tumors, the EPR effect is considered controversial because it is a highly heterogeneous phenomenon. Indeed, it varies with the types and stages of tumors, individuals, and even locations within the same patient [231]. However, EPR is not exclusive to solid tumors. Increasing evidence is showing that an EPR-like process also occurs in other pathologies ranging from infection to even heart failure [232,233]. An example of the use of enzymes for the treatment of infections is the recent work on the use of PLGA-NPs containing pyruvate dehydrogenase as potential therapy for *Pseudomonas aeruginosa*

biofilm-associated infections [201]. The encapsulated enzyme induced biofilm bacteria to disperse and revert to an antibiotic-susceptible state by catalyzing the depletion of pyruvate. These results are promising thinking on the use of enzyme therapeutics for the treatment of persistent non-surface-related infections caused by bacterial aggregates or biofilms such as cystic fibrosis, otitis media, chronic wounds, chronic osteomyelitis, *etc.*

Current knowledge in active targeting demonstrates that functionalization is necessary to enter the target cell once the nanoparticles accumulate in the tumor [234,235]. Nanocarriers can reach the targeted site of action through EPR, however, this strategy does not guarantee intracellular accumulation or adequate localization within these cells. Hence, the functionalization of nanoparticles is crucial for enhancing therapy outcomes by promoting their internalization even when the nanoparticles are intratumorally injected. For some enzyme therapies, internalization of the enzyme carrier is not necessary to trigger the desired therapeutic effect. This is the case, for example, of the horseradish peroxidase as a prodrug-converting enzyme to trigger cell death by toxic oxidative species released upon 3-indole acetic (prodrug) bioconversion. Although the biological active intermediates and free radicals that induce apoptosis of target cells has a potent bystander effect, prodrug activation by HRP internalized by targeted cells ensures the selectivity of the therapy [236]. However, other enzymes must be delivered at a cellular or subcellular level to be able to perform their therapeutic role. This is the case of lumbrokinase [237] in thrombosis, which must reach the thrombus, or β-galactosidase A [238], which must reach the lysosomes to treat Fabry disease.

In this sense, *active targeting* schemes utilize ligands attached to the surface of the enzyme carrier, which can bind molecular structures or antigens that are overexpressed or preferentially present at the site of action (targeted cell or extracellular matrix) [23–25]. Ligand-targeted nanoparticles bind to cell surface receptors and may internalize by receptor-mediated endocytosis. Therefore, active targeting increases the uptake of nanoparticles and their cargo into the targeted cells. Unfortunately, little literature about active transport of these nanoreactors can still be found. Among the molecules used for active targeting biofunctionalization it is possible to find antibodies [239], affibodies [240], polysaccharides [237,241,242], peptides [204,238,243,244], lipophilic cations [245], small molecules [246,247] or even biomimetic membranes [248–251]. Clearly, more research is needed to improve the field of targeted enzyme-nanocarrier therapy. Recently, Lou et al. [252] have opened up an exciting landscape of proteins called interferon-induced transmembrane proteins (IFITMs) with ubiquitous presence in cells and mediated in the uptake pathway of large bitops molecules. These are ligands formed by two distinct molecular features linked together to selectively bind at the same time to the allosteric and to the orthosteric binding site of a receptor [253,254]. This new approach opens a new range of possibilities for targeted therapy. Although still unexplored with nanocarriers, this combination could allow the development of an effective targeting system for complex biological barriers, such as BBB.

Despite the fact, as described, the majority of enzyme-based nanoreactors for therapy that have been reported so far rely solely on the EPR effect to transport the enzyme of interest integrated within nanodevices, the use of physical (*e.g.,* ultrasound and hyperthermia) and enzymatic (*e.g.,* collagenase and hyaluronidase) strategies to modify tumor accessibility *via* extracellular matrix disruption are gaining interest [255]. Indeed, extracellular matrix (ECM) accumulation is crucial for tumor resistance, with hyaluronic acid (HA) being a key ECM component of solid tumors. Therefore, the matrix-degrading Hyaluronidase (Hase) enzyme has emerged as a promising therapeutic adjuvant in systemic cancer therapy. In this line, Smith et al. [256] showed that co-loading chitosan nanoparticles with the anticancer drug 5-fluorouracil and hyaluronidase improved the penetration, distribution, and the cellular uptake of the developed nanotherapeutic using spheroids as 3D culture system. Therefore, in addition to playing a direct therapeutic role, the use of enzymes can also offer an adjuvant role in improving the distribution and penetrability of other therapies, whether they are based on the use of nanocarriers or not.

4.4. Nanomaterials for overcoming biological barriers

As mentioned before, physicochemical instability, susceptibility to enzymatic degradation and immunogenicity are intrinsic disadvantages of enzymes. As already described in Section 2 (*1_Poor biodistribution* challenge), apart from these concerns, enzymes encounter multiple biological barriers (BBs) sequentially before they can reach their target site [233]. An example of how the integration of nanomaterials could help to overcome these barriers is on the use of the recombinant enzyme iduronate 2-sulfatase (IDS) for the ERT based treatment of Hunter Syndrome or Mucopolysaccharidosis II, which is a well-known lysosomal storage disease. IV of IDS is not effective when MPS II affects the brain, which happens in 75 % of the cases, because the recombinant lysosomal enzyme exogenously administered is not able to cross the BBB. In this context, nanocarriers may offer a solution to overcoming this challenging barrier. Their small size allows them to effectively diffuse through various biological environments, and their extensive surface area makes it easier to attach various biomolecules that could grant them the capacity to cross diverse biological barriers. Indeed, it has been shown that PLGA nanoparticles functionalized with a glycopeptide of 7 amino acids (g7) for CNS targeting and loaded with the recombinant lysosomal enzyme IDS can cross the BBB. These IDS-PLGA targeted nanocarriers allowed the reduction of the deposits of glycosaminoglycans at the brain to non-pathological levels both *in vitro* and *in vivo* along with a decrease on the level of neurological damage markers [257].

Another example where nanotechnology aided to cross BBs is the use of enzymes for the treatment of skin conditions. The topical use of the soluble forms of diamine oxidase (DAO) and catalase (CAT) for the treatment of skin allergies is an unviable therapeutic option as the outer layer of the epidermis (stratum corneum) impairs the absorption of enzymes. DAO is the main histamine degrading enzyme and thus its intestine delivery *via* oral administration in combination with enzymes having antioxidant properties (such as CAT), is the common therapeutic approach for the systemic treatment of allergies (*e.g.,* histamine intolerance). Recently, the encapsulation of both enzymes within chitosan NPs have been explored for its potential to reach deeper tissues (skin translocation) and thus be able to explore their topical administration. Chitosan nanoformulations were selected by Leoninda et al. [258] due to their small size and natural muco-adhesiveness that has already been reported to increase the efficiency of skin translocation of other active ingredients. Indeed, the authors reported that the obtained NPs offered protection and increased the bioavailability of both enzymes, thus showing their potential as vehicles for the topical treatment of skin disorders caused by “histamine intolerance” and other inflammatory processes affecting the skin.

As previously stated, once the therapeutic enzyme reaches the target organ, it faces additional challenges to ensure its homogenous penetration within the targeted site. In this sense, tumor tissue displays several challenges including high interstitial pressure, acidic microenvironment, or dense packing of the ECM. In order to overcome this BB, Dai et al. [259] designed a pH and ROS responsive manganese-MOF nanoreactor loaded with glucose oxidase (GOx) and an immune suppressor. Upon reaching the tumor microenvironment, the acidic pH facilitated the reduction in size and reversal of charge of the developed nanosystem, facilitating its tumor penetration and internalization. Besides, the nanosystem also faces the challenge of enhancing cargo bioavailability as it promptly breaks down and releases its cargoes in response to intracellular reactive oxygen species (ROS). Indeed, effectively addressing these biological barriers enables this nanoreactor to achieve enhanced tumor-killing effectiveness through a combination of starvation therapy and immune modulation effects. *Ad-hoc* design of nanomaterials could not only enhance the penetrability of therapeutic

enzymes within the tumor but also facilitate their internalization. Polymeric nanoparticles or liposomes, coated with surfactants such as polysorbate 80 or poloxamer 188, offer a solution to overcome this limitation. The surfactants induce the recruiting of endogenous apolipoproteins from the bloodstream and facilitate transcytosis mediated by LDL receptors present on the apical surface of brain capillary endothelial cells [260–262]. Furthermore, these NP surfaces can be easily customized with targeting peptides (*e.g.,* cyclic transferrin-targeting peptide, apolipoprotein E peptide, and angiopep-2) or antibodies. This enhances the uptake of the enzyme-encoding genes or enzymes in the brain while reducing the nonspecific distribution in the liver or other organs. Del Grosso and colleagues demonstrated the possibility of restoring galactosylceramidase activity in the brain to healthy levels by functionalizing poly-(lactide-*co*-glycolide) (PLGA) nanoparticles with three different peptides (Ang2, g7 or Tf2) [204].

As previously discussed in Section 2 (*Cellular level BBs)*, when it comes to enzyme therapies that require biological activity to take place in cellular compartments other than endosomes/lysosomes, a challenge lies in the capability to evade the endocytic pathway. This pathway is typically the standard route for the internalization of nanomaterials and biomolecules associated with or enclosed within them. In this sense, several cationic nanoparticles for enzyme therapy have been described in the literature that successfully achieves endosomal escape by exploiting the proton sponge effect. Briefly, under acidic conditions some cationic molecules can sequester protons, keeping the proton bump going which in turn leads to the retention of chlorine ions and water for each proton that enters the lysosome. Eventually, this process causes lysosomal swelling and rupture, leading to particle release in the cytoplasm [263]. Yang et al. [264] incorporated ferrocene (Fc) into polymeric nanoparticles loaded with glucose oxidase (GOx). Besides initiating cancer starvation therapy, GOx raises H_2O_2 levels above their natural, endogenous levels. This elevation in H_2O_2 intensifies the rate of radical ·OH generation *via* the Fenton reaction, which is catalyzed by ferrocene (Fc). In addition to its cytotoxic role, ·OH plays a crucial role in facilitating the escape of lysosomes by breaking down the endosomal membrane. Simultaneously, the oxidation of Fc to Fc^+ during ·OH production triggers the formation of cationic nanoparticles. These nanoparticles disrupt the integrity of the lysosomal membrane through a proton sponge effect, working in tandem with the membrane-disrupting properties of ·OH. Also recently, Ren et al. [265] introduced an innovative nanomotor designed to take advantage of the elevated levels of hydrogen peroxide within tumors for self-propulsion. This propulsion is induced by the oxygen gradient generated during hydrogen peroxide decomposition by catalase (CAT). The core of this nanosystem consisted of calcium carbonate nanoparticles (NPs) coated with the positively charged polymer polyethyleneimine (PEI) and CAT, achieved through a layer-by-layer self-assembly process. This autonomous motion not only promoted substantial uptake of the nanomotors by tumor cells *via* active-targeted mediated endocytosis but also facilitated their escape from endosomes. This escape was achieved in collaboration with the proton sponge effect induced by PEI and the release of CO_2 resulting from the degradation of $CaCO_3$ nanoparticles. This enabled paclitaxel (PTX) and siRNA to efficiently reach their intracellular targets, ultimately leading to a highly effective induction of tumor cell apoptosis, both *in vitro* and *in vivo*. As in the previous examples where positively charged molecules were used to induce the proton sponge effect, Du et al. [266] reported a multifunctional mesoporous silica nanoparticle. In this case, the NP was coated with poly (L-lysine) as an alternative positively charged polymer instead of PEI. This positively charged NPs was used for the codelivery of GO and the anticancer drug paclitaxel with successful endosomal scape.

4.5. Nanomaterials for spatio-temporal control of enzyme therapy

Another significant benefit of employing nanomaterials lies in the potential for creating stimuli-responsive nanodevices. These devices can respond to internal cues such as temperature, pH, redox states, or enzymatic activity [267] as well as external triggers like magnetism, light, ultrasound, and electricity [268] (Fig. 5). These responsive nanodevices offer the advantage of spatial and/or temporal control over enzyme activity or the precise release of enzymes at targeted locations. This relatively new field of research aims to develop nanotherapeutics that can respond to internal and/or external factors, thus surpassing the limitations of traditional static designs in nanomedicine. Although the development of such smart nanotherapeutics for site-specific drug release is widely reported [268,269], there are still few examples of their use in the spatiotemporal control of enzyme-based therapies. However, the examples found in the literature illustrate that the creation of stimuli-responsive enzyme-based nanoreactors in their design has the potential to enable synergistic, multimodal, and more precise therapies.

Endogenous stimuli-responsive enzyme nanotherapies have been designed to respond to internal biological stimuli such as pH, specific redox conditions or even temperature changes. As a result, these nanomaterials can effectively adapt and react to the internal biological environment, with pH being the most commonly employed endogenous stimulus. An example is the polymersome nanoreactor developed by Mukerabigwi et al. [23]. It encapsulates GOx, camptothecin, and paclitaxel prodrugs (ProCPT and ProPTX). The prodrugs are linked to self-assembled diblock copolymers forming the polymersome through an H_2O_2-responsive self-immolation linker, rendering them inactive. A pH-sensitive segment within the copolymer enhances permeability to small molecules under acidic pH conditions. During circulation and in normal tissues, *co*-loaded GOx and prodrugs in the polymersomes remain inactive. Upon reaching the tumor site with lower pH (approximately 6.5-6.8), the nanoreactor's membrane becomes more permeable. This allows glucose and oxygen to diffuse across the membrane, facilitating their reaction catalyzed by GOx located in the membranes and cavities of polymersomes. As a result, *in-situ* generated H_2O_2 by upstream GOx converts H_2O_2-responsive prodrugs into active drugs by cleaving the caging groups. This stimuli-responsive nanoreactor demonstrated effective therapy both *in vitro* and *in vivo*. It addresses several limitations in enzyme prodrug therapy, including variations in pharmacokinetics between nanoreactors and prodrugs and the risk of off-target prodrug activation causing unintended toxicity in normal tissues (Fig. 6). Based on the same concept, but using sodium polystyrene sulfonate (PSS) and polycation poly(allylamine hydrochloride) (PAH) as pH sensitive nanomaterials, Cheng et al. [24] were able to develop pH-responsive hollow mesoporous silica nanoparticles loaded with doxorubicin and GOx. Furthermore, Wang et al., [270] developed a nanosystem that responded to the acidic pH generated by a bacterial infection during wound healing. The nanosystems were based on Cu_2O/Pt nanozyme covered by GOx and layered with calcium phosphate. Calcium phosphate serves as an effective nanocarrier for pH-responsive delivery due to its ability to dissolve under acidic conditions. The acidic environment in infected wounds triggered the structural breakdown of the system, which resulted in the release of the GOx and Cu_2O/Pt nanozyme.

Other enzyme-based nanosystems have been designed to react to intracellular ion concentrations or metabolites. For instance, Peng et al. [247,271], engineered the metal-organic framework (MOF) MIL-101 (Fe), sensitive to high phosphate concentrations. MOFs are created by combining metal ions and organic ligands. In this case, iron (Fe) was chosen as the divalent transition metal because it can convert endogenous hydrogen peroxide (H_2O_2) into highly toxic hydroxyl radicals (·OH) *via* the Fenton reaction. To enhance the production of H_2O_2, glucose oxidase (GOx) was incorporated into these MOFs, overcoming low endogenous H_2O_2 levels and inducing a starvation therapy effect by depleting glucose. To target tumor cells selectively, GOx-MOF hybrids were decorated with folic acid for active targeting of cancer cells with overexpressed folate receptors (FA). Recognizing mitochondria's vulnerability to ROS, triphenylphosphonium (TPP) was used to functionalize the MOFs, enabling mitochondria-targeting capabilities. As a

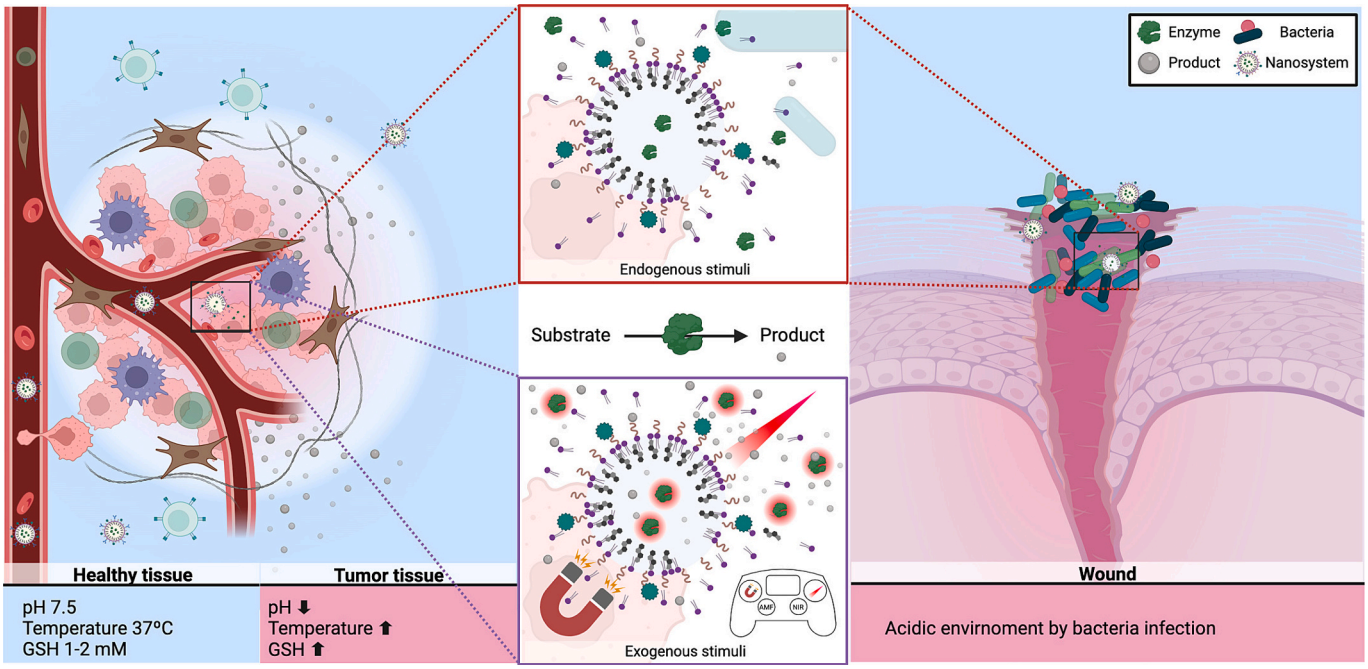

Fig. 5. Scheme of endogenous and exogenous stimuli for controlled release and activation of therapeutic enzymes conjugated with nanomaterials in cancer treatment and wound healing.

result, nanoparticles specifically accumulated in tumor cells, internalized into the cells and reached the mitochondria. Additionally, this nanosystem is sensitive to high phosphate concentrations commonly found inside cells. Thus, upon internalization, its structure collapsed, leading to the site-specific release of co-loaded doxorubicin (DOX) for chemotherapy. In another example, hollow Fe-tannic acid nanocapsules (HFe-TA) were designed to also release both GOx and DOX upon cell internalization, responding however to the high intracellular concentration of ATP. This ATP-responsive platform not only enables combinational chemo- and starvation therapy but also reduces multidrug resistance to DOX. The starvation therapy, triggered by elevated ATP levels, leads to the inhibition of ATP production, ultimately restraining the efflux of DOX by inhibition of multidrug resistance proteins. Another endogenous stimulus used for drug release is glucose. In another example, a glucose responsive nanocarrier was designed to enable the controlled release of the antivascular endothelial growth factor aptamer or insulin for patients with macular disease and diabetes respectively. For that, the cargo was loaded on zeolitic Zn^{2+}-imidazolate cross-linked metal-organic framework nanoparticles (ZIF-8 NMOFs), which degrade under acidic conditions. To modulate the local pH within the nanosystem, GOx was immobilized onto the ZIF-8 NMOFs. The mechanism of release was based on the presence of glucose. GOx catalyzed the oxidation of glucose, generating gluconic acid and thus inducing the dissolution of the NMOFs as a result of pH changes. The authors demonstrated that as glucose concentration increased, localized acidification of the NMOFs occurred, driven by the GOx-catalyzed glucose oxidation and gluconic acid production. This ultimately led to an augmented release of the therapeutic cargos [272].

Nanomaterials can also be engineered to guarantee the controllable release of cargos regarding glutathione concentration. For instance, He et al. [273] developed a pioneer system utilizing glutathione (GSH)/pH dual-responsive supramolecular hybrid vesicles for a combined GOx-based starvation therapy and docetaxel (DTX)-based chemotherapy. This hybrid system exhibited a high tumor inhibitory rate, resulting in a 100 % survival rate in mice bearing a hepatocarcinoma tumor model. Zhou et al., [274] developed a GSH-responsive nanoplatform based on porous hollow Prussian Blue nanoparticles (PHPBNs) and GOx. The surface of the nanosystems was covered with hyaluronic acid using disulfide bond. This bond is cleaved by GSH after cellular uptake for GOx release.

Exogenous stimuli-responsive enzyme nanotherapies, on the other hand, are engineered to respond to an external stimulus. Few examples in the literature describe these smart enzyme-based nanoreactors, as it remains challenging to use physical stimuli for enzyme nanoactuation without compromising its activity and three-dimensional structure. In general, endogenous stimuli enable the regulation of therapeutic enzyme release, whereas exogenous stimuli can govern and enhance the catalytic activity of the therapeutic enzyme. The most described exogenous stimuli for enzyme activation is the alternating magnetic field (AMF). Zhang et al., [36] immobilized GOx on ferromagnetic vortex-domain nanorings, demonstrating its effective use for remote activation of combined starvation and ROS-based therapy in both *in vitro* and *in vivo* settings. These nanorings, besides supporting GOx immobilization, exhibit peroxidase-like activity, converting H_2O_2 from GOx into toxic oxygen radicals. They also serve as heat sources, becoming hotspots when activated by an AMF, providing spatiotemporal remote control over GOx and nanorings' peroxidase activity (Fig. 7). The applied AMF conditions (345 kHz, 300 Oe) stayed within clinical safety limits. The article also showed the importance of precisely adjusting the distance between GOx and the nanorings' surface to fine-tune the local magneto-heating effect, regulating enzyme activity. Armenia et al. [35] showed that AMF can activate thermophilic α-amylase (T_{opt} = 100 °C) and L-aspartate oxidase (T_{opt} = 70 °C) when linked to superparamagnetic iron oxide NPs without raising the overall reaction temperature. This study reported the first successful use of superparamagnetic iron oxide nanoparticles for enzyme activity tuning by magnetic heating. Besides, the article emphasized that proper orientation of the enzyme on the NP surface is crucial for optimizing this effect. These findings expand the application of nanoactuation, as superparamagnetic MNPs do not retain magnetization after the external magnetic field is removed, making irreversible aggregation a non-issue for nanobiocatalyst reuse. In a recent study, Torres-Herrero et al. [133] demonstrated that co-encapsulating magnetic nanoparticles (MNPs) with therapeutic enzymes using biomimetic silica as an entrapment matrix enables remote control and fine-tuning of enzyme activity, even when the enzyme is not directly bound to the MNP

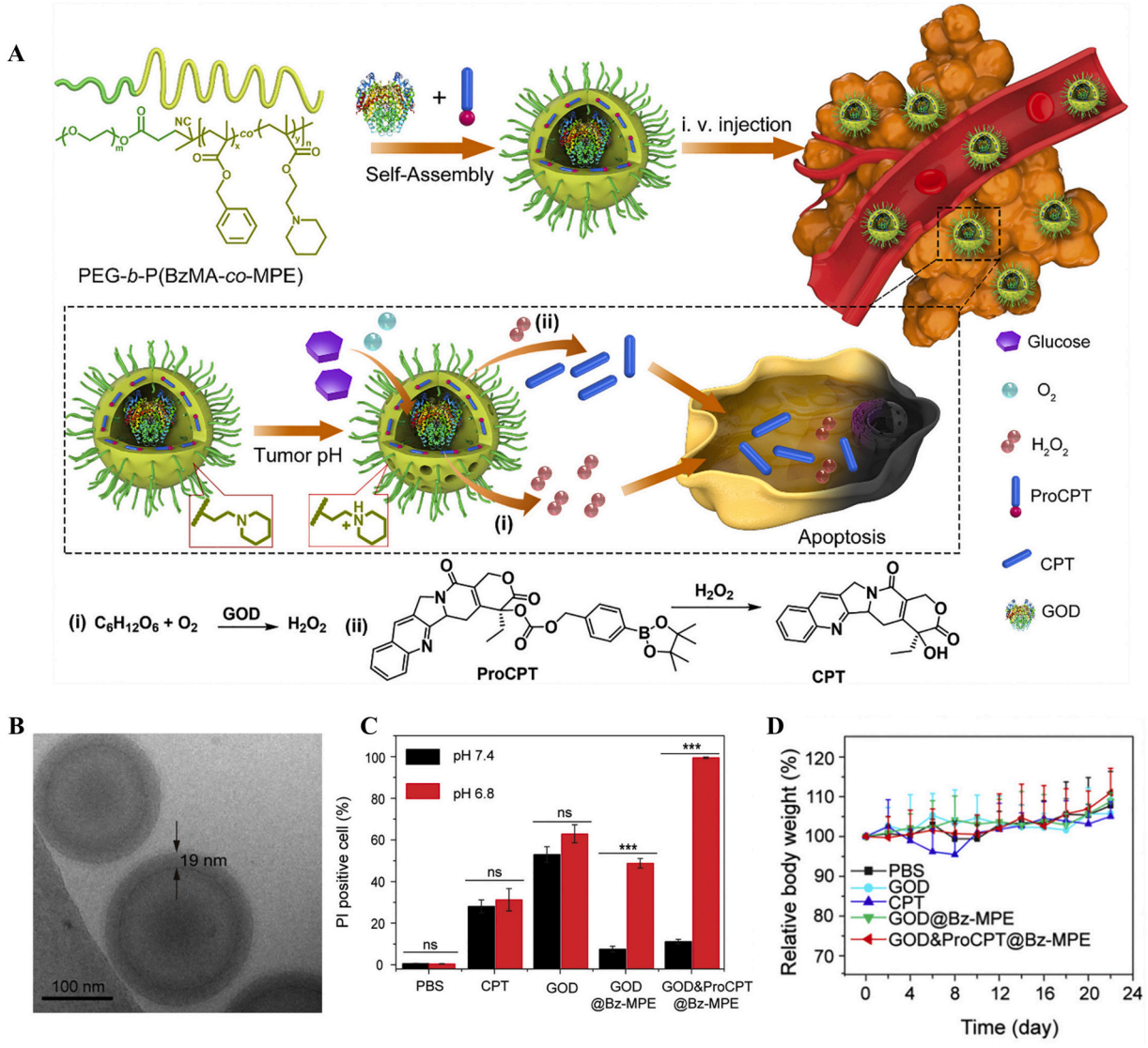

Fig. 6. A) Schematic synthesis and antitumoral action of the GOD&ProCPT@Bz-MPE nanoreactor. B) Representative cryo-TEM image of the nanoreactor. C) Cytotoxicity evaluation of the nanoreactor at pH 6.8 and 7.4. D) H22 tumor growth profiles of the mice under different treatments. Adapted with permission of [23].

surface. The silica matrix acts as a thermal insulator, preventing heat dissipation from the MNPs functioning as hotspots, which are activated by AMF, to the surrounding environment. Consequently, the environment surrounding the entrapped enzyme heats up, significantly affecting enzyme activity due to AMF, even without direct MNP attachment. This approach was applied to remotely control horseradish peroxidase (HRP), leading to enhanced bioconversion of the prodrug 3-indole acetic acid (3-IAA) into toxic oxygen radicals. The therapeutic effectiveness of these remotely controlled nanohybrids was notably improved by AMF application, as demonstrated *in vitro* on pancreatic carcinoma cells (MIA PaCa-2) and confirmed *in vivo* using xenograft models.

Another remote stimulus that can be employed to gain control over enzyme activity is near-infrared light (NIR). Lu et al. [246] developed mesoporous silica nanorods with controlled width-to-aspect ratio, loaded with doxorubicin, glucose oxidase (GOx), and Siram (a lysosomal dysfunction drug). These nanorods featured a polydopamine (PDA) shell that prevented loaded element leakage and converted light into thermal energy under NIR irradiation. GOx sensed the NIR-induced temperature increase, accelerating intracellular glucose consumption and generating cytotoxic H_2O_2, enhancing both starvation and oxidative therapies. Siram inhibited lysosomal metabolism, further complementing GOx for dual-enhanced starvation therapy. Additionally, DOX entered the nucleus, cleaving DNA and triggering synergistic chemotherapy. Drug delivery was controlled by lysosomal pH following nanorod endocytosis and NIR irradiation. A recent strategy for remotely controlling enzyme activity on macromolecular substrates involves achieving regulation through localized heating to control substrate access, rather than directly affecting the enzyme's intrinsic activity [275]. Initially, the enzymes are employed to synthesize ultrasmall platinum nanoparticles within their structure. These Pt/enzyme hybrids are then coated with a thermosensitive polymer. Below the upper critical solution temperature (UCST) of the polymer, the enzyme-polymer hybrids are insoluble. However, upon NIR irradiation, the local heating of the Pt nanoparticles renders the polymer soluble. This ability to switch the polymer's solubility from soluble to insoluble remotely allows for precise control of access to the macromolecular substrate, thereby regulating enzyme activity. The concept's versatility was demonstrated using enzymes with various substrates such as proteins (proteinase K), starch (glucoamylase), and DNA (deoxyribonuclease I), resulting in enzyme activity increases of up to 61-fold upon laser irradiation.

Exogenous stimuli could also be employed not to tune enzyme activity but to gain control over the nanosystem mobility, as demonstrated by Wu et al. [276]. They incorporated GOx into $CaCO_3$ microparticles,

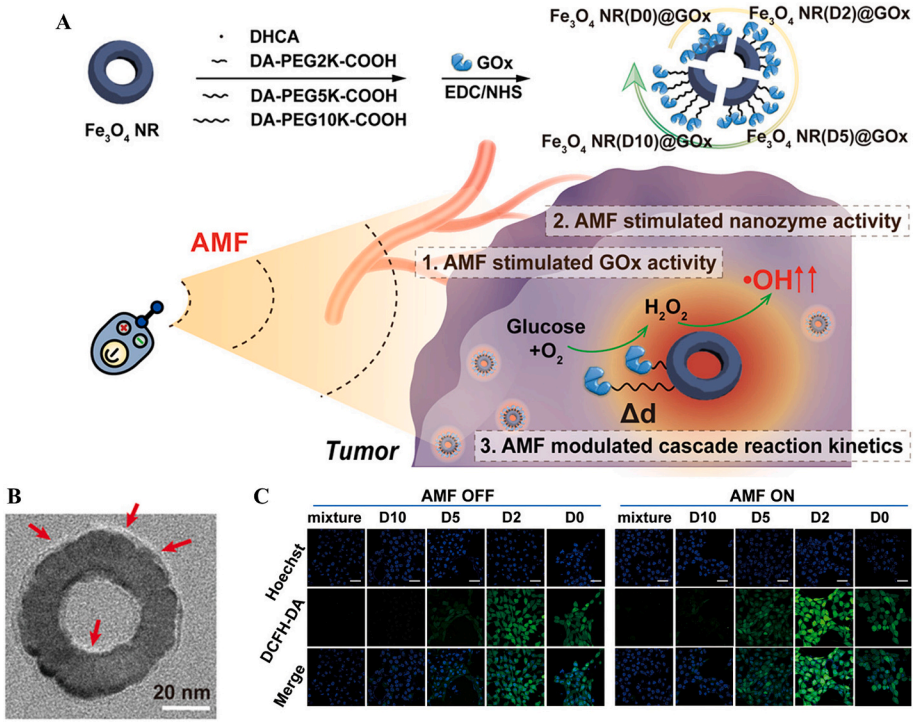

Fig. 7. (a) Schematic diagram of the nanocatalysts with varying linking and hydroxyl radical production through the AMF-induced cascade activity for cancer treatment. B) TEM image of the nano-catalyst. Red arrows indicate the organic layer coating the Fe_3O_4 NR. C) Intracellular ROS production and *in vitro* cytotoxicity of the prepared Fe_3O_4 NR@GOx without and with AMF exposure. Blue: nuclei (Hoechst dye), Green: ROS (DCFH-DA dye). Adapted with permission from ref. [36]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

not only for triggering glucose consumption for tumor starvation therapy but also as a chemical engine for self-propulsion. Magnetic nanoparticles were co-integrated to guide these self-propelled nanoreactors using an external magnetic field. Additionally, DOX was encapsulated within the microparticles. As $CaCO_3$ dissolved under acidic pH conditions, chemotherapy was activated in conjunction with endocytosis, augmenting the system's anticancer efficacy. This dual-engine approach enhanced cellular uptake in targeted areas, resulting in improved motion behavior and synergistic therapeutic strategies.

4.6. Synergistic therapies

The high cargo capacity of nanomaterials, owing to their exceptionally large surface area, enables not only dual therapeutic approaches by combining therapeutic enzymes with conventional drugs, genetic tools or the nanomaterial itself, but also facilitates biocatalytic transformations catalyzed by multienzyme cascades within spatially confined microenvironments [247,277]. From the earlier mentioned examples, it is evident that GOx is primarily employed in enzymatic therapy in association with nanomaterials. Typically, GOx is combined with doxorubicin (DOX) for the controlled release of the latter *via* different stimuli, whether endogenous or exogenous [24,246,247,278]. As an example, to mitigate side effects resulting from the uncontrolled release of DOX due to its encapsulation, Ren et al. [279] synthesized DOX prodrugs loaded into mesoporous silica nanoparticles, along with palladium, which mediated the biorthogonal bond-cleavage activation of the prodrug, and GOx for glucose starvation. Another strategy, different from the typical combination of enzymes with chemotherapeutics, involves combining therapeutic enzymes with genetic tools, as demonstrated by Fan et al. [280]. They developed a gold nanorod-based nanosystem with GOx and siRNA targeting the pro-tumoral transmembrane glycoprotein B7-H3. In this nanosystem, glucose deprivation sensitized cancer cells to photothermal therapy generated by the nanorods and inhibited tumor progression through gene silencing.

Therapeutic nanoplatforms exhibit versatility and high efficiency in delivering multiple synergistic effects, but they predominantly depend on solutions based on a single enzyme. While the field of enzymatic nanotherapeutics that coordinate the actions of multiple enzymes is in its early stages, numerous studies aim to develop enzymatic cascades. In fact, Huo et al. [281] probed that two enzymes (apyrase and 5'-nucleotidase) bound to reduced graphene oxide (RGO) can synergistically catalyze procoagulant adenosine diphosphate (ADP) into anticoagulant adenosine monophosphate (AMP) and adenosine successively under physiological conditions. These RGO-enzyme complexes were utilized to coat the surface of collagen-coated vascular matrices, resulting in the production of tissue-engineered blood vessels (TEBVs) without endothelial cells. *In vitro* and *in vivo* testing showed that the developed RGO-multienzyme-coated TEBVs exhibited excellent antiplatelet and antithrombotic functions. They also accelerated the endothelization process, ensuring that the clot-preventing function and openness of TEBVs are maintained even after the RGO-enzyme complex lost its activity. Another example of multienzyme cascade nanoreactor was developed by He et al. [282], combining horseradish peroxidase (HRP) and GOx. Both enzymes were loaded in mesoporous silica nanoparticles along with a prodrug photothermal agent. The cascade functioned as follows: glucose starvation catalyzed by GOx inhibited the generation of ATP which directly downregulated the heat shock protein-70 (HSP-70) levels. Low HSP-70 levels sensitized cells to heat, reducing their thermoresistance and enabling their ablation at temperatures below 50 °C. Then, HRP oxidized the prodrug TMB (PTA prodrug) into an active agent with an excellent photothermal effect (PTA) This innovative bienzymatic nanoplatform achieved complete thermal tumor ablation at approximately 48 °C while minimizing heat damage to healthy tissues, resulting in reduced inflammation.

Starving therapeutic strategies relying on GOx are contingent upon oxygen availability. Therefore, they are influenced by tumor hypoxia. Additionally, the substantial generation of H_2O_2 may result in DNA damage and, thus in including DNA damage, cell proliferation,

metastasis and angiogenesis [283]. A strategy to address this issue is the coupling of the enzymes GOx and catalase (CAT). In one example, GOx and catalase (CAT) have been immobilized in a zeolitic imidazolate framework-8 (ZIF-8) template, and then coated with tannic acid and poly(ethylenimine) to create hollow nanocapsules loaded with DOX. Tannic acid's affinity for collagen in the tumor stroma retained the nanosystems within the tumor. Inside the tumor, the encapsulated GOx decomposed glucose into glucuronic acid and H_2O_2, further decomposed by catalase into water and oxygen. This cascade effect promoted glucose consumption, enhancing the efficiency of starvation therapy. Additionally, glucose bioconversion by GOx induced an acid pH, facilitating DOX release at the tumor site. *In vivo* tests demonstrated the nanoreactors' excellent antitumor efficacy, combining starvation therapy and chemotherapy [277]. In another example, the GOx and CAT were covalently cross-linked *via* a pH-responsive polymer. To prevent the premature exposure of these enzymes while circulating in the bloodstream, a protective outer shell made of bovine serum albumin (BSA) was incorporated into the system. Indeed, during *in vivo* experiments, it was observed that administering free GOx led to the rapid death of the mice. Combining free GOx with free CAT did not alleviate the toxicity associated with GOx, primarily due to their separation in the bloodstream without the chemical cross-linkage. Additionally, the nanosystem without the protection of albumin shell caused rapid death of mice due to the disassociation of clustered structures and the subsequent release of free GOx. In contrast, the nanosystems where GOx and CAT were co-located, and enclosed within the BSA core shell, exhibited biocompatibility and sustained their ability to consume glucose and oxygen. Furthermore, these nanosystems displayed a remarkable capacity for accumulating within tumors and proved successful in eliminating them in mice. This example highlights the critical importance of maintaining close spatial proximity between the two cascaded enzymes, GOx and CAT, to achieve the desired therapeutic outcomes [284] (Fig. 8). Xiao et al.,[285] similarly adopted this approach combining the co-delivery of three enzymes: GOx, lactate oxidase (LOx), and CAT within a fluorinated polymer. GOx and LOx deprived glucose and lactate, respectively, enhancing tumor starvation; while CAT catalyzes H_2O_2 into O_2. This oxygen release alleviated tumor hypoxia and ensured a continuous supply of oxygen to support the ongoing nutrient depletion by GOx and LOx. This nanosystem exhibited the remarkable ability to completely inhibit glycolysis and induce severe mitochondrial dysfunction in cancer cells. Furthermore, relief from tumor hypoxia was achieved within 8 h after IV administration in mice. The synergistic effect of depleting both glucose and lactic acid, coupled with oxygen compensation through CAT, led to a remarkable reduction in the tumor growth rate.

In addition, the combination of enzymes and nanozymes is currently being explored [286]. However, it is essential to note that current nanozymes primarily mimic a limited set of mammalian enzymes, primarily performing redox reactions. This limitation restricts the application of enzyme-nanozyme cascades, especially when redox reactions are involved [36]. An example is the nanosystem reported by Du et al. [287], where cholesterol oxidase (CO) was immobilized onto a MOF

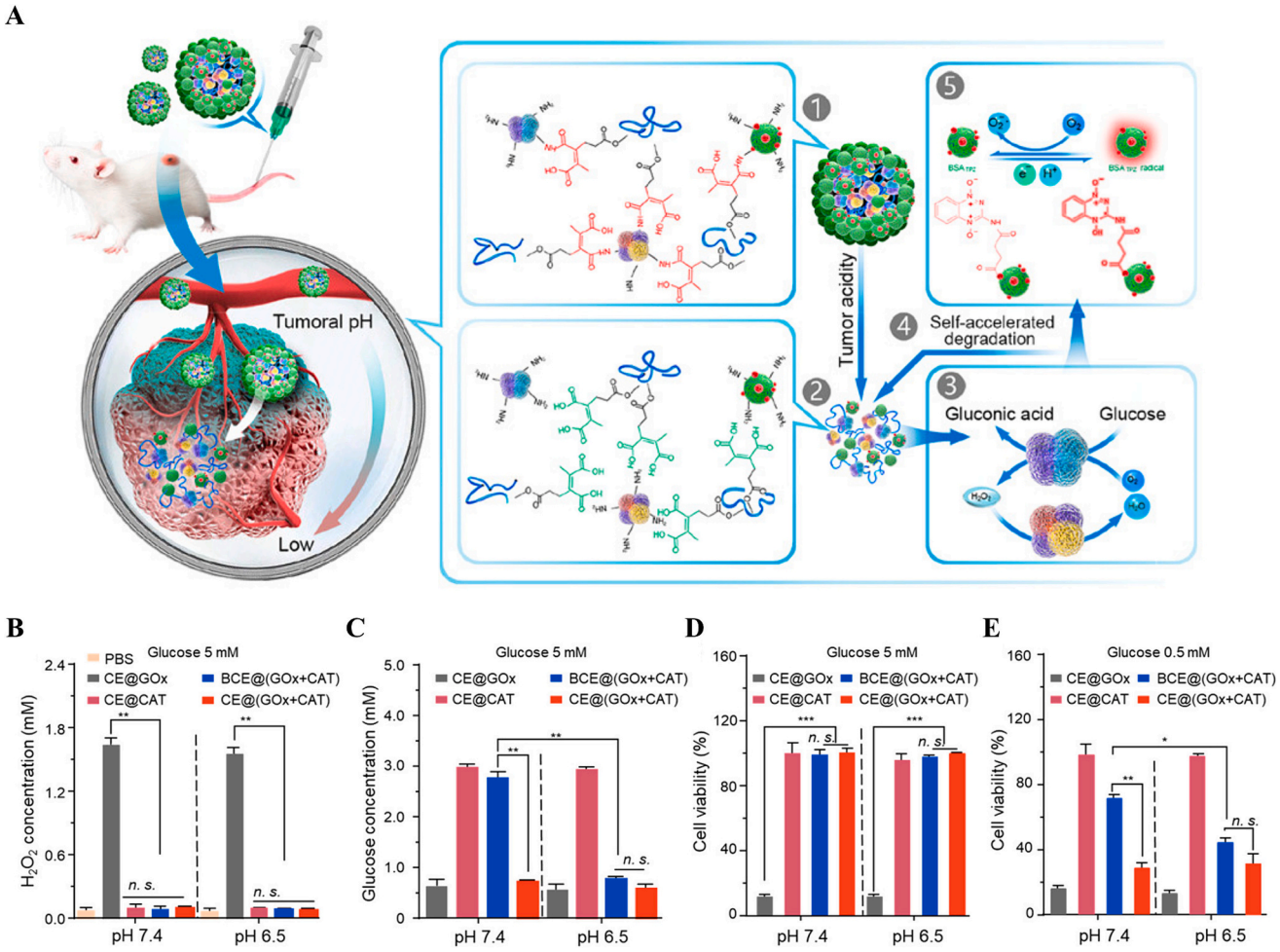

Fig. 8. A) Scheme of synthesis, pH-sensitive enzyme release and reaction of the nanoclustered glucose oxidase (GOx) and catalase (CAT). B—C) Concentrations of (A) H_2O_2 and (B) glucose in DMEM medium containing 5 mM glucose after incubation with different clustered enzymes. D, E) Cell viability after incubation with different nanoclustered enzymes in DMEM medium containing C) 5 mM and D) 0.5 mM glucose. Adapted with permission from [284].

with intrinsic peroxidase-like activity. CO catalyzed cholesterol into H_2O_2, further catalyzed by MOF nanoenzyme to produce toxic ·OH. Additionally, DOX was loaded in the system and released upon its endocytosis, combining ROS-based therapy with chemotherapy. Besides, reducing cholesterol levels in drug-resistant cell membranes, which often have increased cholesterol content, also contributes to overcoming multidrug resistance. Wang et al.,[270] combined the properties of the enzyme GOx and the nanozyme Cu_2O/Pt to treat chronic wound healing. GOx catalyzed the oxidation of glucose to generate glucuronic acid and H_2O_2. Thus, a significantl decrease in glucose level allows starving therapy. Since the nanozyme Cu_2O/Pt displayed peroxidase and catalase like activity, the generated H_2O_2 was subsequently converted into highly active hydroxyl radicals ·OH for chemodynamic therapy. Furthermore, the phothermal conversion capability of the nanoenzyme allowed increasing the temperature of the nanosystems under NIR laser irradiation improving the activity of the GOx. As a result, Wang developed a nanosystems able to coordinate starving, chemodynamic and photothermal therapies to kill bacteria and inhibit their growth during wound healing (Fig. 9). Zhou et al., [274] introduced a GSH-responsive nanoplatform engineered to starve tumors of nutrients while simultaneously applying low-hyperthermia photothermal therapy. This platform involved the integration of porous hollow Prussian Blue nanoparticles (PHPBNs) with GOx. PHPBNs functioned as a nanozyme with catalase-like activity and as a photothermal agent. GOx rapidly depleted intratumoral glucose by facilitating oxygen replenishment through the decomposition of H_2O_2 catalyzed by PHPBNs. Additionally, when exposed to NIR radiation, PHPBNs absorbed incident light photons and converted them into thermal energy, resulting in a mild temperature elevation in the tumor area.

5. Key aspects to ensure a good integration between enzymes and nanomaterials

The catalytic properties of enzymes supported in nanomaterials are influenced by a complex interplay of factors, including the enzyme's characteristics, the choice of nanomaterial as a carrier, and the method used for the integration. The interaction among these elements can have a substantial influence on the performance of the immobilized enzyme system, affecting not only its kinetic parameters but also its selectivity, specificity, and stability. Due to the numerous factors influencing the performance of enzymes immobilized on nanomaterials, systematic studies are challenging. Nevertheless, a thorough literature review unveils several key points that clearly impact the catalytic properties of the final hybrid: i) the choice of nanomaterial as a carrier; ii) size and shape

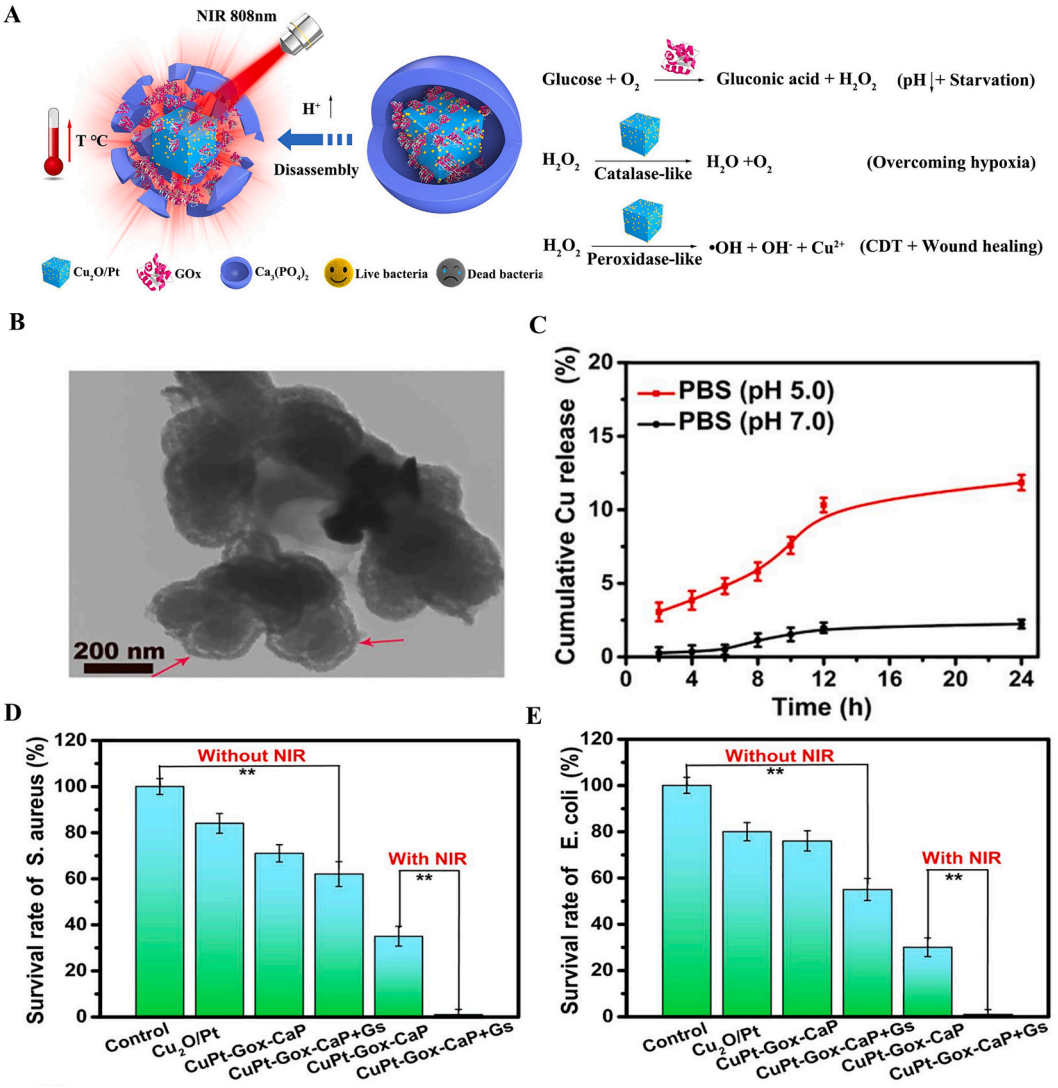

Fig. 9. A) Schematic representation of the laser induced disassembly of the nanosystem. B) Enzymatic cascade induced by pH and laser irradiation. C) Release profiles of Cu in two different pH conditions. D-E) Gram-positive and gram-negative survival rate after NIR irradiation. F) Representative SEM images of *S.aureus* and *E.coli* upon NIR irradiations in the presence of the nano-catalyst. Adapted with permission from [270].

of the nanocarrier; iii) the nanomaterial physical and chemical properties. iv) the methodology used for attaching enzymes to nanocarriers; v) the enzyme/nanomaterial ratio.

A clear example of how the nature of a carrier nanomaterial influences the catalytic properties of an enzyme upon immobilization was demonstrated by Pudlarz et al. [288] Using the same Ag and Au nanoparticles size and under the same immobilization conditions, a catalase significantly decreased its activity when immobilized on AuNPs while it retained its full activity when AgNPs were used as support. Yet, these outcomes are specific to the enzyme-nanosupport pairing and therefore cannot be extensible to the immobilization of other enzymes. The same group immobilized a manganese superoxide dismutase (Mn-SOD) on the same gold and silver NPs. While successful immobilization was achieved through direct adsorption onto AuNPs with no influence on the Mn-SOD activity, no immobilization was observed onto AgNPs following the same strategy as with the catalase [288].

On the other hand, Hetmann et al. [289] examined the impact of different carbon nanomaterial structures on the activity of adenylate kinase (AK). AK is a phosphotransferase responsible for interconverting adenine nucleotides (ATP, ADP and AMP) and plays a vital role in nucleotide homeostasis, monitoring cellular energy levels, purinergic signaling. Alterations in nucleotide concentrations are linked to various diseases (*e.g.,* thrombosis, inflammatory processes). To make AK suitable for biological systems, its enzymatic activity, stability, and catalytic properties need enhancement, along with efficient biodistribution and overcoming cell barriers when bound to potential nanocarriers. To obtain an effective enzyme-nanosystem, they selected graphene oxide (GO), carbon quantum dots (CQD), and carbon nanoonions (CNO) as support for AK immobilization to their surfaces. The study found that all tested nanomaterials increased enzymatic activity and stability upon immobilization, with AK-nanosystems remaining active even after lyophilization. Different nanomaterials had varying adsorption capacities and biocompatibilities: GO exhibited the highest adsorption and biocompatibility, while CNO was less favorable due to toxicity and low adsorption, similar to CQD. *In vitro* experiments suggested GO as a better choice for extracellular environments, ensuring AK operation while preventing cellular internalization. Conversely, CQD's small size made it preferable for intracellular enzyme delivery. The article clearly highlighted that the enzyme's interaction with nanomaterials depends on factors like size, curvature, aspect ratio, morphology, crystal structure, and surface chemistry of the nanocarrier.

Furthermore, several studies emphasize the importance of surface functionalization and surface chemistry in the immobilization efficiency and activity of different enzymes on various nanostructures. For example, Mu et al. [290] immobilized the enzyme L-asparaginase on MNPs functionalized with polymer brushes made of poly(2-vinyl-4,4-dimethylazlactone) (PVDMA) *via* the ring opening of azlactone, without the need for activation or pretreatment of the PVDMA-MNPs. They highlighted that the amount of immobilized enzyme increased with longer polymer chains, as longer chains provided more reaction sites. Besides, the immobilized enzyme exhibited a higher affinity for L-asparagine compared to the soluble one. These findings suggest the potential use of the enzyme-MNPs for developing extracorporeal shunt enzyme reactor systems for clinically treating acute lymphoblastic leukemia. Czechowska et al. [291] observed that immobilization method used with AuNP significantly affects both the adsorption degree and the ultimate enzymatic activity. Immobilizing catalase on AuNPs through adsorption resulted in higher surface coverage but lower enzymatic activity when compared to oriented immobilization using a stable conjugate formed between the poly-his tag of the enzyme and the NTA-Ni complex introduced to the surface of AuNPs. This latter method allowed for immobilization without compromising the enzyme's activity.

Lastly, different studies underline that catalytic properties of the enzymes depend also on the enzyme to nanomaterial ratio. An example of this effect was studied for a catalase (CAT) immobilized on graphene [292]. To partially preserve CAT's enzymatic activity upon immobilization, it was important to achieve maximum coverage of the graphene surface, ensuring that all the enzyme subunits were anchored to the same sheet of graphene. *In silico* experiments revealed that anchoring subunits to two or more graphene sheets lead to destabilization of the enzyme's secondary and quaternary structure, resulting in complete biological inactivation. Despite the partial activity loss, the immobilization significantly enhances enzyme stability, as it remained active for over one month in buffered solutions at 37 °C. Similarly, Naderi et al. demonstrated that chrondroitase ABC I, an enzyme that cleaves glycosaminoglycan chains and may aid in axon regeneration, retained most of its activity upon complexation with a minimal amount of gold nanorods (GNRs) [293].

In summary, to achieve active nanomaterial integrated enzymes with the desired properties for biomedical applications tailored immobilization protocols have to be developed. The piled-up information has shown that the catalytic properties of enzymes immobilized on nanomaterials are highly dependent on nanomaterial properties, immobilization methodology, and the biochemical properties of the particular enzyme to be immobilized. Although so far empirical approaches are needed to tailor the integration of enzymes into nanomaterials for better properties, understanding the factor affecting the properties for each enzyme-material pair may help in the desired of rational approaches. Further studies are needed to better understand how to create a synergistic environment that allows for the development of the best enzyme-nanosystem, with minimal cytotoxicity and maximum therapeutic efficacy.

6. Conclusions and prospects

Enzymes have been explored for therapeutic application since the 1970s. Their potential is still being studied to improve their application in clinics by reducing their side effects and enhancing their significant advantages. Their use in clinical settings still poses various challenges, including poor colloidal stability, loss of enzyme activity, immune responses, and clearance by organs due to difficulties in overcoming biological barriers.

The use of nanomaterials as carriers presents promising solutions to these challenges. Nanomaterials can stabilize enzymes in biological environments, extending their activity over time. Furthermore, they introduce unique capabilities that enzymes alone lack, such as remote control over enzyme activity and the ability to traverse biological barriers without the need to produce recombinant enzymes with complex modifications (*e.g.,* mannose 6-phosphate-based post-transductional modification). Thus, enzyme therapy combined with nanomaterials holds immense potential, not only for enhancing existing enzyme-based treatments but also for developing innovative therapeutic approaches using adaptable nanoreactors that respond to environmental cues and remote stimuli. These nanoreactors could integrate multiple therapeutic mechanisms, promoting synergistic actions and expanding their therapeutic utility.

While the application of enzyme therapy with nanomaterials extends beyond cancer treatment (including metabolic disorders, fibrosis, Alzheimer's, virus infections, wound healing, inflammation, hyperuricemia, *etc.*), it has primarily focused on cancer starvation therapy, often utilizing well-characterized enzymes like glucose oxidase (GOX). Current research efforts aim to expand the use of nanomaterials in combination with other therapeutic enzymes for a wider range of applications.

Despite the advantages of nanomaterial integration, challenges remain, including the complexity of large-scale production and characterization of multicomponent enzyme-hybrid materials with enhanced properties, the need for comprehensive *in vitro* and *in vivo* studies, and rigorous risk assessments, encompassing biocompatibility, long-term impacts, and life-cycle analysis. These concerns are actively addressed by the research community, funding agencies, and regulatory bodies [261,282,283]. The future of enzyme therapy will undoubtedly

be shaped by advancements in nanotechnology, and this collaborative synergy holds the promise of overcoming significant therapeutic hurdles.

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CRediT authorship contribution statement

Beatriz Torres-Herrero: Writing – original draft, Investigation, Conceptualization. **Ilaria Armenia:** Writing – original draft, Investigation, Conceptualization. **Cecilia Ortiz:** Writing – review & editing. **Jesús Martinez de la Fuente:** Writing – review & editing, Funding acquisition. **Lorena Betancor:** Writing – review & editing, Supervision, Funding acquisition. **Valeria Grazú:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

None.

Data availability

No data was used for the research described in the article.

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