The nanobiocatalyst (NBC) is an emerging innovation that synergistically integrates advanced nanotechnology with biotechnology and promises exciting advantages for improving enzyme activity, stability, capability and engineering performances in bioprocessing applications. NBCs are fabricated by immobilizing enzymes with functional nanomaterials as enzyme carriers or containers. In this paper, we review the recent developments of novel nanocarriers/nanocontainers with advanced hierarchical porous structures for retaining enzymes, such as nanofibres (NFs), mesoporous nanocarriers and nanocages. Strategies for immobilizing enzymes onto nanocarriers made from polymers, silicas, carbons and metals by physical adsorption, covalent binding, cross-linking or specific ligand spacers are discussed. The resulting NBCs are critically evaluated in terms of their bioprocessing performances. Excellent performances are demonstrated through enhanced NBC catalytic activity and stability due to conformational changes upon immobilization and localized nanoenvironments, and NBC reutilization by assembling magnetic nanoparticles into NBCs to defray the high operational costs associated with enzyme production and nanocarrier synthesis. We also highlight several challenges associated with the NBC-driven bioprocess applications, including the maturation of large-scale nanocarrier synthesis, design and development of bioreactors to accommodate NBCs, and long-term operations of NBCs. We suggest these challenges are to be addressed through joint collaboration of chemists, engineers and material scientists. Finally, we have demonstrated the great potential of NBCs in manufacturing bioprocesses in the near future through successful laboratory trials of NBCs in carbohydrate hydrolysis, biofuel production and biotransformation.

1. Introduction

Advances in biotechnology have witnessed a growing interest in the development of green and sustainable bioprocesses using enzymes. The exquisite activity, specificity and selectivity of enzymes have made them promising biocatalysts for numerous fascinating applications including in biocatalysis, biosensors and biomedicine. Biocatalysts promote green processes due to low chemical consumption and absence of toxic by-products. Nevertheless, the major challenges associated with the enzyme-catalysed bioprocesses are high operational costs due to low stability and reusability of the enzymes when extended to large-scale industrial processes. Enzyme immobilization on support materials has been rapidly gathering pace in recent years. It has been commonly recognized that such technology is able to shelter and/or stabilize enzymes against chemical and environmental attacks, and importantly the immobilized enzymes could be recovered and re-used in a large-scale continuous process [1]. The development of immobilization techniques as well as the materials from natural/synthetic polymers or inorganic materials as enzyme supports have been extensively studied recently, as reported in the review paper by Datta et al. [2]. However, employment of the immobilized enzymes in a bioreactor system may lead to a reduction of enzyme activity due to the change of the unique and native structures of enzymes upon immobilization [3], although the enzyme activity and stability can be maintained through optimal immobilization by extensive studies to identify the most suitable immobilization protocols [4]. The methodologies for
recovering biocatalysts are also poorly developed. It is, therefore, a focal point to identify enzyme carriers with biocompatible, robust and separable features.

Recent development in nanotechnology has provided a wealth of diverse nanoscale carriers that could be applied to enzyme immobilization. Immobilization of the enzymes on nanostructured materials has been recognized as a promising approach to enhance enzyme performances. The nanobio catalyst (NBC) is an emerging innovation that synergistically fuses nanotechnology and biotechnology breakthroughs. The NBC formation encompasses the assembling of enzyme molecules onto nanomaterial carriers to favour desirable chemical kinetics and selectivity for substrates. The functionalized nanocarriers allow enzymes to be assembled in ordered structures, which act as a nanoscale information storage and processing system. Research has shown that nanostructured materials possess the requirements of the NBCs and provide large surface areas that allow a higher enzyme loading and reduced mass transfer resistance for substrates [1]. The NBC is a specifically functionalized enzyme–nanocarrier assembly, which promises exciting advantages in improving enzyme stability, capability and engineering performances, and allowing creation of a microenvironment surrounding the enzyme catalysts for maximal reaction efficiencies. Enzyme immobilization using nanostructured carriers can significantly increase life cycles of the biocatalyst, hence reducing the cost of the biocatalytic process. Betancor & Luckarift [5] described enzyme immobilization onto the nanoscale material as a versatile new technology, which offers advantages including low cost, rapid immobilization and reaction, similarity of nano size, mild conversion conditions, robust activities, mobility, high loading, minimum diffusional limitations, self-assembly and stability. A revolutionary class of biocatalysts can be developed by introducing the unique properties of the nanoscale material such as mobility, confining effects, solution behaviours and interfacial properties into NBCs. The preparation, catalytic efficiency and application potential of NBCs are significantly different from conventional immobilized enzymes. To date, functional nanomaterials have been used for the development of NBCs such as nanofibre (NF) scaffolds [6], nanotubes (NTs) [7], nanoparticles (NPs) [8], nanocomposites (NCs) [9] and nanosheets (NSs) [10]. The increasing interest of NBCs has become a driving force for the fabrication of nanocarriers with unique properties and structures. Advanced nanocarriers such as NFs, nanopores and nanocontainers can significantly enhance the engineering performances of enzymes.

Retaining the enzyme functionality is a major challenge associated with the development of NBCs for bioprocessing applications. Over the last decade, a number of technologies for fabrication of NBC assemblies have been intensively reported. Among the binding mechanisms studied, covalent attachment is exclusively reliable for specific binding sites and circumventing enzyme leaching [11–13]. Meanwhile, in spite of the simplicity of physical adsorptions, their execution in industrial scale is largely challenged due to critical enzyme leakage [14,15]. The immobilization strategies are varied depending on the physical and chemical characteristics of both enzymes and nanocarriers, and their interfacial interactions. The NBC assembly should also be applicable in the bioprocessing environment without affecting the native enzyme properties. Therefore, NBCs with tremendous biochemical and engineering performance such as enhanced enzyme activity and stability as well as reusability and processability are to be pursued. The development of functional nanomaterials as enzyme carriers and supports, and immobilization techniques have been intensively studied during last two decades, which have been comprehensively reviewed in recent reports [4,16–19]. However, to our best knowledge, the engineering performances of NBCs and their contributions in the bioprocesses appear to be less reported. Bioprocess operations endow a special niche for the production of valuable and marketable products. With attractive performances in regards to productivity and recyclability, the NBC is a promising candidate for substrate pre-treatment [20], biofuel production [21] and biotransformation [22]. An integrated process for industrial bioprocesses using the NBCs is proposed as sketched in figure 1, which illustrates the research approach from the synthesis of nanocarriers and enzyme immobilization strategy to execution of a specific function and evaluation of bioprocessing performances of the NBCs.

This article aims to review recent developments and applications of the NBCs in industrial bioprocesses. We first discuss the advancements of up-to-date nanocarriers including NFs, nanoporous carriers and nanocontainers. We highlight the unique structures of these nanocarriers and their physicochemical properties which are essential for enzyme immobilization. It is also noted that porous microbeads can be classified into these nanocarriers for preparation of biocatalysts as the porous environment is highly compatible with the biological activity of the immobilized biomolecules although the pore size is larger than the mesoporous materials. New strategies for the fabrication and functionalization of NBCs are then addressed for different enzyme nanocarriers and immobilizations. We summarize and evaluate the biocatalytic activities and bioprocessing performance of resulting NBCs. In closing, case studies of NBC bioprocesses for carbohydrate hydrolysis, biofuel production and biotransformation are reported.

2. Advancements in nanocarriers for nanobiocatalysts

NBCs integrate a biological entity for biocatalysis (i.e. enzyme) with a nanomaterial carrier with unique electronic, optical, magnetic and external-stimuli-responsive properties. There have been many reported technologies for production of specific and processable enzymes, such as recombinant DNA technology to bring the commercial enzyme cost down, directed evolution for pursuing specific traits and widening substrate repertoire and bioprocesses for making recyclable and durable enzymes for industrial applications. These techniques have been reviewed in detail by Illanes et al. [23] and Lopez-Gallego & Schmidt-Dannert [24]. Here, we will focus on the development of nanostructured and nanoporous carriers for the NBC assembly fabrication.

The development of novel nanocarriers with unique functions and characteristics comprises (i) the introduction of functional groups on the surface of the nanocarriers for immobilizing various enzymes or responding to external stimuli, (ii) construction of special structures for increasing the surface area, facilitating substrate diffusion, recycling nanocarriers or confining enzymes inside nanocages, and (iii) improving processability of nanocarriers such as mechanical and thermal stability. Hierarchical nanostructures play a significant role in improving the performance of immobilized enzymes in terms of their activity, functionality and stability. A range of advanced
nanocarriers have been developed and reported in recent studies, including NPs, nanospheres, nanogels, nanocages, nanowires, nanocubes, nanorods, NFs, NTs, NSs and nanofilms. Enzyme immobilization onto the NPs has been reviewed by Ansari & Husain [16]. Here, we will only focus on recent research progress for the development towards the application of NFs, nanoporous carriers, and nanocontainers for NBC fabrication.

2.1. Nanofibres
Among the nanostructured materials examined for NBC assembly, nanostructured fibre (NF) offers a number of outstanding characteristics, including high enzyme loading and homogeneous dispersion in a liquid phase. Furthermore, the high porosity and interconnectivity endow NF with low hindrance for mass transfer. The specific surface characteristics, discrete nanostructures and self-assembling behaviours of NFs provide exciting opportunities to develop NBCs for bioprocesses using bioreactor systems. The integration of enzymes with NFs leads to a hybrid assembly that combines the bio-catalytic ability of the enzymes with the unique functions of NFs within the nanostructure network. One-dimensional NFs have been proved as promising nanocarriers in improving biocatalyst performance by considering the factors such as surface area to mass ratio, loading capacity and efficiency, mass transfer resistance and recyclability. Electrospinning is a commonly used technique to synthesize NFs, due to its simplicity in operation. Fabrication through the electrospinning generates various sizes of fibrous mats, with scales ranging from nanometres to a few micrometres [25–27]. Besides the single fibre configuration, hybrid structures such as side-by-side, core-shell and hollow NFs can be fabricated from this technique [28]. Side-by-side hybrid fibres (figure 2a) are synthesized by merging two polymer solutions into a single jet using a dual-opposite-spinneret electrospinning. Well-aligned and uniform side-by-side fibres may potentially be used for carrying two enzymes at the same site.

Feng et al. [28] fabricated core-shell NFs by incorporating non-electrospinnable materials into NFs as a core, while the shell template was made from electrospinnable polymers. This was achieved by using a facile coaxial electrospinning process, consisting of two syringes for inner (non-electrospinnable) and outer (electrospinnable) solution, respectively. Removal of the core left the inner part empty so that a hollow structure inside the NFs was generated. These core-shell NFs were applied to a multiple enzyme-catalysed reaction, involving 3α-hydroxysteroid dehydrogenase, diaphorase and NADH-dehydrogenase. The enzyme mixture solution was fed into the hollow area, while a N,N-dimethylacetylamide solution of 30 wt% polyurethane sheath served as a template [33]. This novel polymer NF carrier affords high performance in the multi-enzyme biotransformation. Core-shell NF carrier alleviates biomolecule diffusion limitation by controlling the thickness of the sheath and the size of the hollow, which can be tuned by the ratio of inner–outer injection speed during the electrospinning process.

The electrospinning technique can also be useful to fabricate NFs with a hybrid of inorganic/organic material to improve the robustness and mechanical strength of materials. For example, silica NPs were inserted inside the hollow ultrafine polymer fibres to synthesize a silica-embedded NF composite [34,35]. This hybrid NC can withstand the shear stress during bioprocessing without significant deformation. This feature allows enzyme-loaded NFs to be used in a semi-continuous or continuous bioprocess. Hwang et al. [36] reported that uniform quantum-dots distribution on polymeric NFs induced a higher degree of compactness and shape rigidity, resulting in efficient enzyme immobilization.

Figure 1. Flow diagram demonstrating an integrated process of using advanced nanobiocatalysts for industrial bioprocessing applications. (Online version in colour.)
NFs offer a high surface-to-volume ratio to show a high adsorption capacity of enzyme loading. The porous structures of the NFs allow easy penetration of the molecules into all available surfaces. However, the overlapping surfaces of NFs block the enzyme penetration, while some enzyme molecules shielded inside the NFs may not be attacked by the substrate molecules, which reduce enzyme activities. Ros et al. [37] reported a chemical modification strategy to increase the surface area of polymer NFs by treating NFs with oxidizing agents such as nitric acid and sulfuric acid. Chemical modification plays an important role in fine-tuning the porous structure of NFs for easy penetration of biomolecules. As the alteration yields an accessible and penetrable surface, enzymes can attach into the inner region of NF carriers. This functional structure also allows optimal substrate diffusion. As a result, the bioconversion of substrates in bioprocessing reactions can be performed effectively without diffusion constraints.

2.2. Nanoporous carriers
Mesoporous silica and carbon materials are designed with uniform nanopores and tuneable periodic nanostructures, which are promising for controlling release of small and
large molecules [38]. The porous structures can offer a high surface area as well as their ability to encapsulate enzymes within the pores to provide a more suitable microenvironment than a planar surface. However, the pore sizes could be vital for enzyme entrapment. In contrast to immobilization protocols, the entrapment does not involve any bond between the enzyme and carrier surface. It has been observed that the enzyme exhibits higher activity and thermal stability when the pore size of the carrier is equivalent to the hydrodynamic size of the enzyme. The enzyme can preserve its native conformation in such a pore size, so that the active sites of the enzyme molecules can facilitate interfacial reactions with substrate molecules. When the pore size of the enzyme carrier is smaller than the enzymes, enzyme molecules are incapable of entering the pore, resulting in a low enzyme loading. On the other hand, nanocarriers with pore sizes larger than the enzyme may reduce the enzyme’s activity, which may be attributed to enzyme leaching or reduction in the stability of the enzyme [39,40].

Recently, Du et al. [29] developed dendrimer-like silica NPs with hierarchical pores. These silica NPs were synthesized with centre-radial and multiple-scale pores. Large pores were favourable for hosting large biomolecules, while uniform mesopores acted as nanocarriers for encapsulating small molecules. Such unique properties are pertinent across a spectrum of the domains, which are significant in coordinating the diffusion of a range of molecules of different sizes to the interior part of NPs (figure 2b).

The porous structures of the nanocarriers can also be beneficial for hybridizing other components to form multiple functional NCs or assemblies. For example, silica mesoporous nanomaterials can be co-synthesized with magnetic NPs to allow the enzyme/nanocarrier complexes to be easily recovered from the aqueous reaction media. Magnetic silica NPs can be synthesized in several ways, including fabricating in a rattle type, and encapsulating magnetic NPs into mesostructured silica nanospheres, aligned mesoporous shells and pore channels [38]. Multifunctional mesoporous silica has been employed in an electrochemical immunosensor [41].

An integrated multifunctional intelligent nanocarrier developed by Motorow et al. [42] could be another futuristic biocatalyst support. The stimuli-responsive nanocarriers are able to be triggered by light [43,44], temperature [45,46], pH [47,48] and ionic strength [49]. Stimuli could be applied either to the material surface or to the biomolecules which encourage or discourage the enzyme–surface interactions. These switchable systems provide unprecedented potential in bioprocess applications [50].

A hybrid of core-shell intelligent particles as an artificial biological cell was developed with an environmental-responsive feature [42]. The core compartments were designed by accommodation of functional composites composed of polymers, enzymes and NPs, while the shell was responsive to environmental stimuli through opening or closing the selective gates. Special functions were performed by responding to different environmental stimuli. Release of small molecules from the cargo made from enzyme-responsive mesoporous silica was reported in the trials for colon-specific drug delivery [51]. Capped with bridged silsesquioxane as a gatekeeper, the mesoporous MCM-41 were able to open the gate and release entrapped drugs in the presence of a specific enzyme. Rica et al. [52] proposed a concept of enzyme-responsive NPs to develop a synergistic biocatalyst-medicine device. They used a cleavable enzyme-linker to modify the polymer-based NPs. The release of the enzymes at the targeted tissue as well as therapeutic biomolecules can be reduced significantly through chemical transformation. This is an exciting futuristic nanoporous complex that can be potentially applied to biocatalysts for bioprocessing applications.

2.3. Nanocontainers

Enzymes can be attached to the fibre surface or enclosed inside the pores. However, enzyme leaching can be an issue if only the enzymes are attached by physical adsorption. Enzyme reactivity could be affected due to conformational change or is reduced by exposing to cross-linking reagents. To encapsulate the enzyme molecules inside a nanoscale container is a very promising approach to maintain enzyme activity, while substrates can freely diffuse into and out of the container.

A nanocontainer with a ‘ship-in-a-bottle’ pore structure was used to prevent enzyme leaching (figure 2c). The mesoporous silicas had spherical mesocellular cages (around 40 nm) connected by 13-nm-sized mesopores. After the enzyme was adsorbed into the spherical cages with a high degree of enzyme loading, the enzyme was covalently cross-linked by the glutaraldehyde to form enzyme aggregates. These enzyme aggregates had a size range which was less than the cage size (40 nm) but larger than the pore size (13 nm). In this way, the enzyme aggregates were retained inside the nanocages, resulting in stabilized enzyme activity [30]. The mesoporous silica was also modified to show a cage-like porous structure so as to retain enzyme molecules without cross-linking, such as SBA-16 (cubic) and FDU-12 (cubic). FDU-12 had isolated cages (about 17 nm) which were connected in three dimensions by small pore windows (less than 4 nm). Liu et al. [31] discovered that when the enzyme molecules were trapped inside the nanocages, the pore entrance was reduced to prevent the possible release of the enzyme molecules, while the pore size was large enough for entry of substrate molecules and exit of products (figure 2f). The nanocages could be formed from biological molecules like DNA, which could assist us in understanding complicated enzyme reactions inside biological species and even human bodies. Through the DNA self-assembly process, DNA molecules could form various cage structures, including tetrahedra, cubes, octahedra, dodecahedra and icosahedra shapes. Juul et al. [53] conducted experiments to control the encapsulation and release of horseradish peroxidase in a DNA nanocage through temperature change. The nanocavity (approximately 10–12 nm) was covalently closed by 12 double-stranded DNA helices as the edges of the structure. The entrapped enzymes showed catalytic activity inside the DNA cage, and the substrate molecules were able to penetrate the apertures in the DNA lattice and move into the DNA cages for biocatalytic reactions.

Another exciting enzyme nanocarrier was fabricated as hybrid organic–inorganic nanoflowers, which was an innovative discovery reported in Nature Nanotechnology [32]. The authors characterized that the resulting microspheres had an average size of less than 2 μm and showed a relatively smooth surface, in which enzymes were mainly located in the core of the nanoflower (figure 2r). The crystal structured nanoflowers were used for immobilization of α-lactalbumin, laccase, carbonic anhydrase and lipase. The hybrid nanoflower exhibited enhanced enzymatic activity and stability compared with free enzymes, which may be attributed to the confinement of the enzyme in the core of the nanoflower.

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This nanoflower may withstand the harsh environment in the bioprocess operations.

3. Strategies for the development of nanobiocatalysts

NBCs can be fabricated through the immobilization of enzymes on or encapsulated in a multitude of nanomaterials including polymers, silicas, carbons and metals. Different techniques and methodologies have been explored and developed successfully to retain the enzymes on or in the nanomaterials. These approaches include physical adsorption via electrostatic interactions, hydrophobic interactions, hydrogen bonding or van der Waals forces, covalent binding, cross-linking of enzymes or physical entrapment or encapsulation. Physical entrapment is mainly determined by the sizes of nanocarrier pores and enzyme molecules, which has been discussed in detail in §2.3. Other approaches are based on the functional groups on the surfaces of enzymes and nanocarriers employed in the formation of NBCs. The enzyme itself possesses amino (-NH2), carboxylate (-COOH), thiol (-SH) and hydroxyl (-OH) groups located in lysine, arginine, glutamic and aspartic acid residues [54] which are widely used to interact with functional groups from nanocarriers, while the functional groups on the nanocarriers are introduced via surface modification. Physical entrapment is mainly determined by the sizes of nanocarrier pores and enzyme molecules, which has been discussed in detail in §2.3. Other approaches are based on the functional groups on the surfaces of enzymes and nanocarriers employed in the formation of NBCs. The enzyme itself possesses amino (-NH2), carboxylate (-COOH), thiol (-SH) and hydroxyl (-OH) groups located in lysine, arginine, glutamic and aspartic acid residues [54] which are widely used to interact with functional groups from nanocarriers, while the functional groups on the nanocarriers are introduced via surface modification. The methodologies for the fabrication of the NBCs can be varied, depending on physical and chemical properties of the nanocarriers and enzymes, as well as applications and processes of the resulting NBCs. These are the focus of this section. A range of nanocarriers and immobilization procedures for fabrication of the NBCs in the bioprocessing applications are tabulated in Table 1. The schematic presentations for fabricating NBCs and SEM/TEM images for some of the resulting NBCs are depicted in figures 3 and 4, respectively.

3.1. Polymer nanocarriers

Compared to other enzyme nanocarriers, polymer-based materials can be easily produced in large quantities, and importantly the polymer nanocarriers can provide a number of reactive groups for fabrication of NBC assemblies with high stability and functional activities [26,67,68]. Polymers have been widely used as enzyme supports. However, their huge geometric size is unfavourable due to diffusion resistance [69]. The molecular structure of conventional polymer materials is uncontrollable, and their physical characteristics such as porosity, pore size or thickness may make the polymer materials unfavourable for enzyme immobilization. Unlike conventional polymers, nanostructured polymers hold many distinctive properties which benefit enzyme functionality and stability. The polymer nanocarriers remain the feature of modifiable surfaces of polymers for further biomolecule conjugation. They can be easily fabricated in nanometre-scale, ranging from 30 to 500 nm with a large surface area [27]. Therefore, they accommodate high enzyme loading and consequently enhance the biological activity [61]. The immobilization of multi-enzymes on polymer nanocarriers builds a molecular bio-network, and thus creates an interfacial microenvironment, which could affect the accessibility of active sites, configuration of enzymes and biochemical mechanisms. Nanogels, NPs, nanocrystals and NFs from polymers have been examined as enzyme hosts in bioprocessing applications [27,70,71].

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resistance through the nanogel to reach the enzyme molecules. This issue can be overcome by implementing porous structures of the nanogels [72]. Covalent immobilization can be achieved via formation of a covalent linkage between the functional groups of nanogels and enzymes (figure 3a). The enzyme α-chymotrypsin was immobilized on mesoporous magnetic nanogels through photochemical in situ polymerization [73]. The enzyme was anchored to the carboxyl-containing support by 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide (EDC) activation. Two or more enzymes also have been co-immobilized on the surface of nanogels [74]. One novel approach was to introduce polymer monomers onto the surface on a single enzyme, and then induce in situ polymerization to form a porous polymeric network with a thickness of a few nanometres [75]. This method can facilitate substrate entry into the nanogel and their interfacial reactions with the enzyme. The nanogel can also provide a shield for the enzyme itself.

Polymeric NPs are also attractive due to their simple synthesis, high stability, uniform size distribution and abundance of functional groups for enzyme immobilization. Covalent binding is often employed for stabilizing enzyme molecules on NPs. Lipase on magnetic Fe3O4–chitosan NPs was realized by using coupling agents, namely EDC and N-hydroxysuccinimide (NHS) [76]. Enzyme binding onto cellulose nanocrystals was initiated by attacking the nucleophilic amino groups of the activated matrix. Mahmoud et al. [77] presented such a binding technique in employing cyclodextrin glucanotransferase (CGTase) on cellulose nanocrystals. The attacks on the amino groups occurred after matrix pre-functionalization with carboxylic groups while prior to further activation by NHS/EDC. Meanwhile, carbohydrate or polyethylene glycol (PEG), generally known as an enzyme-surface connecting agent [3], were introduced to provide additional platforms of hydrogen bonding and enzyme protection [78].

Nanofibrous hosts have received increasing attention because of their unique interconnectivity, permeability and separability [79]. Physical adsorption is the most commonly used method for enzyme immobilization onto the NFs. However, enzyme leaching can be a severe issue for bioprocessing applications. As an alternative, successful immobilization is often employed through covalent binding as confirmed by SEM images (figure 4a) [63]. The hydrophobic surfaces of the polymer NFs can be modified to increase the biocompatibility. For nitrile-rich polymer nanocarriers such as polyacrylonitrile (PAN), enzyme binding onto them is
normally activated through imidoesterification between imido-
esters of the NFs and amino groups of enzymes [6]. However, their covalent conjugation was inefficient as less than 30% of the initial activity was retained after 10 repeated cycles. Li et al. [26] upgraded this technique by using an epichloro-
hydrin coupling agent when they conjugated catalasase onto PAN NFs. Rapid enzyme immobilization was achieved using this cross-linking procedure (figure 3b). Experimental data revealed that the amount of attached catalasase remained stable after 3 h reaction, indicating an equilibrium state was reached. However, drastic activity reduction was simultaneously observed, which was believed due to the detrimental impacts of the epichlorohydrin. Therefore, more effective and non-toxic cross-linking agents are pursued for enzyme immobilization onto PAN NFs.

Co-synthesizing with functionalized polymers is a recently developed strategy to create nanocarrier reactive sites. Co-polymer NFs have been prepared from maleic anhydride and styrene as a new aptasensor platform [67]. The abundance of maleic anhydride promoted high loading of aptamers to ensure sufficient capacity to capture enzymes. For example, Stoilova et al. [80] employed this technique for acetylcholin-
esterase binding. They initially optimized the weight ratio between two polymers to obtain considerable amounts of reactive anhydride moieties. The reactive groups potentially formed a facile binding with other functional molecules for specific reactions. The reaction with polyethers, for instance, can form flexible spacers that allow efficient enzyme mobility and reduce steric interference. Consequently, the resultant favourable environment enhanced the storage and thermal stability of the native enzyme.

A combination of enzyme aggregate coating and co-
valent enzyme attachment has been explored for fabricating enzyme–polymer NF assemblies. A thin layer of enzyme molecules were covalently linked to the surface of polymer NFs. When more enzyme molecules were added, they were cross-linked to the first layer of molecules, leading to a cross-linked enzyme aggregate coating (figure 3c) [81]. Enzyme molecules can also be encapsulated inside the polymer NFs by co-electrospinning the enzyme in solution with a water-soluble polymer. Consequently, successful enzyme immobilization is achieved by cross-linking the polymer. Alternatively, NBCs can be formed by coaxial electrospinning with one or multiple aqueous enzymes in the core and a water-insoluble polymer in the shell [82].

3.2. Silica nanocarriers
Enzymes can be physically adsorbed onto mesoporous silica NFs (MSNs) (figure 4b), mainly through hydrophobic/ hydrophilic and electrostatic interactions. By tuning the surface properties including the pore geometry, surface charge density and functional groups, mesoporous silicates have been used to adsorb a variety of enzymes [29,54,83]. We have listed several examples of cross-linking enzymes inside the pore and physical encapsulation or entrapment inside a nanocontainer made from silica-based nanocarriers in §2.2 and 2.3.

Covalent attachment is realized by using cross-linking agents such as glutaraldehyde and carbodiimide derivatives. Zhang et al. [54] studied the functionalization of silica NFs by introducing aldehyde, cyanogen, epoxy or carbodiimide groups. The –NH₂ and –COOH groups in the lysine residues of enzymes react with aldehyde- and epoxy-activated silica NFs. Multiple covalent attachments (figure 3d) are formed in the reactions using these functional groups. Such modifications were applied for immobilization of Rhizobium etli CFN42 xylitol dehydrogenase to synthesize various rare sugars. Rekuc et al. [84] experimentally revealed that laccase immobilization was also enhanced by readily available functional groups such as –NH₂, -OH and oxirane rings from the MSN support.

Using the covalent binding technique, the enzyme is anchored to the surface through any one or more reactive sites. The enzyme displays high efficiency in biocatalysis on some active sites, while the enzyme’s activity on other sites could be lost. Targeted immobilization has been investigated through specific and focused binding (figure 3e). For example, affinity binding encourages specific molecule bioconjugation onto silica-based supports. It has been implemented on ferric silica NFs by grafting long alky groups for lipase binding [9]. The affinity binding resulted in an excellent lipase binding efficiency up to 97%, leading to enhanced stability without major loss of transesterification activity. Besides affinity grafting, metal introduced onto the MSN surfaces can specifically bind to a histidine tag on the enzyme. Cu²⁺ was employed to attach laccase onto mesoporous silica [15]. Consequently, affinity binding showed a higher adsorption capacity (98.1 mg g⁻¹ particles) and activity recovery (92.5%) than the physical adsorption procedure. Cu- and Ni-modified silicas were also used to specifically bind His-tagged lipase [85,86] and a protease inhibitor [87].

Another immobilization technique is to form a spacer arm between the enzyme and activated matrix [88]. One approach for spacer arms is carried out through amine activation using glutaraldehyde as a coupling agent. However, although the bridge molecules reduce enzyme leaching, their strong binding may decrease biological activity due to enzyme denaturation.

3.3. Carbon nanocarriers
Carbon-based nanocarriers have been widely employed for enzyme immobilization owing to their inertness, biocompat-
ibility and thermal stability [89,90]. Among them, carbon NTs (CNTs), nanodiamond and graphene derivatives appeared to be the most attractive nanocarriers for producing the NBC assembly. A variety of covalent binding and physical adsorp-
tion methods have been studied using pristine or functional CNTs. CNTs possess a hydrophobic nature which is essential for driving enzyme binding. Enzyme deposition onto single-walled CNTs can be achieved via simple physical adsorption without chemical modifications through sequential steps [14]. Using an ionic adsorption technique, α-amylase and urease immobilized onto two-layer alumina silicate halloysite NTs have shown promising stability and reusability [91]. The different charges between the NT surface and enzymes enhanced the binding. Both enzymes retained more than 55% of their initial activity after seven cycles of separation and reaction, and preserved more than 90% of their activity after 15 days of storage. This simple physical adsorption can also be applied to un-modified multi-walled CNTs surfaces, such as lipase adsorption. Prainovic et al. [60] found that the time required for complete enzyme adsorption depended on the ionic strength of the medium. Based on their observation, the ionic strength enhanced hydrophobic interactions which led to successful enzyme immobilization. In addition, lipase
itself contains approximately 28–33% of hydrophobic amino acids, and thus promotes the enzyme to be in the open-lid position [92], which can be a driving force for initiating the adsorption process.

Covalent binding has been reported for modified CNT carriers via inducing the reaction of free amine groups on the surface of a protein with carboxylic acid groups on CNTs, confirmed by TEM images in figure 4c. The carboxylic acid groups were introduced by oxidation of CNTs and activation using carbodiimide [93,94], Lipase [95,96], organophosphorous hydrolyase [97,98] and other enzymes have been successfully immobilized onto CNTs. Alternatively, enzyme molecules are covalently attached to linking molecules on the CNT surface via hydrophobic or π–π interactions. Linking molecules can provide specific sites for CNTs to immobilize enzymes, exemplified by 1-pyrenenbutanoic acid succinimidyld ester for horseradish peroxidise [99], a PEG-based spacer for perhydro-lase SS4 [100] and Na,Na-bis(carboxymethyl)-t-lysine for His-tagged NADH oxidase [101].

Other carbon-based nanomaterials show similar chemical and physical properties of CNTs and, therefore, similar methods can be used for immobilizing enzymes onto these materials. For example, trypsin was covalently immobilized onto detonated nanodiamonds (3–10 nm) [102], and noncovalent bonds were formed between the co-enzyme of glucose oxidase (GOx) and glucoamylase and chemical reduced graphene oxide nanocarriers [103].

### 3.4. Metal-based nanoparticles or composites

Metal-based nanomaterials have been widely used for fabricating NBCs. Among them, magnetic NPs are one exceptional example due to their high recyclability for biocatalysts. Functional groups, such as amino, carboxylate, thiolate or phosphate, are introduced onto the metal NP surfaces to create strong interfacial reactions with enzyme molecules, and consequently to enhance enzyme immobilization. Excellent enzyme immobilization on nanoporous gold (NPg) biocomposite (figure 4f) resulted in an increase in both biocata-lytic performance and enzyme stability [104]. Carbodiimide linkage, for instance, was applied to activate magnetic NPs for cholesterol oxidase immobilization [105] and to modify gold NPs for GOx immobilizers [106,107]. Multifunctional groups allow enzyme and cofactors, or multi-enzymes to bind onto one single metal NP, facilitating multireaction in a one-pot medium [108]. This catalyst mixture circumvents the laborious and multi-stage operations by shortening long reaction pathways. Amine and carboxylic acid were grafted on multifunctional Fe3O4 NPs using robust silane linkages [109]. Mediator thionine, enzyme horseradish peroxidase and secondary anti-human IgG antibody were loaded concurrently onto the modified Fe3O4 NPs to produce a highly sensitive label for diagnosing pancreatic cancers.

A thin layer of polymers can be coated onto the surface of metal NPs, on which functional groups are then interacted with enzyme molecules to achieve stable immobilization. Chitosan, PEG, polyvinyl alcohol and polyethyleneimine (PEI) have been explored for the coating purpose [110,111]. Targeted immobilization onto metal NPs can be achieved through affinity interactions, such as antibody–antigen, and streptavidin–biotin. Biotin groups are first introduced into metal NPs, and then streptavidin is bound to biotin-coated NPs and acts as a spacer arm for the conjugation of biotinylated enzymes. Here are a few excellent examples for immobilization of catalase on activated Au NPs [112] and GOx on apolisin NPs [58]. Affinity peptides are often used for modification of the metal NPs as well. The gold-binding peptide was the first example of engineered inorganic-binding peptides. Multiple repeats of the peptide were used for directing enzyme self-immobilization on the gold surface [113]. Ligand as a bridging unit in protein bioconjugation can also be modified to promote multifunctional features. Susumu et al. [114] modified ligand molecules with dihydrioplic acid and PEG terminal ends, promoting a simultaneous coupling of biomolecules and dyes to quantum dots. Metal NPs can also be incorporated into CNTs, mesoporous silicas and polymer nanogels or NPs to synthesize NCs. Fabrication strategies for enzyme immobilization methods onto these NCs are similar to those described above.

There are no universal strategies for constructing NBCs in different processes. The characteristics and relative benefits of the different categories of NBCs are summarized in table 2. Selection of a specific immobilization strategy depends on many factors, including enzyme molecular structure, the application process, and nanocarrier and surface functional groups. However, any strategy should aim to maintain or improve enzyme functional properties (activity, stability, selectivity and specificity) and should be applicable for the NBCs in a bioprocessing environment.

### 4. Engineering performance of nanobiocatalysts

Exploitation of NBC technologies is still in the infant stage in the bioprocessing industry. Process-related traits of the NBCs are not fully understood. The success of NBC technology in the large-scale manufacturing processes relies on four key features: (i) specific activity under the process conditions; (ii) stability of the NBCs when exposed to pH/temperature variations, organic solvents, high shear stress and other harsh environments; (iii) reusability of biocatalysts and (iv) high throughput for large-scale processes. It is economically and technically crucial that the NBCs are able either to maintain stable activities in the long term in a continuous process, or to be recycled for re-use in a batch operation process for many runs in which the NBCs are separated from the reaction media after the reaction is completed. For a continuous operation, stability of enzyme activity as well as reduction of enzyme leakage are the main targets for immobilization, while for batch operations, recyclability and constant enzyme activity of NBCs are the key challenges.

#### 4.1. Enzyme activity and stability

It is believed that immobilization onto solid supports could reduce enzyme activity, such as bovine serum albumin [89], penicillin acylase [115] and β-galactosidase [56]. This belief may not be true when enzymes are immobilized onto nanocarriers. There are many studies on the enhancement of enzyme activity after the enzyme is bioconjugated with nanocarriers. The mechanisms of enhancement of enzyme activities through nanocarrier immobilization have been discussed in detail in a recent review paper [116]. The comparison of the kinetic parameters between free and immobilized enzymes, as well as the key contributions for performance improvement is summarized in table 3. It is generally accepted that the enhancement of enzyme activity and stability may be due to
enzyme conformation change. Immobilization of enzymes onto nanocarriers results in stabilization of active conformation, which promotes the interfacial reactions between the substrate and enzyme reactive sites (figure 5a), while the soluble enzyme in the solution has freedom of conformation change. Multiple point covalent bonding via short spacer arms on nanocarriers may be a powerful strategy to improve enzyme activity [9,56], while physical adsorption of lipase onto nanocarriers via hydrophobic interactions significantly increases enzyme activity because the open form (active in catalysis) is stabilized when the large hydrophobic groups are exposed to hydrophobic surfaces of nanocarriers as evidenced by Palomo et al. [121] and Chen et al. [122]. Conformation change may also be induced via some molecules presented in the surfaces of nanocarriers. α-Amylase underwent a conformation change by binding to Ca\textsuperscript{2+} inside CaHPO\textsubscript{4} nanocarriers, resulting in a large fraction of enzymes in their allosterically activated form and enhanced enzyme activities [120].

Increased and prolonged enzyme activity can be ascribed to the enhanced stability of nanocarrier–enzyme complexes as well. Enzymes may become denatured due to interactions with hydrophobic interfaces. The enzymes may also become deactivated due to formation of aggregates at the isoelectric point, at high temperature, or in the presence of salts or solvents. Changes in enzyme structure tend to decrease enzyme activity. Through immobilization onto nanocarriers, enzymes are shielded from denaturing microenvironments [123], resulting in a higher enzyme activity than the free enzyme. For example, the curvature of CNTs reduced the detrimental interactions between enzyme molecules due to the longer distance between molecules, thereby leading to higher enzyme stability on single-walled CNTs in comparison to flat graphite [124]. Covalent binding mediated by carbodiimide formed a stable bond between amino groups of enzyme and carboxyl groups of functionalized supports [125]. Consequently, it reduced the mobility of protein structure. The rigid structure of the NBCs could promote the enzyme stability to withstand relatively harsh system and operation conditions compared to free enzymes. Stabilization of β-galactosidase through multipoint covalent attachments was obtained on magnetic Fe\textsubscript{3}O\textsubscript{4}–chitosan NPs [56]. Jordan et al. [126] has revealed a thermal improvement by stabilizing the weak ionic forces and hydrogen bonds through NP activation using a carbodiimide cross-linker. A remarkable stability of cellulases was observed at a broader range of temperatures with an optimum enzymatic activity at 50°C. Trypsin-coated NPs showed a super capability to maintain the enzyme activity after the NBC assembly was repeatedly used for 1 year and to exhibit great resistance to proteolysis, paving the way for industrial applications [127].

Localized nanoenvironment is an important approach to the improvement of enzyme activity. The nanoenvironment surrounding enzyme molecules may prevent enzyme deactivation. For example, a hydrophilic environment may reduce the concentration of hydrophobic organic solvents or some gases near to the enzyme may prevent loss of enzyme activity, while a hydrophobic environment may tremendously reduce the detrimental effect of very hydrophilic deactivating molecules, like hydrogen peroxide [128]. The nanoenvironment may provide localized optimal reaction conditions for the immobilized enzyme, such as pH, ionic strength and temperature. The presence of peptides on Au NPs helped construct a local pH of 7.7 in comparison to the pH of the bulk solution of 7.0, thus lipase reaction rates were 3–180 times higher than controls [129]. Gold and silver NPs acted as a conduction centre to facilitate transfer of electrons. Immobilization of redox enzymes onto colloidal gold or silver NPs may help the protein to make possible conducting channels between the prosthetic groups and the gold/silver surface [130]. The nanoenvironment may also facilitate the entry of substrate molecules into reactive sites of enzymes through a favourable partition or molecular

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### Table 2. Characteristics of different types of nanomaterials.

<table>
<thead>
<tr>
<th>Source</th>
<th>Polymer nanocarriers</th>
<th>Silica nanocarriers</th>
<th>Carbon nanocarriers</th>
<th>Metal-based NPs or composites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Natural and synthetic polymers</td>
<td>Silica</td>
<td>CNTs, diamond and graphene</td>
<td>Magnesium, quantum dots, gold and metal oxides</td>
</tr>
<tr>
<td>Configuration</td>
<td>NFs, nanogels, NPs, and nanocontainers</td>
<td>NPs, mesoporous structures and nanocontainers</td>
<td>Single-wall NT, multi-wall NT and nanofilm</td>
<td>NPs</td>
</tr>
<tr>
<td>Contributor of nanocarriers for NBCs</td>
<td>Functional groups available from polymers</td>
<td>Highly porous structures, introducing external functional groups</td>
<td>Hydrophobic surfaces, introducing external functional groups</td>
<td>Functional group or thin layers of polymers</td>
</tr>
<tr>
<td>NBC fabrication</td>
<td>Covalent binding; entrapment, cross-linking</td>
<td>Physical adsorption, entrapment, cross-linking, spacer arm</td>
<td>Physical adsorption, covalent binding</td>
<td>Introduced onto metal surfaces</td>
</tr>
<tr>
<td>Recycle</td>
<td>Attached to macroporous structures or magnetic particles</td>
<td>Attached to magnetic particles</td>
<td>Attached to magnetic particles or separated by density differences</td>
<td>Magnetic or centrifugal separation</td>
</tr>
<tr>
<td>Mechanical strength</td>
<td>Weak</td>
<td>Strong</td>
<td>Strong</td>
<td>Strong</td>
</tr>
</tbody>
</table>
### Table 3. Engineering performances of NBCs.

<table>
<thead>
<tr>
<th>nanocarries</th>
<th>enzyme</th>
<th>binding strategies</th>
<th>kinetic parameters of NBCs</th>
<th>kinetic parameter of free enzyme</th>
<th>contribution for improvement</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au NPs</td>
<td>GOx</td>
<td>carbodiimide</td>
<td>$K_m = 3.74$ mM</td>
<td>$K_m = 5.85$ mM</td>
<td>conformation change</td>
<td>[107]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$V_{max} = 1.42$ $\mu$M $\text{min}^{-1} \text{mg}^{-1}$</td>
<td>$V_{max} = 0.25$ $\mu$M $\text{min}^{-1} \text{mg}^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>magnetic NPs</td>
<td>cholesterol oxidase</td>
<td>carbodiimide</td>
<td>$K_m = 0.45$ mM</td>
<td>$K_m = 2.08$ mM</td>
<td>conformation change</td>
<td>[105]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$V_{max} = 1.64$ $\mu$M $\text{min}^{-1} \text{mg}^{-1}$</td>
<td>$V_{max} = 0.67$ $\mu$M $\text{min}^{-1} \text{mg}^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ferric silica NGs</td>
<td>lipase</td>
<td>hydrophobic interaction</td>
<td>$K_m = 3.65$ mM</td>
<td>$K_m = 0.09$ mM</td>
<td>multipoint binding</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$V_{max} = 131.4$ U mg$^{-1}$</td>
<td>$V_{max} = 133.3$ U mg$^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Au NPs</td>
<td>dehydrogenase (MDH) and/or</td>
<td>physical adsorption</td>
<td>$K_m$ (MDH) = 0.05 mM</td>
<td>$K_m$ (MDH) = 0.02 mM</td>
<td>cascade reaction</td>
<td>[117]</td>
</tr>
<tr>
<td></td>
<td>citrate synthase (CS)</td>
<td></td>
<td>$K_m$ (CS) = 6 $\mu$M</td>
<td>$K_m$ (CS) = 18 $\mu$M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$V_{max}$ (MDH) = 5.3 U mg$^{-1}$</td>
<td>$V_{max}$ (MDH) = 8.1 U mg$^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$V_{max}$ (CS) = 2.7 U mg$^{-1}$</td>
<td>$V_{max}$ (CS) = 73.6 U mg$^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Au NPs</td>
<td>lipase</td>
<td>electrostatic interaction</td>
<td>$K_m = 9.10$ $\mu$M</td>
<td>$K_m = 23.91$ $\mu$M</td>
<td>nanoenvironment</td>
<td>[118]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$V_{max} = 1.99$ $\mu$M $\text{s}^{-1}$</td>
<td>$V_{max} = 1.97$ $\mu$M $\text{s}^{-1}$</td>
<td>(presence of salt)</td>
<td></td>
</tr>
<tr>
<td>nickel-impregnated silica NPs</td>
<td>diastase</td>
<td>physical adsorption</td>
<td>$K_m = 8.414$ mM</td>
<td>$K_m = 10.176$ mM</td>
<td>affinity to substrate</td>
<td>[119]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$V_{max} = 4.92$ $\mu$M $\text{min}^{-1} \text{mg}^{-1}$</td>
<td>$V_{max} = 2.71$ $\mu$M $\text{min}^{-1} \text{mg}^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaHPO$_4$ nanocrystals</td>
<td>$\alpha$-amylase</td>
<td>allosteric modulation</td>
<td>$K_m &gt; 0$ mM</td>
<td>$K_m = 0.028$–$0.073$ mM</td>
<td>conformation change</td>
<td>[120]</td>
</tr>
</tbody>
</table>
gatekeepers. Cationic substrates were favoured over anionic substrates when Au NPs were coated with anion peptides for α-chymotrypsin [131].

Multisubunit and coenzyme immobilized on the same nanocarriers may also contribute to the enhanced enzyme activity. The activity and specificity of multimeric enzymes depend on the architectural structure of subunits. Immobilization of subunits on the same nanosupports can prevent subunit dissociation. In a cascade reaction, two enzymes immobilized in a short distance can accelerate the biocatalysis reactions since the diffusion paths of intermediates are reduced (figure 5b). Glucose oxidase–horseradish peroxidase immobilization onto magnetic NPs was used to demonstrate the concept [132].

Nanocarriers may also restore the enzyme activity of a functionally impaired enzyme. An isolated extracellular pectate lyase had a very low native state activity, but the activity was regained by immobilization of the enzyme onto hydroxyapatite NPs. The activity increased 27.7-fold with a 51-fold increase in half-life at a temperature of 90°C as compared with untreated one [133].

4.2. Reusability of nanobiocatalysts

Feasible reutilization of the biocatalyst is an extremely important requirement in translating NBCs into commercialization. Recovery of NBCs can be a significant issue and downstream processing for their recuperation after the reaction is very complex. Microcarriers or microbeads, however, can be easily separated from reaction medium due to the density difference. Integration of nanostructure with micro- or macrostructures can facilitate the separation of NBCs. Nanostructured microparticles can offer the nanoenvironment for enzymatic catalysis and reusability for easy separation. Integration of magnetic technology with the enzyme immobilization on the nanocarriers can enhance recoverability and reusability of the NBCs [134]. NBCs can be separated from the reaction media by simply turning on the magnetic field and attracting magnetic nanocarriers onto magnetic sources. However, some NBCs may be carried away by the reaction media due to weak magnetic forces. Ngo et al. [135] further improved this technique through controlling the size of magnetic silica NPs. The particles were dissociated for enzyme loading and reaction, while those particles were re-clustered for easy magnetic separation. This magnetic technique can also be applied to polymer-based nanocarriers. Excellent magnetic response was obtained by coating the magnetic particles with polyaniline [61].

Reutilization is not only applied to nanocarrier–enzyme conjugates, but also to the nanocarriers. Many research findings are focused on the reuse potential of immobilized enzymes. However, most of the supports are discarded after enzyme inactivation. Nanocarrier recyclability can lead to a significant reduction in the bioprocessing cost. The synthesis of Au-doped magnetic silica NPs for enzyme immobilization demonstrated nanocarrier recyclability [136]. Cysteine-tagged cellulase, endo-glucanase, exo-glucanase and β-glucosidase were co-immobilized on the Au NP surface and easily removed by rinsing with water as the enzyme activities were decayed (figure 5c). The NPs could be recycled and re-used over four times, signifying their robustness against harsh surroundings. Although recycling biocatalysts and nanocarriers through magnetic NPs discussed above can reduce the operation costs, the technique is still being trialled and challenges remain for industrial-scale operations. Meanwhile, microcarriers are easily separated through sedimentation or centrifugation due to their high density. Nevertheless, in contrast to nanocarriers, surface modification such as fine pore adjustment remains the key challenge in microcarriers.

4.3. Processability of nanobiocatalysts

Although an NBC can achieve a much higher enzyme loading capacity, enzyme activity and stability, as well as mass transfer efficiency, processability of NBCs is limited by the availability of the materials for enzyme carriers and costs for manufacturing nanocarriers. Most nanocarriers as enzyme immobilizers reported in the literature were produced in laboratory trials in small quantities. Further studies on economic analysis of overall costs of NBCs for a large-scale production process are needed. Among all nanocarriers discussed above, polymer-based NFs have the tremendous potential for scaling up and meeting the requirement for industrial applications in terms of throughput, reproducibility and functionality [137]. Another approach to reduce the enzyme purification-associated cost is to combine the immobilization and purification in the same
step. Functional groups of nanocarriers allow selectively binding to target enzymes for immobilization and removing impurities [21]. A high transesterification activity was obtained through simultaneous enzyme purification and immobilization. The NPs were first coated with PEI since PEI was rich in amino groups, which led to a greater binding selectivity and twofold purification of the enzyme. As a consequence of the purification, this technique tremendously increased the initial activity to be about 110 times that of the unpurified free enzyme.

Weak mechanical strength of nanocarrier–enzyme bioconjugates is another significant issue which limits NBC technologies for wide industrial bioprocesses. A suitable material with strong mechanical strength is needed to support nanocarriers when the bioconjugates are located inside a reactor. Cellulose acetate-based NPs are robust materials with a comparatively high modulus and tensile strength, which are able to bear harsh environments such as agitation and shear force [138]. A protein digestion column has been developed for trypsin–nanoporous silica but trials have not been successful due to a short lifetime of the column [139]. Operation at a low hydrodynamic shear is preferable, for example, using a packed-bed reactor or monolith column [140]. Four packed-bed reactors have been developed to accommodate bioconjugates in biodiesel production, which provided a longer residence time to enhance the bioconversion rate [141].

Aggregation or clustering of nanocarriers in the reaction medium can potentially reduce the surface area of the nanocarriers, leading to low enzyme loading and mass transfer efficiency. It can be a technical challenge to homogeneously distribute enzyme on the nanocarriers during the enzyme immobilization process. Shear may be inserted in the system to prevent aggregation, but this may also damage the fragile structure of enzymes–nanocarrier bioconjugates. For example, polystyrene-co-maleic anhydride) NPs are hydrophobic and thus are unlikely to be dispersed in aqueous solution. Nair et al. [142] reported an immobilization procedure to use ethanol solution to wash the NPs before immobilizing enzymes. After enzyme loading, the enzyme–NF complexes were well dispersed in the solution and a continuous flow biocatalysis reactor can be further constructed.

5. Applications of nanobiocatalysts in processes

Immobilized enzymes have been used for large-scale industrial processes, such as glucose isomerase for production of fructose corn syrup (10^5 tons per annum), lipase for transesterification of food oils (10^5 tons per annum) and penicillin G acylase for antibiotic modification (10^3 tons per annum) [143]. However, the reported studies on the development and application of nanocarrier-based NBCs for bioprocesses are still carried out in laboratory-scale bioreactor or biodevice system. A successful case using NBCs in industrial bioprocesses has not been found in the literature so far. Here, we report the areas which have benefited from these advances, signifying the prospect of designing and operating NBC-based systems in an industrial bioprocess.

5.1. Carbohydrate hydrolysis

Cellulose, starch, empty fruit bunch and microalgae are the commonly used and abundant carbohydrates, which can be potentially hydrolysable substrates for production of valuable chemicals and marketable products. Hydrolysis of these carbohydrates into lower sugars is an essential step. Conventional hydrolysis can be conducted by physical, chemical and/or physio-chemical processes using strong acids or bases under a high temperature and/or a high pressure. Enzymatic hydrolysis has been recognized as an ideal alternative to alter the structure of lignocellulosic, cellulosic and starch materials. Cellulase mixture, which is composed of endoglucanases, cellobiohydrolases and β-glucosidase, has been explored to breakdown lignocellulosics [126,136]. Nanocarriers allow simultaneous multiple enzyme co-immobilizations and enhance enzyme stability and activity.

Physical adsorption of cellulase onto superparamagnetic NPs generated a binding efficiency of nearly 100%, and the adsorption capacity reached about 31 mg g⁻¹ support [144]. Immobilization on the magnetic nanobeads has led to great pH tolerability of cellulase particularly in alkaline conditions, as well as great stability for long-term storage. Nevertheless, the immobilized enzyme showed a lower biological activity than free enzyme at all tested operating temperatures. Verma et al. [65] attached β-glucosidase onto magnetic NPs via glutaraldehyde activation, yielding an immobilization efficiency of about 93%. The cellulose hydrolysis was significantly increased at 70°C in comparison to the free enzyme. Immobilization of the cellulase mixture onto Au-doped magnetic silica NPs was trialled for the degradation of cellulose [136]. The binding of Au NPs on the surface of functionalized silica NPs created attachment sites for cysteine-tagged enzymes. Although the optimal pH curves were quite similar between the immobilized enzyme and free counterpart, greater pH stability of the immobilized enzyme was demonstrated. The most noteworthy observation was the extension of the lifetime of the enzyme during thermal inactivation measured at 80°C from 24 to 36 h (figure 6). The immobilized enzyme demonstrated enhanced stability and reusability for up to seven cycles.

Starch hydrolysis is performed by two enzymes at different operating conditions. Initially, α-amylase hydrolyses starch via a gelatinization and liquefaction process at 105°C [147]. The gelatinized starch is further saccharificated using glucoamylase at a lower temperature (50–60°C) to form reducing sugars mainly composed of glucose. The conjugates of NPs and α-amylase have been used for fast degradation of starch [148]. The product formation rate was increased 1.5 times and the enzyme activity was found to be about 4.7-fold higher than the free enzyme, respectively. The presence of starch during the AgNPs synthesis process was a probable reason for the fast reaction. The starch initially converted silver nitrate into Ag NPs to embed Ag NPs in the helical core of starch molecules. The enzyme degraded the starch helical structure and immobilized onto Ag NPs while the catalysing sites were kept open towards free starch molecules, which led to a rapid degradation of 9.9 mg free starch within 5 min. High productivity and fast reaction make this discovery economically viable for large-scale production of reducing sugars. In addition, the resulting thiol linkages were able to prevent the particles from forming aggregation. Amyloglucosidase, another starch-hydrolysing enzyme, was conjugated onto single-walled CNTs by Goh et al. [14]. The production cost was defrayed through recycling magnetic NPs-assisted NBC assembly. This study demonstrated that amyloglucosidase retained 40% of its biomass hydrolysis activity within 10 cycle operations. Significant stability was also observed after at least one month storage at 4°C.
5.2. Biofuel production

Environmental concerns and fossil fuel shortages have profoundly driven the development of a green and sustainable bioprocessing strategy for biofuel generation through enzymatic technologies. Biofuels derived from bioconversion, such as biodiesel, bioethanol, biohydrogen and biogas, are sustainable and renewable energy sources. Enzymes have been used to replace conventional chemical catalysts. For example, biodiesel production by lipase-based bioprocess is a less energy intensive and more environmentally friendly technology compared with its production by conventional alkaline catalysed processes. Another attractive example is the use of enzymes for the hydrolysis of cellulose to produce fermentable sugars for bioethanol production. Nanocarriers have been explored to immobilize enzymes for enhancing their activity and stability.

Biodiesel is normally synthesized in the presence of organic solvents at a high operating temperature. Nanocarriers have been demonstrated to form chemical and thermal-tolerance NBCs with a high enzyme loading and enhanced catalytic activity. Core-shell ferric silica NCs have been fabricated as a host for Burkholderia sp. lipase [9]. Alkyl groups were grafted onto the NCs to create an affinity linker. Binding efficiency was determined to be nearly 100%, demonstrating the affinity can successfully facilitate the binding of lipase. The transesterification activity of biodiesel production was sustained up to 10 cycles. The activity enhancement was also demonstrated by confining Pseudomonas cepacia lipase onto PAN NFs [57]. Using the physical adsorption method, the enzyme activity was 23-fold higher than that of the soluble enzyme. The lipase consistently retained 80% of its initial activity for up to 10 batch reactions. Using the same support, Li et al. [145] managed a higher transesterification activity through covalent binding. They initially activated the PAN nitrile groups using amidination reactions prior to reacting with the amino-carrying enzyme. Surprisingly, the immobilized lipase retained almost 100% of the transesterification activity over 10 repeated cycles. Furthermore, the system successfully maintained the same productivity after 20 day storage at 30°C, demonstrating that the enzyme's stability was significantly improved (figure 6b). To produce a solvent and temperature friendly nanosystem, Wang et al. [104] fabricated NPG biocomposites that showed exceptional enzymatic durability against thermal and organic environments. Constructed at a pore size of 35 nm, the enzyme-carried composites allowed encapsulating a large number of biomolecules at various sizes either via adsorbing within the pores or on the external surfaces. The NBC-driven system demonstrated a high conversion rate of soya bean oil into biodiesel at a longer reaction time for up to 240 h and retained enzymatic activity.
up to 10 successive batch reactions (figure 6c). The tolerability test over various organic solvents and operating temperatures demonstrated the confined lipase exceeded the free enzyme outstandingly. The enzyme was protected inside the small pores of the NPG composites, which provided a sufficient space for substrate molecular mobility as well as retention of enzyme molecules with less leaching. These remarkable performances on the laboratory-scale pave the way to commercialization of this technology.

β-glucosidase is often used to relieve the product inhibition of cellobiose in cellulose ethanol production by converting cellobiose into glucose. The enzyme was coated on NFs to form enzyme aggregates that increased the productivity [149]. The activity of enzyme aggregates was approximately 36-times higher than that of covalent-linked enzymes. The ethanol production from enzyme aggregates was 2.1 times higher. More importantly, after 20-day incubation under a vigorous shaking condition, the activity of enzyme aggregates turned out to increase drastically from 33 to 91%. The tremendous improvement in stabilization may be attributed to the multipoint linkages which may prevent denaturation. Although the development of NBCs is in the infant stage, their contribution in biofuel production has become the major driving force in future research and innovation for bioprocessing engineering.

5.3. Biotransformation

Biotransformation through enzymatic reactions has been explored for production of valuable products including drug intermediates and functional food ingredients [22]. β-Galactosidase is generally used to convert lactose-rich dairy wastes into galacto-oligosaccharide (GOS), lactulose and lactosucrose. The bioprocessing performances of the enzymes immobilized onto conventional supports are reported to be less promising. Nanocarriers have been explored for improving the enzyme loading capacity and activity. To improve the GOS production rate, Liu et al. [150] used covalent immobilization of the enzyme onto magnetic poly(GMA-EDGMA-HEMA) nanospheres. Epoxy groups on the nanosphere surfaces were reacted with enzyme nucleophilic through a condensation method. A total of 145.6 mg enzyme g⁻¹ support was successfully adsorbed, indicating a large surface area was available for enzyme attachment. Approximately 2240 g of GOS per gram adsorbed enzyme was produced. Instead of using the condensation technique, they further grafted PEI onto the nanospheres as a platform for ionic adsorption. Despite relatively lower enzyme adsorption (86.7 mg g⁻¹), the ionic binding however resulted in a higher GOS yield of 4500 g per gram adsorbed enzyme (figure 6d). The latter retained about 84.6% of its initial activity up to 15 cycles of reactions, whereas the former only showed 81.5% within 10 operations. In a continuous lactulose synthesis, glutaraldehyde was applied to attach β-galactosidase onto the NT microchannel surface [151]. The system successfully mimicked the current industrial practice at a high lactose conversion rate of 78.3%. Moreover, the system constantly maintained the product concentration at about 1.29 g l⁻¹ for 48 h, operating at a flow rate of 2.5 μl min⁻¹. The continuous process is economically viable and very promising for large-scale production of lactulose.

NBCs enable catalytic cascade processes in one site to reduce laborious and multistep reactions and promote active reactions in non-aqueous media, mainly in the production of chiral drugs and their precursors. Malate dehydrogenase (MDHase) and/or citrate synthase (CSase) have been co-immobilized onto Au NPs with a diameter of 30 nm for conversion of malate to citrate [117]. The bioconjugates were prepared either by directly adsorbing MDHase followed by CSase, or vice versa, or co-adsorbing two enzymes in the same solution. Higher specific activities and favourable kinetic parameters were obtained by bioconjugating CSase to the NPs before MDHase. In this condition, the individual specific activity for MDHase and CSase were 5.3 U mg⁻¹ and 2.7 U mg⁻¹, while the $K_m$ values were 0.05 mM and 6 μM, respectively. The system promoted efficient sequential reactions by one-step biotransformation, in which the products of MDHase served as substrates for the subsequent CSase reaction. This co-enzyme immobilization leads to a low cost and clean process due to intense reduction in the usage and release of organic solvents.

6. Conclusion remarks

NBCs, in which enzymes are incorporated into nanostructured materials, have emerged as a rapidly growing R&D field of nanobiotechnology, which employs and integrates two advanced technologies: nanotechnology and biotechnology. The perspectives of the NBC technology have a bright future as well as exciting challenges. These fascinating challenges require joint interdisciplinary collaboration of chemists, engineers and material scientists. Recent development in nanotechnology has provided a wealth of diverse nanoscale scaffolds that could potentially be applied to the flourishing of NBC-driven industrial bioprocesses. A specifically functionalized nanocarrier–enzyme assembly promises exciting advantages for improving enzyme stability and activity by creating unique nanoenvironments surrounding the enzyme catalysts for maximal reaction efficiencies. Furthermore, enzyme immobilization using nanostructure carriers can significantly increase life cycles of the biocatalyst for its reuse, hence reducing the cost of the biocatalytic process. The integration of enzymes with nanocarriers leads to a hybrid assembly that combines the biocatalytic and specific properties of enzymes with the unique functions within the nanostructure network. However, how to immobilize multi-enzymes on the functionalized nanocarriers to form NBC assembly for a continuous process still remains a challenge. Their small size means that the downstream processing for their recuperation after the reaction is highly complex. Nanostructured microparticles may offer great advantages from both the nano and micro worlds for future enzyme carrier trends. Furthermore, there is a lack of fundamental understanding of the generic mechanisms and interfacial reactions associated with the nanomaterial–immobilized enzyme system. Relationships between the enzyme molecules and nanocarrier properties have not been studied systematically so far. Such fundamental information is significantly important in the development of the multi-enzyme biocatalytic process. Especially for the cofactor-dependent multireaction systems, how to achieve dynamic interactions among cofactor, enzymes and substrates still awaits fundamental in-depth studies. The exploration of multistep enzyme-based bioconversion for large-scale operations, particularly involving cofactor-dependent enzymes, presents a profound research interest. It seeks advanced knowledge and innovative technologies for (i) regenerating and reusing the
NBCs to minimize the operation costs, (ii) enhancing the stability and performance of the NBC assembly and (iii) manipulating multi-enzyme-catalysed reaction kinetics for maximizing enzyme activity and product yield.

During the catalysis of a number of bioprocesses, biocatalysts promote green processes due to low chemical consumption and releasing non-toxic by-products to the environment. A range of nanocarriers derived from polymers, silica, carbon and metal have been explored to immobilize enzymes or multi-enzymes, making functional and active NBCs. The NBCs demonstrated enhanced enzyme activity and stability, high enzyme loading capacity and recyclability for bioprocesses. Critical challenges still remain for commercialization. Firstly, cost reduction is the major hurdle for large-scale trials. The major cost is associated with enzyme preparation, immobilization and nanocarrier synthesis. Recent advances in protein engineering, fermentation and purification technology allow large-scale production of enzymes, while most nanocarriers are still synthesized on the laboratory scale. Secondly, long-term operation of NBCs is required to demonstrate the feasibility of a large-scale operation process. Most NBCs developed in laboratory trials only demonstrate proof-of-concept and very few are run up to 10–15 cycles. Stability, activity, leakage and mechanical strength should be evaluated for long-term operation. Thirdly, bioprocess engineers should play a significant role in translating these bench-scale technologies into commercial practices. Most NBCs are developed by material scientists and the potential of such NBCs has been raised. However, the realization of the potential relies on bioprocess engineers. Engineering issues should be considered in the early stage of assembling NBCs. With efforts from material scientists, bioprocess engineers and biochemists, advanced multifunctional NBCs could be effectively commercialized in the very near future.

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