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REVIEW ARTICLE

New nanostructured carriers for cellulase immobilization

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Abstract

Objectives. Cellulase is a multienzyme complex that breaks down cellulose contained in plant cell walls. Cellulase consists of three types of enzymes: endoglucanase, exoglucanase, and β-glucosidase, each of which is involved in the destruction of certain chemical bonds in cellulose. Nanobiocatalysts based on cellulase immobilized on nanostructured carriers are used for catalytic hydrolysis of biomass waste, as well as in the food industry and for environmental protection. This article reviews scientific developments in the immobilization of cellulase on nanostructured carriers.

Methods. The article analyzes scientific papers published over the past five years that concerned the main aspects of immobilization of cellulase, an enzyme for processing cellulose biomass waste, on nanostructured carriers. The article examines methods of cellulase immobilization, the morphology of nanostructured carriers, and the factors affecting the enzyme activity and allowing one to achieve maximum conversion of cellulose-containing waste of plant origin.

Results. Nanostructured carriers have a large surface area, providing high immobilization efficiency, and also create a favorable environment for activating cellulase and increasing its stability. This allows one to create nanobiocatalysts for efficient conversion of cellulose substrate. The conducted analysis of the latest trends shows that positive changes have occurred in immobilization methods and carrier compositions over the past five years. The article describes such nanostructured carriers as graphene layers, polymer nanoparticles, nanohydrogels, nanofibers, silica nanoparticles, hierarchical porous materials, and magnetic nanoparticles.

Conclusions. Magnetically separable carriers increase the reliability of the biocatalyst and facilitate biocatalytic processes. The use of magnetic nanoparticles is especially advantageous due to their easy separation and the possibility of extracting the nanobiocatalyst for reuse.

Keywords

cellulase, immobilization methods, nanostructured carriers, processing of lignocellulosic biomass

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ОБЗОРНАЯ СТАТЬЯ

Новые наноструктурированные носители для иммобилизации целлюлаз

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Аннотация

Цели. Целлюлаза — мультиферментный комплекс, расщепляющий целлюлозу, содержащуюся в клеточных стенках растений. В состав целлюлазы входят ферменты трех видов: эндоглюканазы, экзоглюканазы и β-глюкозидазы, каждый из которых участвует в процессах разрушения определенных химических связей в целлюлозе. Нанобиокатализаторы на основе целлюлазы, иммобилизованной на наноструктурированных носителях, используются для каталитического гидролиза отходов биомассы, а также в пищевой промышленности и для защиты окружающей среды. Цель настоящего исследования — представить обзор научных разработок по иммобилизации целлюлазы на наноструктурированных носителях.

Методы. Проанализированы опубликованные за последние пять лет научные работы, касающиеся основных аспектов иммобилизации целлюлазы — фермента для переработки отходов целлюлозной биомассы — на наноструктурированных носителях. Рассмотрены методы иммобилизации целлюлазы, морфология наноструктурированных носителей, а также факторы, влияющие на активность ферментов и позволяющие достичь максимальной конверсии целлюлозосодержащих отходов растительного происхождения.

Результаты. Наноструктурированные носители обладают большой площадью поверхности, обеспечивая высокую эффективность иммобилизации, а также создают благоприятную среду для активизации целлюлазы и увеличения ее стабильности. Это позволяет создавать нанобиокатализаторы для эффективного превращения целлюлозного субстрата. Проведенный анализ последних тенденций показывает, что за последние пять лет в методах иммобилизации и составах носителей произошли положительные изменения. Описаны такие наноструктурированные носители, как слои графена, полимерные наночастицы, наногидрогели, нановолокна, кремнеземные наночастицы, иерархические пористые материалы и магнитные наночастицы.

Выводы. Магниторазделяемые носители повышают надежность биокатализатора и облегчают биокаталитические процессы. Использование магнитных наночастиц особенно выгодно ввиду их легкого отделения и возможности извлечения нанобиокатализатора для повторного использования.

Ключевые слова

целлюлаза, методы иммобилизации, наноструктурированные носители, переработка лигноцеллюлозной биомассы

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1. INTRODUCTION

The scarcity of conventional fuels and trend toward their gradual abandonment due to environmental concerns necessitate the development of processes for processing plant waste into biofuels. Current technologies for processing lignocellulosic biomass into biofuels include many stages, of which the decomposition of cellulose to fermentable sugars (providing a substrate for bioethanol production) is the most important [1]. To ensure environmental safety in the processing of lignocellulosic biomass, biocatalytic processes involving enzymes that decompose cellulose to glucose are widely used [2–5]. However, the industrial use of such enzymes is limited by their low thermal stability, the presence of a wide range of impurities in enzymatic preparations, and the difficulty of separating enzymes from processing products, as well as the impossibility of their reuse. These disadvantages can be minimized or completely eliminated by immobilizing enzymes on various carriers. Such carriers preserve the secondary and tertiary structure of the enzyme to create favorable conditions for its interaction with the substrate. The optimal carrier can be selected based on the enzyme type and the features of the technological process [6, 7].

The attention of researchers is particularly drawn to the processes of enzyme immobilization on nanostructured carriers to form nanobiocatalysts [8–13]. Nanostructured carriers are materials containing nanometer-sized

elements (usually from 1 to 100 nm), including nanoparticles (NPs) of various shapes, nanorods, and nanofibers. Unlike conventional granular carriers, nanostructured materials minimize diffusion limitations of substrate mass transfer. In addition, their developed surface area favors the effective immobilization of enzymes to improve their location on the surface, which increases enzymatic activity [14, 15].

The present study reviews scientific developments in the immobilization of cellulase on nanostructured carriers. Cellulase is a multienzyme complex that breaks down cellulose contained in plant cell walls. Cellulase consists of three types of enzymes: endoglucanases, exoglucanases, and β-glucosidases, each of which is involved in the destruction of certain chemical bonds in cellulose [10, 16].

Lignocellulosic biomass contains polysaccharides (cellulose and hemicellulose), as well as the aromatic polymer lignin, the presence of which suppresses cellulose hydrolysis. Immobilization of cellulase on nanostructured carriers not only stabilizes the biocatalyst and makes it possible to reuse it, but also changes the surface charge of the enzyme, reducing its nonspecific binding to lignin and increasing its affinity for cellulose [17, 18]. In order to use cellulase to effectively hydrolyze cellulose [19], lignocellulosic biomass wastes generally require delignification using laccase from *Trichoderma asperellum* (another enzymatic catalyst) or by co-immobilizing several enzymes on a single carrier [20–22].

This review discusses publications of the last five years that study cellulase immobilized on nanostructured carriers. Particular attention is paid to cellulase immobilization methods, as well as to the types of nanostructured carriers, including magnetically separable ones. The advantages of using immobilized cellulase are described based on the analysis of biocatalytic processes for processing cellulose-containing biomass to obtain sugars.

2. CELLULASE IMMOBILIZATION METHODS

Known methods of immobilization of cellulase on nanostructured carriers include adsorption, encapsulation, entrapment in a polymer matrix, covalent attachment, and cross-linking [23]. The most common method is covalent binding by the formation of chemical bonds between functional groups in the cellulase molecule and reactive groups on the carrier surface. Due to its strong covalent bonds, the immobilized cellulase is highly stable and reusable while maintaining a sufficiently high activity. This immobilization method is necessary in industries where the stability of cellulase and the possibility of its reuse are especially important. At the same time, in industries where simplicity and cost-effectiveness are of primary importance—e.g., in small-scale agriculture or the food industry—preference is often given to physical adsorption, cross-linking, or entrapment methods [24]. Ultimately, the choice of cellulase immobilization method should be guided by specific application requirements and a reasonable balance between performance, stability, and cost.

2.1. Adsorption

The physical adsorption method has been used to immobilize cellulase on metal–organic frameworks (MOFs) [25, 26], composites based on iron oxide and acid-activated montmorillonites [27], as well as multiwalled carbon nanotubes (MWCNTs) [28]. Important adsorption factors are high surface area, a suitable carrier pore size, and opposite net charges of the enzyme and the carrier [29].

Costantini *et al.* [30] showed that, while pretreatment of lignocellulosic biomass with ionic liquids can be used to facilitate its hydrolysis, such ionic liquids may lead to the enzyme's destruction. Zhou *et al.* [25] studied the resistance of nanobiocatalysts based on several MOFs (with different metals) and physically adsorbed cellulase to ionic liquids. Zhou *et al.* [26] also found that the most effective way to protect immobilized cellulase from the negative effect of ionic liquids on enzymatic activity or to reduce desorption is to modify the carrier surface before adsorption. Enzyme desorption can be reduced by surface treatment with zeolitic imidazolate frameworks (ZIF-8, i.e., MOFs that consist of Zn^{2+} and 2-methylimidazole ligands), charge-changing compounds (e.g., chitosan), or hydrophobicity-changing macromolecules (such as polyethylene glycol) (Fig. 1).

Modification of Zr-containing MOF UiO-66[1] with amino groups increases the enzyme content on the carrier (from 220 to 350 mg/g) due to the formation of additional "anchors" (NH_2 groups) on the carrier surface [31].

Some studies demonstrated the possibility of modifying the biocatalyst following enzyme adsorption. Adsorption of cellulase on carbon nanotubes followed by treatment with sodium alginate provides increased stability of the biocatalyst [28]. In this case, the gradual decrease in activity with each reaction cycle (following seven cycles of repeated hydrolysis of carboxymethyl cellulose, the activity of immobilized cellulase remains

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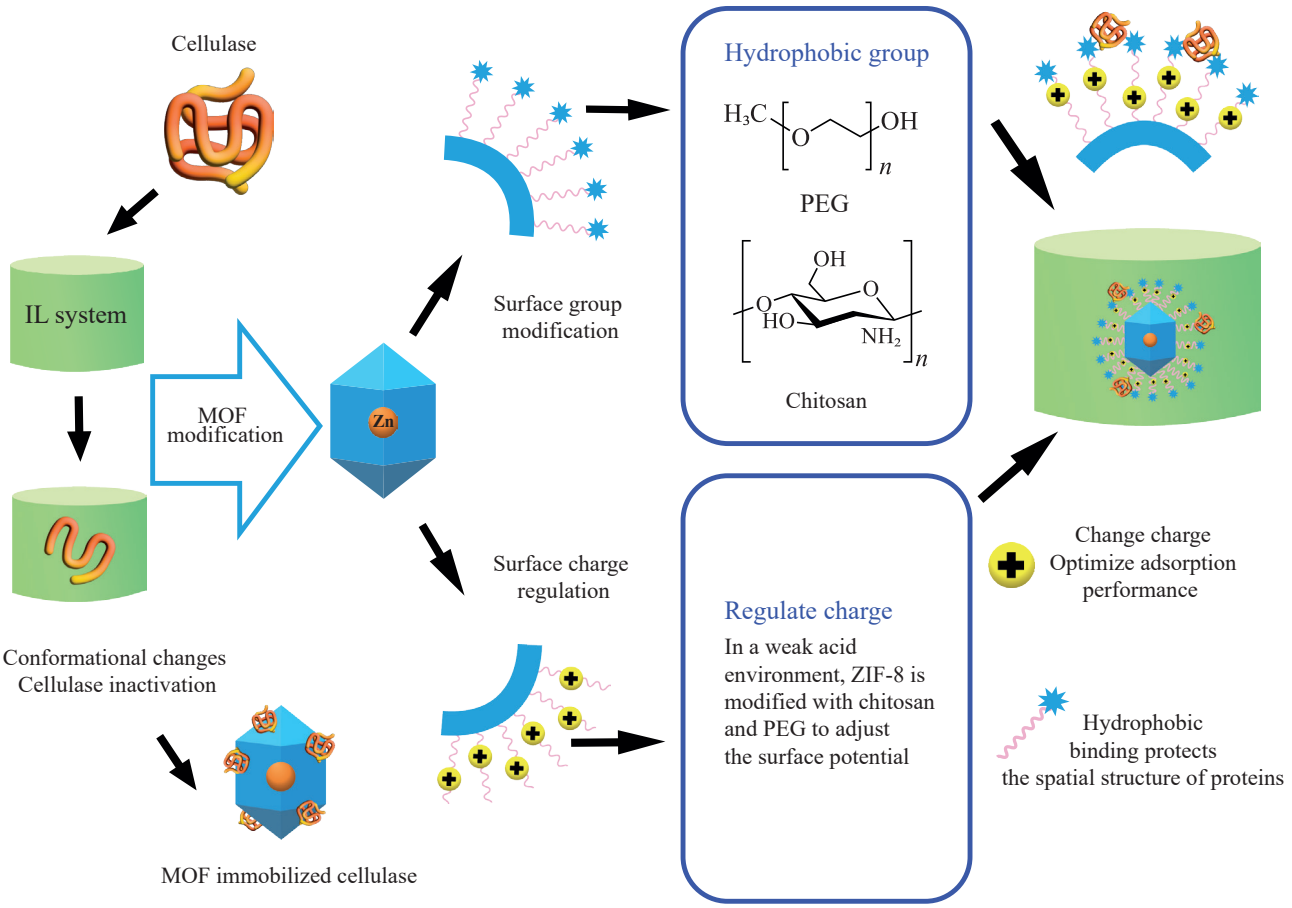

Fig. 1. Functional modification of ZIF-8 (modification of surface groups/regulation of surface charge) and its effect on immobilized cellulase (protection of the spatial structure of the enzyme) [26]. PEG is polyethylene glycol; IL system is ionic liquid system; MOF are metal-organic frameworks

at the level of 71.5%) is caused by weak noncovalent interactions between cellulase and the carrier (MWCNTs). After 30-day storage of immobilized and soluble cellulase, the enzyme activity remains at the level of 71.2 and 56.8% of the initial activity, respectively. Thus, the slightly increased stability of cellulase during storage following immobilization is considered an important condition for its industrial application. An original method of cellulase immobilization was proposed by Zhu and colleagues. After adsorbing the enzyme on Fe_3O_4+C NPs by means of electrostatic interactions, they coated these NPs with a thin layer of precipitated silica, which enhanced the adsorption of cellulase (the enzyme content on the carrier reached 200 mg/g) at the same time as preserving its enzymatic functions [32].

In order to obtain effective biocatalysts by adsorption of cellulase, carriers may be modified or functionalized. In the case of modification after adsorption, the applied outer layer should be sufficiently porous or swollen to ensure contact of cellulase with the cellulose substrate. Potential disadvantages of the adsorption method are a relatively low immobilization efficiency and the possibility of enzyme desorption during the reaction, leading to contamination of the final product.

2.2. Entrapment/encapsulation

Entrapment represents an irreversible immobilization method in which cellulase is retained in a porous matrix or polymer network without forming direct bonds with the carrier material. According to this approach, only the molecules of substrates and products are allowed to move in the pores, while the enzyme is prevented from leaching out of the biocatalyst. Therefore, a suitable pore size is critical for the selection of the carrier. Entrapment can enhance enzyme stability, reduce enzyme leaching, and protect the enzyme from adverse effects of the reaction environment, thus preventing cellulase deactivation. In contrast to entrapment, encapsulation separates the enzyme from the reaction environment by means of a permeable and porous organic/inorganic polymer. The creation of a microenvironment by encapsulation protects the enzyme molecules from adverse conditions that are

commonly observed in lignocellulosic hydrolysates due to inhibitory agents [23, 24, 33].

Wu *et al.* [34] immobilized several enzymes including cellulase using Zr-containing MOFs UiO-66 with different mesopores (6.46, 7.55, and 10.80 nm). By using UiO-66 with a pore size of 6.46 nm, the maximum enzyme adsorption for cellulase of 203.9 mg/g was achieved without damaging the enzyme structure. According to the authors, significantly increased stability of the immobilized enzyme cellulase+UiO-66 was achieved due to the optimal ratio of the pore size of the carrier material and the size of the enzyme molecule. Since the cellulase molecule has a prolate ellipsoidal shape with a diameter (minor axis) of 4–6.5 nm and a length (major axis) of 18–21.5 nm,[2] pores of the corresponding sizes provide more effective immobilization. Wu *et al.* [35] also showed cellulase could be firmly retained on the carrier by the addition of 4.6-nm mesopores to microporous UiO-66 to increase the stability of the immobilized cellulase. In particular, the results showed that the UiO-66 carrier provided high cellulase immobilization efficiency (265 mg/g) at a residual enzymatic activity of about 83% after six cycles. Analysis of the Michaelis–Menten equation showed that the Michaelis constant (13.355 mg/mL) and maximum reaction rate (1.311 mg/(mL·min)) of the immobilized cellulase were higher than those of the soluble enzyme (14.525 mg/mL and 0.732 mg/(mL·min), respectively).

Mesoporous Zn-based MOFs were also used for encapsulation of cellulase by coprecipitation of the enzyme and MOF precursors [36]. This increased the immobilized enzyme content to 350 mg/g to improve mass transfer due to the formation of structural defects during the formation of large pores in the MOF. The encapsulated cellulase retained 77% of the initial activity after four cycles.

Self-assembly of chitosan around cellulase by salting out from a mixed solution [30] led to the formation of a nanohybrid, which was deposited on alginate beads. The resulting nanobiocatalyst demonstrated increased stability and efficiency in the hydrolysis of sugarcane bagasse. The Michaelis constant increased by 3% for the immobilized nanohybrid compared to native cellulase (11.9 and 11.5 mg/mL, respectively). This could be due to the alginate matrix forming a barrier to limit the enzyme affinity for the substrate. The maximum rate for the immobilized nanohybrid was reduced compared to soluble cellulase (1.1 and 1.2 mM/min, respectively) due to the concentration gradient created by the nanohybrid within the alginate beads, which decreased the hydrolysis rate. The slight decrease in the maximum rate for the immobilized nanohybrid indicated the possibility for carboxymethyl cellulose to diffuse into the alginate beads due to its water solubility.

Thus, the most important advantage of enzyme immobilization by entrapment/encapsulation is its high robustness, although drawbacks such as co-adsorption and loss of structural integrity may limit its attractiveness. Furthermore, as in physical adsorption, the cellulase entrapment/encapsulation method is effective only if the enzyme is easily accessible within the nanomaterial.

2.3. Covalent attachment

Although covalent attachment is often preferred due to its provision of increased enzyme stability, it requires functionalization of carriers that do not initially contain functional groups [37, 38]. In addition, a suitable coupling agent is needed to maintain the enzyme conformation. The most commonly used bifunctional coupling agent is glutaraldehyde, which does not require the presence of any catalyst due to its interaction with the amino groups of the side amino acid residues and the NH_2 groups of the carrier [39]. Despite the short length of the glutaraldehyde molecule (0.75 nm) [40], this is sufficient to prevent nonspecific adsorption of the enzyme. Although longer-molecular-length coupling agents such as tetradecanedioic and docosandioic dicarboxylic acids with approximate chain lengths of 1.4 and 2.2 nm, respectively, have also been investigated, the interaction of the terminal carboxyl groups with the amino-functionalized carriers (to form a peptide bond) requires elevated temperatures and/or the presence of a catalyst [41, 42]. In the case of carriers such as graphene oxide with carboxyl groups on the surface, the coupling agents (dicarboxylic acids) are first activated by carbodiimide followed by reaction with *N*-hydroxysuccinimide to create a functional group for enzyme attachment [43].

Cellulase covalently immobilized using glutaraldehyde on amino-functionalized magnetic Fe_3O_4 nanoparticles (MNPs) coated with silica (Fe_3O_4+SiO_2) showed an activity of 3341 EA/g in the hydrolysis of carboxymethyl cellulose, which was 83.5% of the activity of the native enzyme [44]. The Michaelis constants and the maximum reaction rates for the immobilized and soluble cellulase were 0.0125 and 0.015 mg/mL, and 5.0 and 0.833 mmol/min, respectively, indicating a slight decrease in the affinity for the substrate and catalytic efficiency of the immobilized cellulase. A study of the stability of the immobilized enzyme in five repeated cycles demonstrated the retention of 44% of the

[2] Worldwide Protein Data Bank, https://www.rcsb.org/#Category-analyze. Accessed February 17, 2025.

initial activity. When using immobilized cellulase for the enzymatic saccharification of pretreated poplar wood [44], the maximum conversion of enzymatic saccharification (at 50°C and pH 4.5) was 38.4% over 72 h.

Targeted functionalization of the carrier may be critical for efficient covalent attachment of the enzyme. Gao *et al.* [45] prepared graphene oxide as a carrier for immobilization of cellulase by covalent binding. Graphene oxide was activated by esterification with 2-[(4-aminophenyl)sulfonyl]ethyl hydrogen sulfate (SESA) as a hydrophobic spacer. After modifying the resulting complex by diazotizing the acid, it was possible to covalently immobilize cellulase on it. Compared with soluble cellulase, the thermal and operational stability of the immobilized cellulase was significantly improved. At 50°C, the half-life of immobilized cellulase (533 min) was six times higher than that of soluble cellulase (89 min). In addition, the affinity between immobilized cellulase and the substrate (2.19 g/L) was more favorable than that of soluble cellulase (3.84 g/L). This suggests that immobilized cellulase has a higher catalytic efficiency. Here, it should be noted that the reliability of covalent attachment of cellulase is hampered by the complex functionalization procedure, which significantly reduces the attractiveness of this method.

Covalently immobilized and catalytically active enzymes on microgel particles can be obtained using reactive groups of amino acid residues (e.g., amino groups from lysine residues, thiol groups from cysteine residues, and carboxylic groups from aspartic or glutamic acids) and reactive groups in microgels (e.g., epoxides, and *N*-hydroxysuccinimide and maleimide esters). The main problem of immobilization is the presence of identical reactive groups in the target enzyme (e.g., several solvent-accessible lysine residues on the surface of cellulase). In this case, multipoint immobilization may occur to reduce the flexibility of the enzyme structure and potentially reduce its catalytic activity. Zou *et al.* [46] proposed a strategy for oriented single-point site-specific covalent immobilization of the enzyme in a microgel. They considered doping with sortase (transpeptidase) as a highly selective tool for conjugation of peptides or proteins [47]. *Staphylococcus aureus* sortase A recognizes the amino acid sequence LPETG in the protein to form a reactive thioether intermediate sortase A–protein (Fig. 2a), which

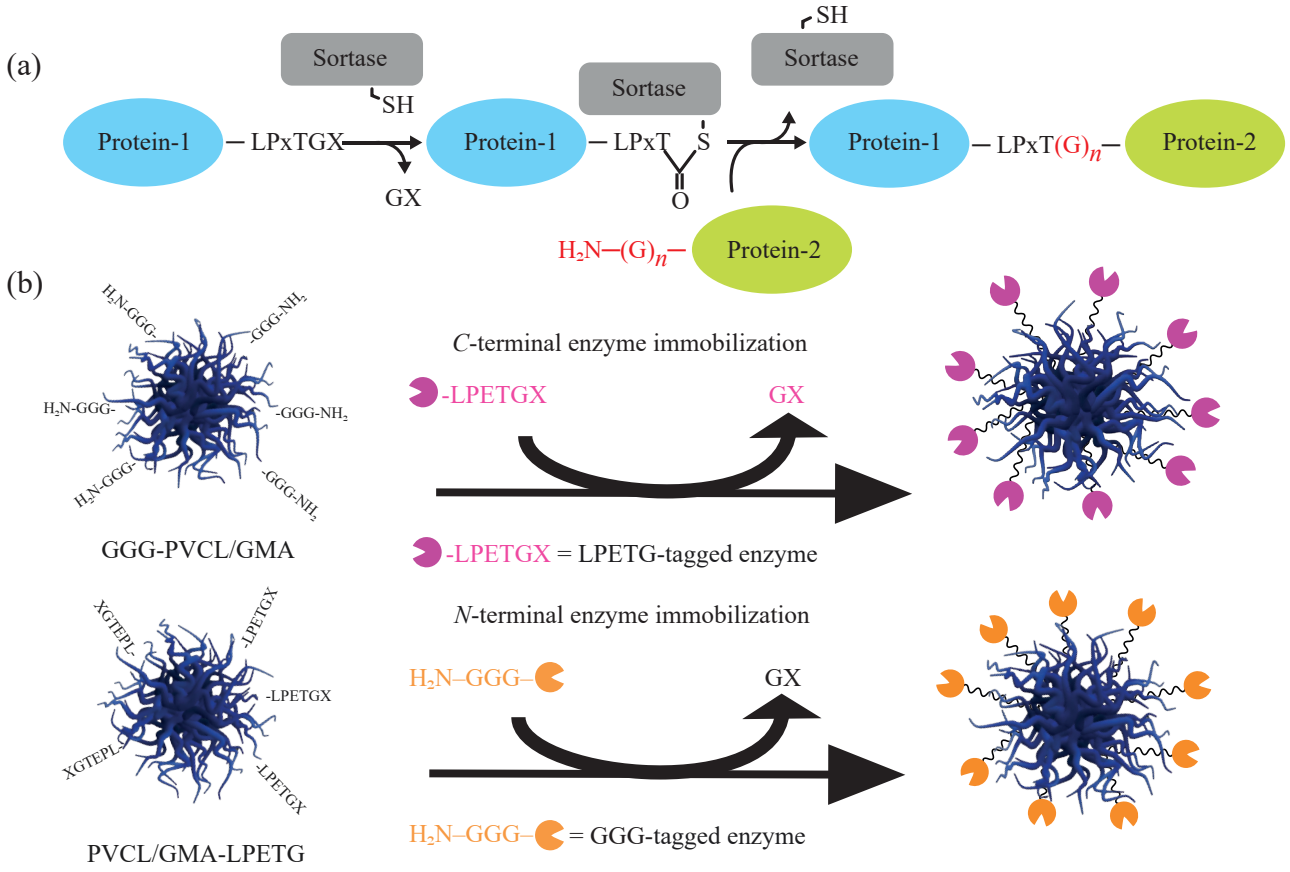

Fig. 2. (a) Sortase-catalyzed protein–protein conjugation using LPETG-labeled and GGG-labeled proteins as substrates. (b) Both conjugation variants (*N*- and *C*-terminal) are shown to confirm the effectiveness of a microgel-based carrier for enzyme immobilization: *C*-terminal immobilization (top); *N*-terminal immobilization (bottom) [46]

is subsequently cleaved by a nucleophile (e.g., the amino group of the *N*-terminal oligoglycine tag in another protein). As a result, a stable amide bond is formed and sortase A is released [46]. In that work, Zou *et al.* improved the principle of peptide synthesis in catalytic microgels to create a universal platform for enzyme immobilization on poly(*N*-vinylcaprolactam)/glycidyl methacrylate (PVCL/GMA) microgels.

To ensure the possibility of enzyme conjugation using both options (*N*- or *C*-terminal immobilization), two immobilization approaches are used. *C*-terminal enzymes labeled with LPETG are immobilized by coupling to H_2N–GGG–PVCL/GMA, whereas *N*-terminal enzymes labeled with GGG are doped with PVCL/GMA–LPETG–COOH microgels (Fig. 2b). For testing covalent immobilization, biotechnologically significant enzymes were selected, including cellulase A2M2 (*N*-terminal enzyme).

According to the presented examples, a possible disadvantage of covalent attachment of cellulase may be the complexity of chemical modification of the carrier and/or enzyme.

2.4. Cross-linking

Cross-linking of enzymes into aggregates (CLEA) permits their immobilization without the use of carriers. Such cross-linking can be achieved by interaction with glutaraldehyde [48]. However, the activity of cross-linked enzyme aggregates was found to depend on the type of cross-linking agent, which can affect the density of CLEA [49].

If precipitated cross-linked aggregates of cellulases are combined with MNPs, an additional advantage consists in the increased ease of manipulation of the magnetic nanobiocatalyst [50].

An original method for obtaining multienzyme hybrid nanoflowers (ECG-NFs) was implemented by Khan *et al.* [51] by cross-linking three enzymes of the cellulase complex: cellobiohydrolase (CBH), endoglucanase (EG), and β-glucosidase (BG). Cross-linking was performed using the recombinant enzymes EG–Linker–ELP (elastin-like polypeptide)–His_6 (EGLEH), CBH–Linker–ELP–His_6 (CBHLEH), and Glu–Linker–ELP–His_6 (GLEH). Figure 3 presents the processes of cross-linking of the enzymes and their catalyzed reaction of cellulose hydrolysis to glucose. The formation of hybrid nanoflowers is assumed to generally occur in two main stages [52]. In the first stage ("nucleation"), GLEH, CBHLEH, and EGLEH bind to Cu^{2+} to form a complex compound involving the appearance of copper phosphate crystals. In the second stage ("growth"), the amino groups of GLEH, CBHLEH, and EGLEH combine with Cu^{2+} by the coordination reaction of protein ions and Cu^{2+} to form nanocrystals, which can provide binding sites for GLEH, CBHLEH,

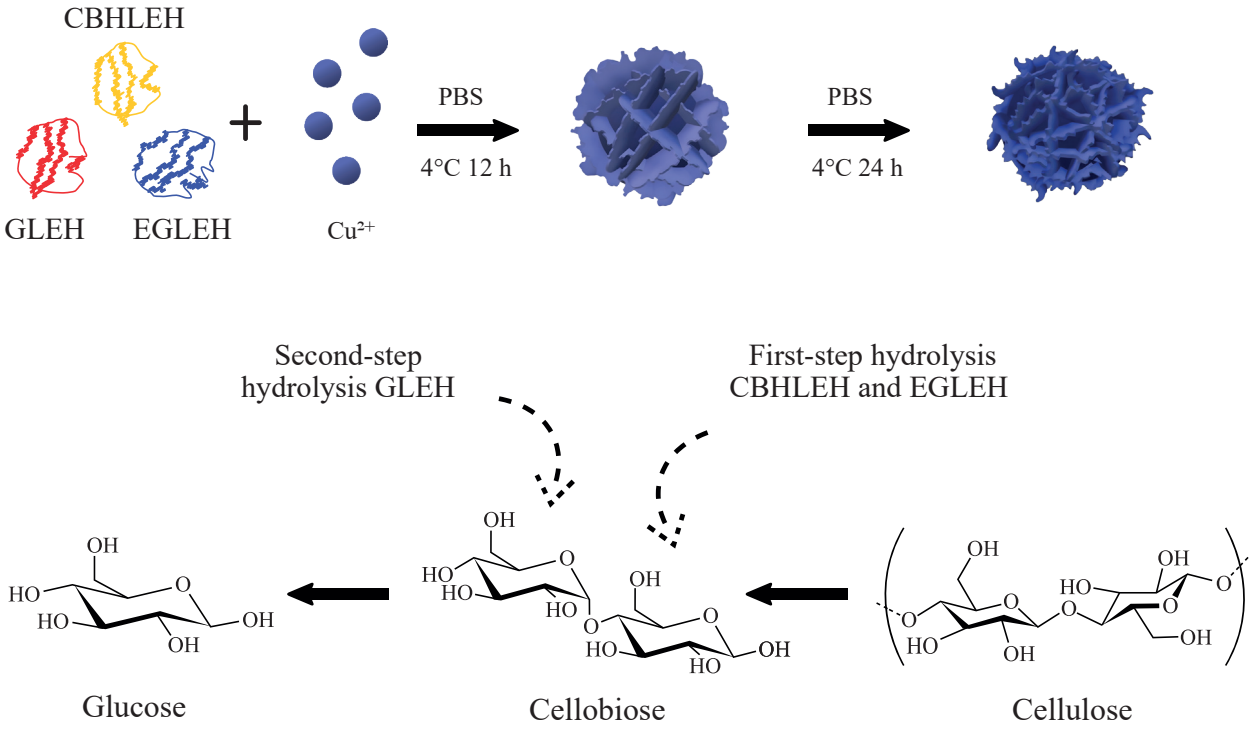

Fig. 3. Diagram of the ECG-NF formation and the functional cellulase action mechanisms. PBS is polybutylene succinate [51]

and EGLEN during the growth process. The continuous growth of NPs leads to the formation of mature nanoaggregates. In this case, the spatial structure of enzyme aggregates is the key to successful catalysis. The constructed system of multienzyme hybrid nanoflowers was used for a one-pot cascade conversion of cellulose to glucose. Compared with the native multienzyme system, the ECG-NFs nanoflowers demonstrated better physical stability, thermal stability, and storage stability. Moreover, the enzymatic activity of ECG-NFs was retained at 61.59% after eight repeated reaction cycles. The kinetic constants of the immobilized and native enzymes (Michaelis constants 9.33 and 9.54 g/L, respectively; maximum reaction rate, 0.0056 and 0.0048 g/(L·min), respectively) also indicate increased cellulase affinity and activity following co-immobilization. The total enzymatic activity of ECG-NFs increased by a factor of 1.12 compared to the native enzyme in the process of converting microcrystalline cellulose into glucose. Thus, it is expected that the effect of co-immobilization of several enzymes will be successfully used in industrial production conditions.

A potential drawback of this immobilization method is a decrease in activity due to limited access to the active site if the cross-linked aggregates are too dense.

3. TYPES OF NANOSTRUCTURED CARRIERS

The main nanostructured carriers used for cellulase immobilization are nanoporous materials (MOF, biochars, porous silica), nanohydrogels, and polymeric NPs and MNPs [10]. Most of these carriers have been used for enzyme immobilization for many years, but over the past five years, significant technological changes have occurred in the production or modification of these nanomaterials to increase the efficiency of immobilization processes and improve the characteristics of nanobiocatalysts created on their basis.

3.1. Nanosized porous materials

Pota *et al.* [53] proposed the co-immobilization of β-glucosidase and cellulase by adsorption on folded mesoporous silica NPs with a hierarchical structure of open pores having a smaller (wrinkled silica NPs, WSN) and larger (WSN synthesized by using pentanol, WSN-p) distance between the folds. The results showed that the best biocatalyst is the one obtained by co-immobilization of β-glucosidase and cellulase at the WSN-p distance. In this case, the fraction of adsorbed enzymes reaches 20%, which corresponds to their content at the level of 100 mg/g of the carrier. During testing in the reaction of hydrolysis of cellulose extracted from the leaves of loquat *Eriobotrya japonica*, the biocatalyst demonstrated a conversion of 82% and an activity of 72 μmol/(min·g). The biocatalyst retained 83% of its initial activity after nine cycles of repeated use. In addition, it had better stability over a wide temperature range than the mixture of soluble enzymes, retaining 72% of the maximum conversion value at temperatures up to 90°C.

Chen *et al.* [54] compared mesoporous silicas (MS) with average pore sizes of 17.6 and 3.8 nm (MS-17.6 and MS-3.8, respectively) and determined that larger pores, the sizes of which are similar to the major axis of the cellulase molecule, provide higher immobilization efficiency: the adsorption value was 410 mg/g for MS-17.6 and 315 mg/g for MS-3.8. On the other hand, the pores of the carrier with a diameter of 3.8 nm, close to the size of the minor axis of the cellulase molecule, provide higher activity (up to 67% of the activity of the native enzyme at 60°C) compared to cellulase on MS-17.6, which after immobilization retained only 26.6% of the activity of the native enzyme under the same conditions. Chen *et al.* [54] assumed that, in the case when the average pore size of the carrier is similar to the minor axes of the enzyme molecules, the immobilized cellulase retains active sites and demonstrates the highest activity. And in the case of the MS-17.6 carrier, the enzyme molecules penetrate into the pores, creating a dense and ordered structure, which probably hinders the conformational flexibility of cellulase necessary for the interaction between cellulase and the substrate.

3.2. Nanogels

Zarei *et al.* [55] proposed a simple two-stage approach to the fabrication of a conductive nanohydrogel composed of poly-ε-caprolactone and the cationic macromolecule of phosphine oxide. For this purpose, a cationic nanohydrogel was synthesized by electrospinning in the form of fibers with a diameter of about 469 nm, followed by *in situ* polymerization of polyaniline nanorods. The resulting nanohydrogel was used to immobilize cellulase, the activity of which was studied in the reaction of carboxymethyl cellulose hydrolysis. High immobilization efficiency (96%) was observed after optimization of such parameters as pH, temperature, processing time, and enzyme concentration in the mixture. The immobilized enzyme retained almost 90% of its initial activity after four weeks of storage and 73% of the initial activity after nine cycles of repeated use. The kinetic parameters (Michaelis constant and maximum reaction rate) showed values of 2.9 g/L and 7.6 g/(L·min) for immobilized cellulase and 1.5 g/L and 6.8 g/(L·min) for the native enzyme, respectively. The increase in the Michaelis constant after immobilization indicates that the reaction reaches its maximum catalytic efficiency

at slightly higher substrate concentrations. This may be due to limited substrate access to the active site of the enzyme and/or changes in the enzyme conformation and a decrease in the possibility of enzyme–substrate complex formation. The increase in the maximum reaction rate for immobilized cellulase compared to the native enzyme may be due to increased enzyme stability after immobilization.

Ariaeenejad *et al.* [56] created efficient nanohydrogel–enzyme systems with excellent stability and activity for practical use of cellulase in the hydrolysis of lignocellulosic biomass, and presented a strategy for the synthesis of new three-dimensional hydrogels from carboxymethyl cellulose copolymers of 2-acrylamido-2-methylpropane sulfonate and acrylamide. Graphene oxide nanosheets were used as a filling and cross-linking agent creating hydrogen bonds between polymer chains to obtain three-dimensional networks. The effect of graphene oxide content on the efficiency of the synthesized structures during conjugation with a model enzyme, cellulase, was studied. Immobilization of cellulase in graphene oxide–reinforced hydrogels resulted in a noticeable retention (at the level of 60%) of its maximum activity at a temperature of 90°C, as well as a significant increase in its specific activity and stability during storage. Compared with soluble cellulase, the immobilized enzyme containing graphene oxide hydrogels showed a noticeable (154.8%) increase in the conversion of alkali-treated sugar beet pulp, while the original cellulase hydrogel showed only a 66.7% increase under the same conditions.

Hedaiatnia *et al.* [22] obtained an efficient immobilized bifunctional enzymatic complex cellulase/xylanase on a hydrogel carrier with increased stability and activity, which was subsequently used for the hydrolysis of lignocellulosic biomass. The initial hydrogel (SA–CH) was synthesized by radical polymerization of chitosan (CH) and sodium alginate (SA) solutions in the presence of a cross-linking agent and acrylic monomers. The hydrogel nanocarrier (SA–CH/NCs) was synthesized by adding nanocellulose (NCs) to SA–CH. The activity and stability of native cellulase and xylanase and immobilized bienzyme complexes (PersiCelXyn1+SA–CH and PersiCelXyn1+SA–CH/NCs) were investigated in the hydrolysis of sugar beet pulp to produce a hydrolysate containing fermentable sugar and serving as a substrate for lactic acid production. The Michaelis constant values for native enzymes and the PersiCelXyn1+SA–CH and PersiCelXyn1+SA–CH/NCs complexes were found to be 2.84, 0.89, and 0.58 mg/mL, respectively. Different values demonstrated different affinities of the enzymes for the substrate. During the immobilization process, the enzyme conformation may change, and various diffusion effects and steric hindrances may alter the enzyme microenvironment, which affects its affinity for the substrate after immobilization. The decrease in the Michaelis constant for the immobilized enzymes compared to their soluble form indicates an increase in the affinity for the substrate after immobilization. This decrease was more pronounced in the case of the PersiCelXyn1+SA–CH/NCs hydrogel compared to PersiCelXyn1+SA–CH. It is possible that the presence of nanocellulose in the matrix of this hydrogel caused a more intense interaction of the substrate with the enzyme, which in turn led to an increase in the affinity of the enzyme for its substrate. The maximum reaction rates for PersiCelXyn1+SA–CH and PersiCelXyn1+SA–CH/NCs were 74.19 and 103.79 μmol/(mg·min), respectively, while for the native form this value was 58.70 μmol/(mg·min). Since the maximum reaction rate parameter represents the rate of enzymatic reaction at saturating substrate concentrations, its higher values for immobilized enzymes indicate that, when saturated with the substrate, they ensure a higher reaction rate than the soluble enzyme. Thus, the addition of NCs to the hydrogel network allowed one to obtain a modified hybrid nanocarrier for immobilizing bienzyme complexes with an increased specific activity compared to the original hydrogel and, ultimately, to increase the catalytic activity of the immobilized enzyme.

3.3. Polymeric particles

Polymeric NPs can be useful for surface covalent attachment of enzymes if the polymer structure contains the necessary functional groups. Wang *et al.* [57] obtained polymeric NPs from a cross-linked copolymer of styrene and maleic anhydride by precipitation polymerization without a stabilizer, followed by covalent attachment of cellulase through anhydride groups.

Self-assembling micelles of the poly(styrene)-*b*-poly(styrene-*alt*-maleic anhydride) polymer modified with nitrilotriacetic acid (NTA), the further modification of which with Ni^{2+} ions made it possible to attach bacterial His_6-cellulase, were successfully used by Lu *et al*. [58] to obtain core–shell NPs with cellulase molecules in the outer layer (Fig. 4).

Lu *et al.* [58] showed that cellulase immobilized on polymer NPs after incubation for 24 h produced approximately twice as much reducing sugar (50 mg/L) as the soluble enzyme (30 mg/L). The stability of the immobilized cellulase was also tested, and it was found that, after two-week storage at 48°C or storage for 48 h at room temperature, the activity of the immobilized cellulase did not undergo significant changes. Lu *et al.* explained the activity and stability of the immobilized enzyme by the specific orientation of proteins on the surface of the

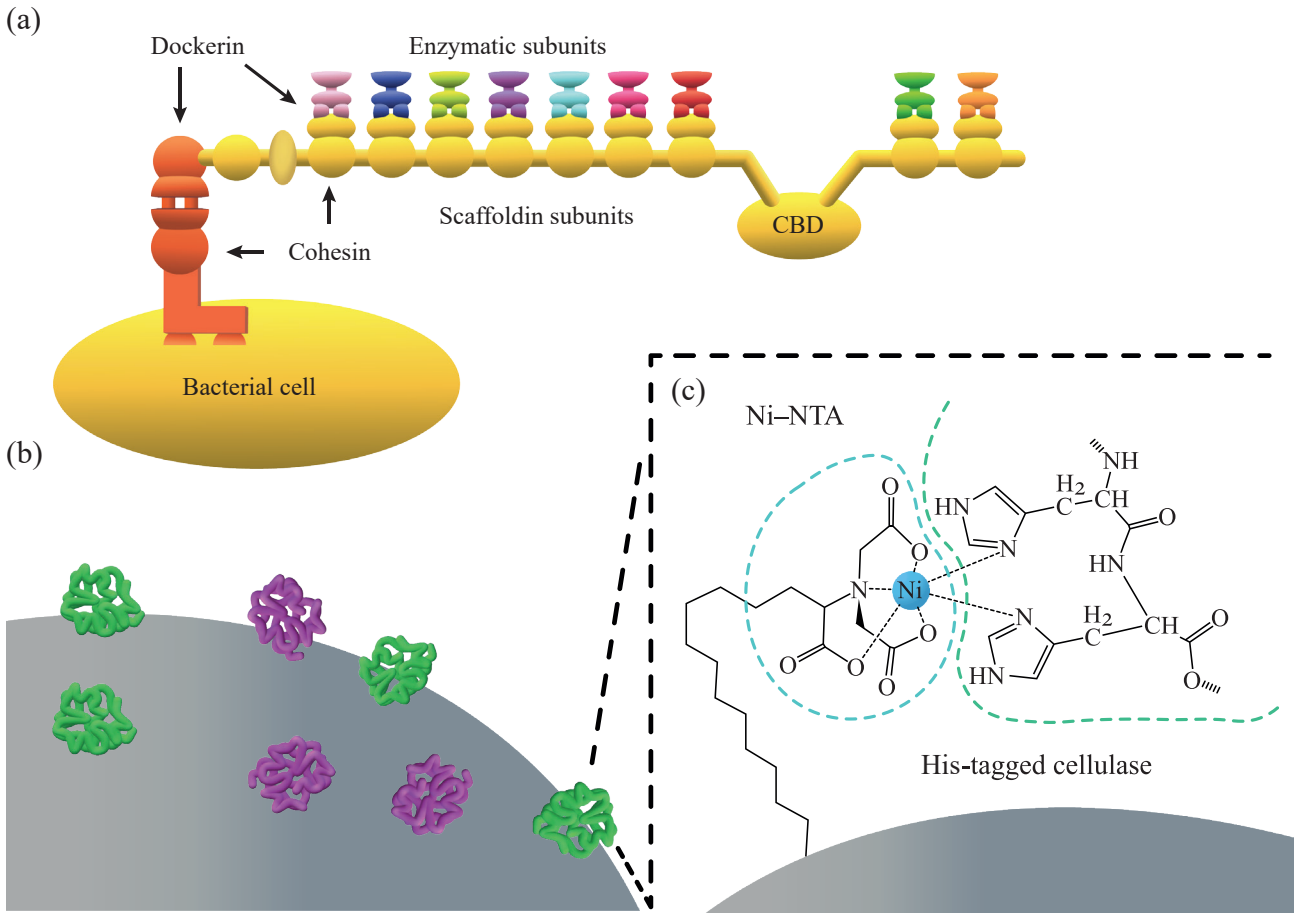

Fig. 4. (a) Structural organization of the cellulosome in *Clostridium thermocellum* cells, (b) functionalized Ni–NTA micelles for cellulase immobilization, and (c) the interaction of Ni–NTA with cellulase molecules tagged with His_6 [58]

NPs and in the active sites, as well as by the more effective action of the immobilized enzyme on the substrate.

3.4. Magnetic nanostructured carriers

Magnetically responsive nanostructured carriers are usually based on MNPs. The use of MNPs for the development of nanobiocatalysts has rapidly increased in recent years due to the simplicity of magnetic separation, which allows for the repeated use of nanobiocatalysts and makes the processes involving them more reliable, as well as economically and environmentally beneficial. MNPs (most often, iron oxide NPs) are usually prefunctionalized to ensure the possibility of enzyme attachment. For this purpose, MNPs are coated either with silicon dioxide followed by the addition of functional amino groups [44, 59–68] or with polymers containing reactive groups (e.g., chitosan or other functional polymers) [69–75]. The addition of metal ions (e.g., copper) to amino-functionalized MNPs improves the immobilization of cellulase due to its affinity for the metal [76]. Under optimal operating conditions (Cu/MNP ratio = 1, cellulose/MNP ratio = 0.11, pH 6), the relative activity and content of immobilized enzyme on MNPs were 91% and 164 mg/g, respectively. Abbaszadeh and Hejazi showed [76] that immobilized cellulase tested in the hydrolysis reaction of carboxymethyl cellulose taken at a concentration of 1% exhibits greater stability than the soluble enzyme. In addition, the immobilized enzyme retained 73% of its initial activity after five cycles of use.

For better protection of iron oxide MNPs, Poorakbar *et al.* [77] first formed a gold shell around the MNPs and then a silica shell, followed by functionalization with polyethylene glycol and L-aspartic acid for covalent attachment of cellulase (Fig. 5). Covalent binding of the enzyme was confirmed by Fourier transform infrared spectroscopy. The binding efficiency was 84% as determined by the Bradford method. During filter paper hydrolysis, the immobilized cellulase showed 88% of the activity of the native enzyme and retained 73% of its initial catalytic activity after 9 h (with an activity of 0.78 mmol/mL) [77].

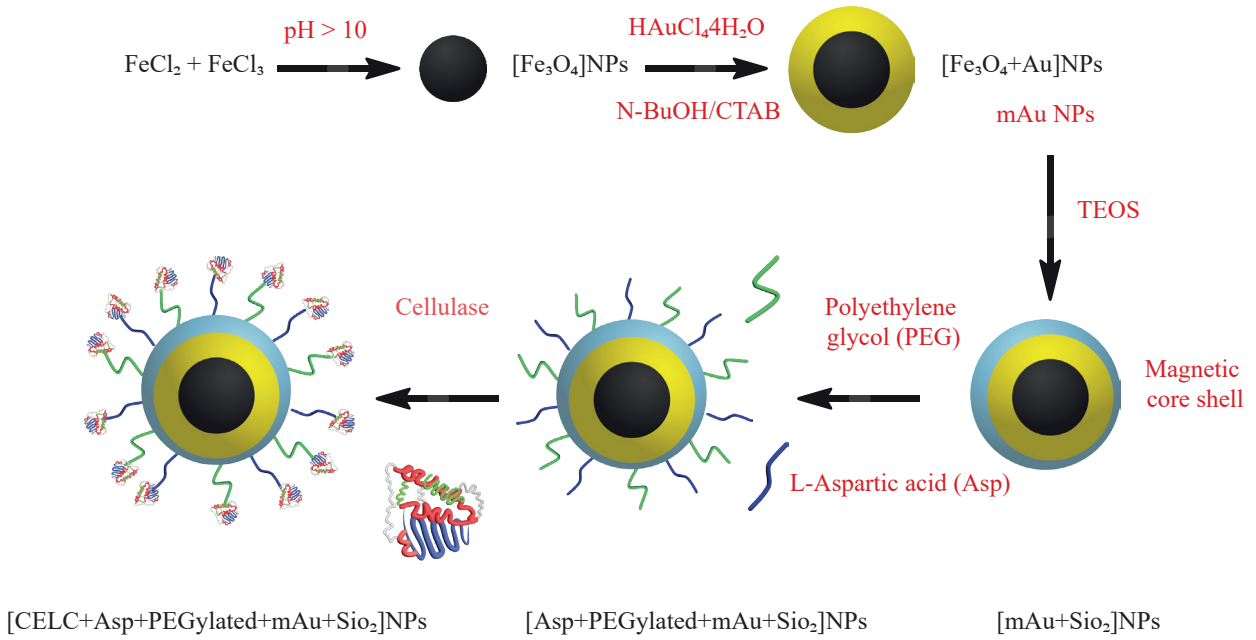

Fig. 5. Synthesis of core–shell gold MNP as a new nanocarrier and the immobilization of cellulase on it to obtain biocatalysts [77]

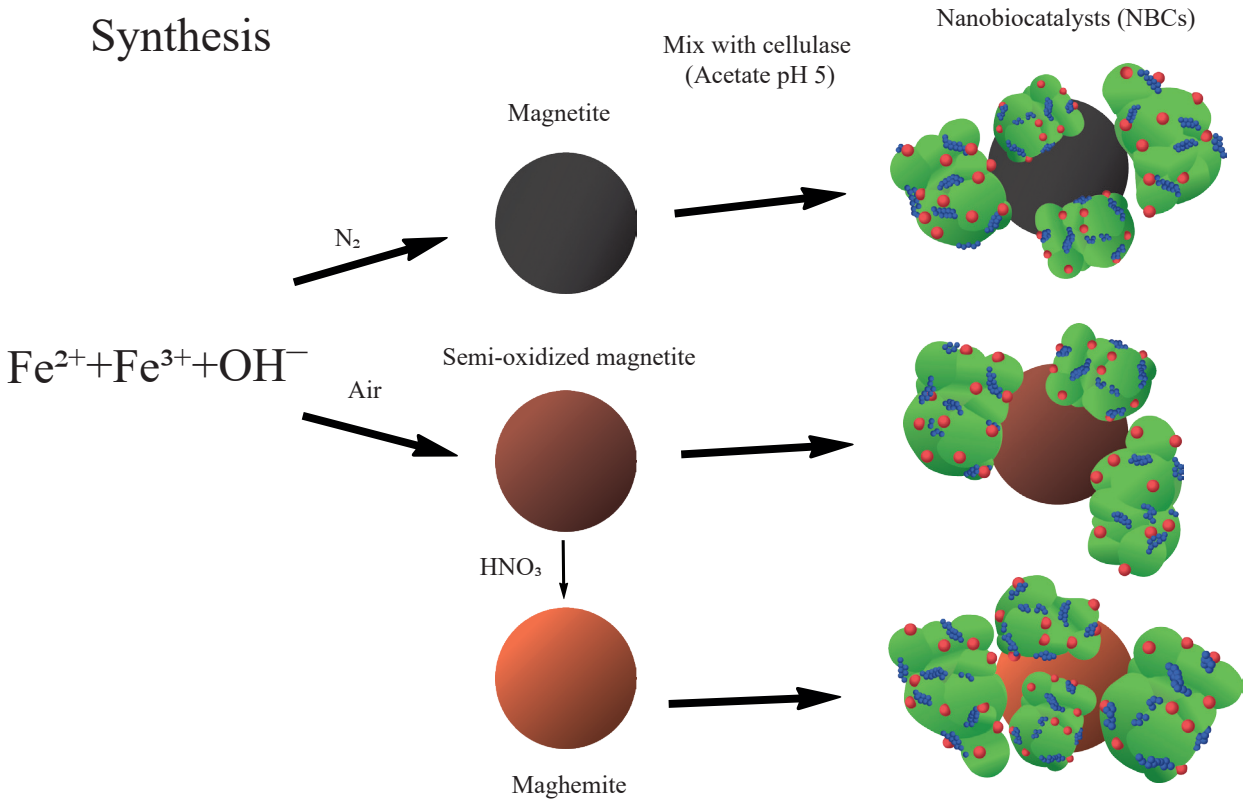

Fig. 6. MNP synthesis for cellulase immobilization [80]

Another method for synthesizing a magnetic nanobiocatalyst [78, 79] involves introducing MNPs into porous or polymeric materials. For example, the porous material used by Zhang *et al.* [79] was mesoporous silicon dioxide SBA-15 with pores 7–9 nm in diameter, inside which Fe_3O_4·MNPs were formed. Cellulase immobilization was carried out by adsorption at pH 4.8 and a temperature of 4°C for 24 h. In the

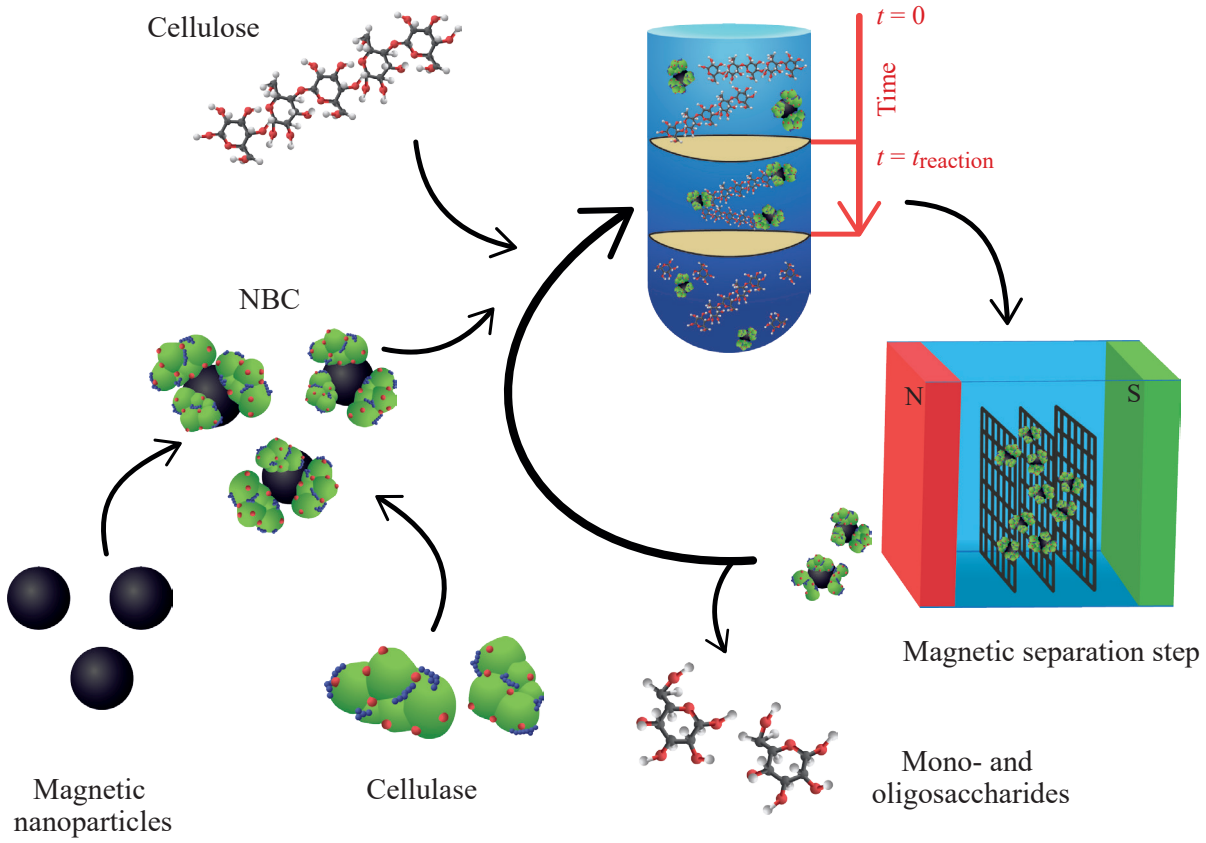

Fig. 7. Cellulose conversion and magnetic separation of nanobiocatalysts (NBC) [80]

hydrolysis of cellulose in the presence of immobilized cellulase, a glucose yield of 86.2% was achieved.

Magnetite NPs without a carrier are also used for cellulase adsorption, as shown in Fig. 6 [80]. For cellulase immobilization, three types of iron oxide NPs were synthesized: nitrogen-synthesized magnetite (MNP-N_2), air-synthesized semi-oxidized magnetite (MNP-Air), and nitric acid–oxidized magnetite NPs (MNP-Ox).

The catalytic activity of immobilized cellulase was investigated in the hydrolysis of carboxymethyl cellulose and filter paper. In all cases, the obtained nanobiocatalyst with magnetite as a carrier demonstrated better absolute activity (4.28 and 0.82 units/(g·h) for the catalyst on magnetite and maghemite, respectively) and relative enzymatic activity at lower pH values, as well as at higher and lower temperatures. This indicates an improvement in the thermal and mechanical stability of magnetite-based nanobiocatalysts, which is a desirable effect in enzyme immobilization.

Another important effect of cellulase immobilization is an increase in long-term catalytic stability. The presented nanobiocatalysts demonstrate long-term stability for 42 days without loss of catalytic activity, while the activity of the native enzyme during this time drops to 40%. However, the most important positive effect is the possibility of reusing nanobiocatalysts by magnetic separation. Schnell *et al.* [80] achieved 95% recovery of magnetic nanobiocatalysts after use in six consecutive reaction cycles (Fig. 7).

Similar magnetite NPs were also synthesized by Ingle *et al.* [81] using glutaraldehyde to immobilize cellulase by covalent binding. During hydrolysis of the cellulose substrate (sugar cane cake powder), cellulose conversion for 24 h was achieved at a level of 93% for the native enzyme and 89% for cellulase immobilized on MNPs.

4. CONCLUSIONS

Nanobiocatalysts based on cellulase immobilized on nanostructured carriers are used for the catalytic hydrolysis of biomass waste, as well as in the food industry and for environmental protection purposes. The analysis of recent trends presented in this review shows that positive changes have occurred in the immobilization methods and carrier compositions over the past five years.

One of the most striking examples involves oriented single-point site-specific immobilization of the enzyme in

a microgel by sortase-catalyzed protein conjugation, which increases the immobilization targeting and improves the subsequent interaction of the enzyme with cellulose.

Another example demonstrates the possibility of using an unusual carrier structure (folded mesoporous silica NPs) for the production of effective and stable nanobiocatalysts by simple adsorption of cellulase due to the unique nature and morphology of the carrier. Cellulase adsorbed in this way turned out to be very stable and active due to preliminary modification of the carrier with macromolecules that change the charge or hydrophobicity–hydrophilicity balance.

Carrier-free multienzyme hybrid nanoflowers could be used to achieve a similar goal by forming combined enzymatic structures with high affinity for cellulose biomass.

The use of magnetically separable carriers increases the reliability of the biocatalyst and facilitates biocatalytic processes, thus providing the possibility of extracting the magnetic nanobiocatalyst.

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Authors' contributions

A.M. Sulman and **V.P. Molchanov**—writing (original draft preparation).

D.V. Balakshina and **O.V. Grebennikova**—literature analysis and preparation of graphic material.

V.G. Matveeva—conceptualization, review, and editing.

All authors have read and agreed to the published version of the manuscript.

The authors declare no conflicts of interest.

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