Materials Advances

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Mater. Adv.*, 2025, 6, 311

Use of supramolecular chemistry based on β-cyclodextrin-grafted chitosan beads to prepare green biocatalytic materials†

Agatha Bastida, *[a] Leoncio Garrido[b] and Alfonso Fernández-Mayoralas *[a]

Received 16th September 2024,
Accepted 1st December 2024

DOI: 10.1039/d4ma00929k

rsc.li/materials-advances

The use of biomass-derived materials as supports for enzyme immobilization is of interest for developing biocatalytical processes based on renewable resources. Reversible immobilization offers a solution to the problem of enzyme activity loss over time, as it allows for the removal of deactivated enzymes and their replacement with fresh ones using a renewable biopolymer. In this work, β-cyclodextrin-grafted chitosan (Ch-CD) has been prepared and used as support to reversibly immobilized adamantane-modified enzymes *via* supramolecular host–guest interactions in an environmentally friendly aqueous medium. The prepared Ch-CD beads were characterized by solid-state ^{13}C and ^{15}N CP/MAS (cross-polarization/magic angle spinning) NMR spectroscopy. The performance of three types of enzymes immobilized by this method has been studied: β-galactosidases in the hydrolysis of β-galactopyranosides, one glucose oxidase in the oxidation of glucose, and one peroxidase in the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). A double sequential enzyme reaction catalyzed by immobilized glucose oxidase/peroxidase was performed, showing the possibility to develop a glucose test by means of this sustainable biocatalysis. The reusability of the biocatalytic materials was dependent on the type, source of enzyme and the linker used in the preparation. The enzymes bound to cyclodextrin-grafted chitosan beads were desorbed after washing with a β-CD solution, thus being able to recycle the Ch-CD beads for a new enzyme immobilization.

Introduction

There is an increasing demand for more sustainable materials and organic reactions with environmentally benign properties.[1–3] The modification of natural polysaccharides may provide novel materials for a variety of applications that could enable a reduction of the dependence on fossil fuels. Chitosan is an abundant polysaccharide derived from many natural sources (shrimp's shells, and exoskeleton of crustaceans, among others), through the deacetylation of chitin. The low toxicity, biodegradability and biocompatibility of chitosan makes it amenable for biomedical applications.[4–6] In addition, chitosan-based materials can be prepared in different shapes and sizes (such as fibers, microcapsules, nanoparticles, beads, coating and gels), which expands its application to many other areas.[7,8] In catalysis chitosan has been used as a support for the preparation of heterogeneous catalyst or as a direct organocatalyst through chitosan derivatives.[9,10] Chitosan has also been used as support for enzyme immobilization under green conditions and due to its nontoxic and biocompatible nature, it is appropriate support for immobilization of biocatalysts with application in the food industry.[11,12] The presence of amino and hydroxyl groups in its structure allows to modulate the physicochemical properties of chitosan-based supports, affording a range of possibilities in the design of biocatalytic materials. However, the presence of these functional groups can also be a drawback, hence the need to choose the best strategy depending on the application of the biocatalyst.[13] On the other hand, a well-stablished strategy for the selective synthesis and transformation of organic molecules in a sustainable and efficient way is the use of enzymes. These biocatalysts are increasingly used in industrial sectors such as pharmaceuticals, fine chemicals, agrochemicals and food industries.[14] The industrial use of enzymes generally requires their immobilization on supports. Immobilization offers the possibility of an easy recovery and reuse of the catalyst.[15,16] However, enzymes are intrinsically labile, and a loss of activity may occur after some prolonged time of incubation, even in aqueous media. In this case, the enzyme must be replaced, which may be a problem in determined systems, such as in a microfluidic channel system where the biocatalyst is attached to the microchannel walls.[17] Reversible enzyme immobilization may be the solution: when the activity is

[a] *Instituto de Química Orgánica General, IQOG-CSIC, c/juan de la cierva 3, Madrid, 28006, Spain. E-mail: agatha.bastida@csic.es, alfonso.mayoralas@csic.es*
[b] *Instituto de Ciencia y Tecnología de Polímeros, ICTP-CSIC, c/juan de la cierva 3, Madrid, 28006, Spain*

† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d4ma00929k

loss the enzyme is detached and the support or microfluidic device reuse with fresh enzyme. Moreover, the same device could be used to immobilize another enzyme or set of enzymes.

One possible strategy for reversible immobilization is the binding of the enzyme to surfaces through hydrophobic, polar or ionic interactions. Although these methods are simple, random immobilization of proteins presents the risk of blocking the active center of the enzyme, thus decreasing catalytic activity. On the other hand, there exist site-specific/noncovalent immobilization by linking a specific functional group or fragment to the enzyme that has affinity for another group displayed on the surface of the support. For example, the use of avidin/biotin affinity hybridizations or the addition of a histidine peptide chain, *via* genetic engineering.[18] Alternatively, using chemical tools, the application of supramolecular chemistry constitutes an interesting approach, which may provide an easy detachment of the biocatalyst from the support.[19] β-Cyclodextrin (β-CD) is a non-toxic cyclic oligosaccharide of seven α-1,4-linked D-glucopyranosyl units. The use of β-CD-based host–guest chemistry has proved to be a powerful tool for many applications, including for enzyme immobilization.[20] The hydrophobic cavity of the seven glucose units of β-CD can accommodate hydrophobic moieties, and one of the groups that forms a strong inclusion is the adamantyl group.[21] The equilibrium constants for complexations of various adamantane derivatives with β-CD is often higher than 10^4 M^{-1}. There are several examples in which the strong complexation constant adamantane-β-CD is used to immobilize enzymes.[20] Thus, adamantane-modified L-phenylalanine dehydrogenase was immobilized on β-cyclodextrin coated gold nanospheres, which retained high catalytic activity.[22] Monolayers of β-CD on gold in combination with a biotinylated bisadamantyl linker were used to generate reusable homogeneous enzyme layers in microchannels.[23] We have described the fabrication of a microfluidic device with a porous surface enriched in β-CD which was used to immobilize adamantane-modified horseradish peroxidase by host–guest interactions.[24] These surfaces showed catalytic activity that remained stable after repeated use.

In the present work we combine the use of a renewable biopolymer, chitosan, to reversibly immobilize enzymes *via* supramolecular host–guest interaction between β-CD and adamantane in water. Thus, β-CD was bound to chitosan beads using different bifunctional isocyanates as linkers, and the enzymes were functionalized with adamantane. We have studied the strategy with 3 classes of enzymes: β-galactosidases in the hydrolysis of *o*-nitrophenyl β-galactopyranoside (β-Gal), glucose oxidase in the oxidation of glucose (GOx), and peroxidase (HRP) in the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Fig. 1).

This biocatalytic system allows for the recovery of the β-cyclodextrin-grafted chitosan beads free of enzyme and available for a new eco-friendly immobilization.

Results and discussion

Chitosan beads (Ch beads) were prepared by adding chitosan power (2 wt%) in acetic acid solution (1%) over a basic solution (1N NaOH) drop by drop under stirring and subsequent washing with water until neutral.[25,26]

The linking of β-CD to chitosan was achieved using three different diisocyanates which provide structures with flexible and rigid linkers between β-CD and chitosan: 1,6-hexamethylene diisocyanate (HMDI) which should provide a flexible linker, toluene-2,4-diisocyanate (TDI) and xylylene diisocyanate (XDI).[25] In a first step, chitosan beads were treated with 2.5% of HDMI, TDI and XDI in DMF solution to form either a urethane (NH–CO–O) or a urea (NH–CO–NH) bond, depending on whether the reaction takes place on the hydroxyl or the amino

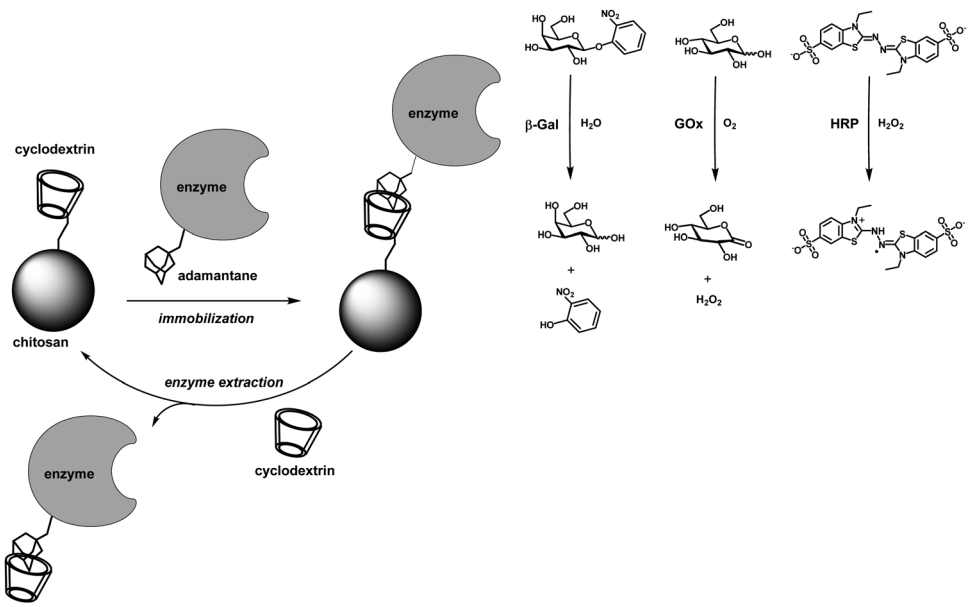

Fig. 1 Supramolecular chemistry applied to enzyme immobilization on β-cyclodextrin-grafted chitosan beads: β-Gal, β-galactosidase; GOx, glucose oxidase; HRP, peroxidase.

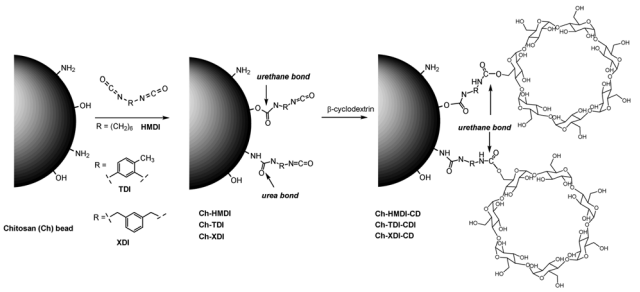

Fig. 2 Preparation of cyclodextrin-grafted chitosan beads.

group of chitosan, respectively (Fig. 2). In a second step, β-CD was allowed to react with the other isocyanate group to form a urethane bond, leading to functionalized beads Ch-HMDI-CD, Ch-TDI-CD and Ch-XDI-CD (Fig. 2).

Cyclodextrin-grafted chitosan beads were analyzed by solid-state ^{13}C and ^{15}N CP/MAS NMR spectroscopy and FTIR spectroscopy. The ^{13}C NMR spectrum of chitosan with the assignment of the glucosamine ring and acetamido group signals is shown in Fig. 3a. In the ^{13}C NMR spectra of Ch-TDI-CD and Ch-XDI-CD (Fig. 3b and c) new peaks at 132.1 and 137.8 ppm in Ch-TDI-CD and between 126.8-141.0 ppm in Ch-XDI-CD, indicated the presence of the aromatic ring of the linkers. In the spectrum of Ch-HMDI-CD (Fig. 3d), a strong peak centered at 30.8 ppm is assigned to the CH_2 carbons of the alkyl chain. The signal at 159.7 ppm in Ch-TDI-CD, 160.3 ppm in Ch-XDI-CD and 159.9 ppm in Ch-HMDI-CD can be attributed to the carbonyl of urethane and urea groups of the newly generated bonds. The signals of the carbons of glucosamine units of chitosan and glucose units of cyclodextrin cannot be distinguished in the spectra of Ch-CD beads, except for C-2 of glucosamine that appeared around 57 ppm.

However, when comparing the relative intensities of pyranose carbon peaks respect to the aromatic peaks of Ch-XDI-CD, with those of the intermediate Ch-XDI lacking the cyclodextrin residue (Fig. 3e), it is observed a decrease in the intermediate Ch-XDI, which indicates that glucose units of cyclodextrin were added in the second step.

The ^{15}N NMR spectra of chitosan, Ch-TDI-CD, Ch-XDI-CD and Ch-HMDI-CD beads are shown in Fig. 4. The spectrum of chitosan consists of two signals at −357.5 and −258.5 ppm which correspond to the nitrogen of amino and acetamido groups, respectively (Fig. 4a). After reaction with linker and subsequent coupling with β-CD the relative peak intensity of the amino group with respect to the acetamido of chitosan was markedly reduced (Fig. 4b–d).

The decrease of this signal indicates that the reaction of chitosan with diisocyanate takes place predominantly through the amino group forming a urea bond. In the spectrum of Ch-TDI-CD the signal at −278.0 ppm was assigned to the nitrogen of the urethane bond, which is downfield due to its link to an aromatic ring, while the resonance of the two nitrogens of the urea was assigned to the peak at −297.8 ppm.[27] In compounds Ch-XDI-CD and Ch-HMDI-CD, with not nitrogen atom directly linked to an aromatic ring, the nitrogen nuclei of urethane and urea bonds appeared upfield between −295 and −305 ppm for Ch-XDI-CD, and one signal at 297.3 ppm for Ch-HMDI-CD.

All the IR spectra of chitosan and β-cyclodextrin-grafted chitosan beads (Fig. S1, ESI†) showed a distinct primary amino group (NH_2) bending vibration at around 1638 cm^{-1}. Compared

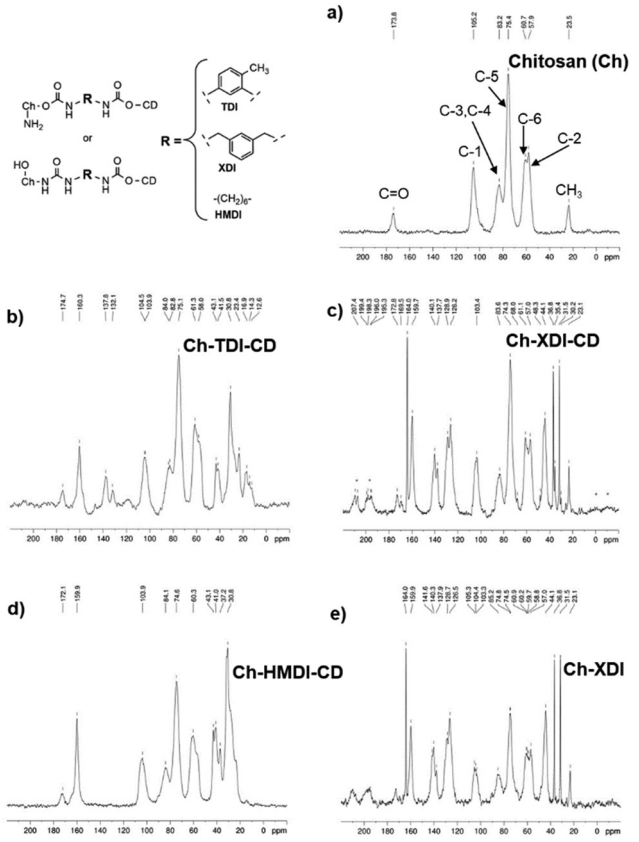

Fig. 3 ^{13}C CP/MAS NMR spectra of chitosan and chitosan derivatives.

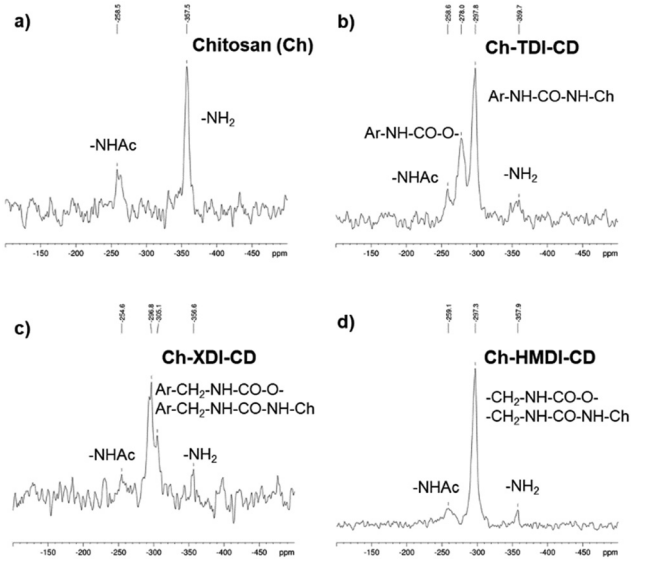

Fig. 4 ^{15}N CP/MAS NMR spectra of chitosan and chitosan derivatives.

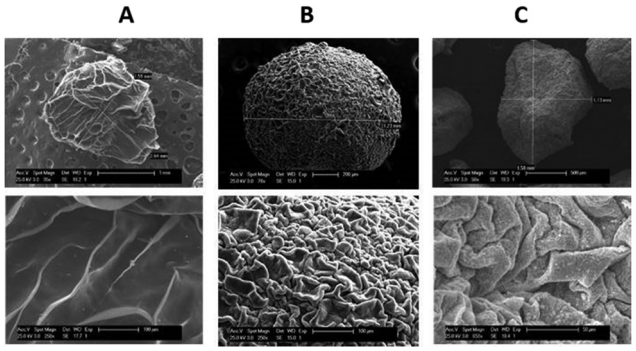

Fig. 5 Scanning electron microscopy (SEM). (a) Ch, (b) Ch-HMDI-CD, (c) Ch-TDI-CD.

with chitosan, β-cyclodextrin-grafted beads presented a new band corresponding to stretching vibration of carbonyl (CO) of urethane and urea: at 1578 and 1615 cm^{-1} for Ch-HMDI-CD, 1547 cm^{-1} for Ch-XDI-CD, and Ch-TDI-CD. On the other hand, Ch-HMDI-CD showed two bands at 2856 and 2933 cm^{-1} corresponding to the C–H stretching vibration of the hydrocarbon chain. A medium band at 1254 cm^{-1} for Ch-HMDI-CD, 1253 cm^{-1} for Ch-XDI-CD, and 1553 cm^{-1} for Ch-TDI-CD, can be attributed to asymmetric stretching of NH–CO–O group.

The morphology and size of β-cyclodextrin-grafted chitosan beads were analyzed by scanning electron microscopy (SEM) and the results are illustrated in Fig. 5. It could be observed that the grafting of hydrophobic reactants to the chitosan beads contributes to soften the angular edges and increase their roundedness. Once the Ch-CD beads were characterized the enzymes were chemically modified with adamantane following reported procedures.[21]

The functionalization involves the reaction between the amino groups of lysine residues present at the protein surface and adamantane carboxylic acid. Functionalization and subsequent immobilization on Ch-CD beads was first studied with β-galactosidases (β-Gal) from *E. coli* and from *B. bifidum* (Saphera). The enzyme activity was determined by measuring the release of *o*-nitrophenol from *o*-nitrophenyl β-galactopyranoside substrate (Fig. 6a). For both enzymes, the modification with adamantane (ada) resulted in loss of activity compared to unmodified enzymes (42% residual activity for *E. coli* and 38% for *B. bifidum*, respectively, Table S1 (ESI†) suggesting that the reaction could affect lysine residues located near the catalytic site). For enzyme immobilization on β-cyclodextrin-grafted chitosan beads, the modified biocatalysts were incubated with the beads (0.1 mg enzyme/200 mg bead) and the β-galactosidase activity in the supernatant was measured over the time (Fig. 6b). The binding to the supports bearing XDI or TDI as linker was faster than with HMDI. After 24 h the enzyme activity in the supernatant declined totally (with TDI) or almost completely with XDI and HMDI, indicating that the percentage of the offered enzyme immobilized on the support was very high. In Table 1 the catalytic activity of the enzymes immobilized on Ch-CD beads bearing the three different linkers are shown. Ch-CD beads incubated with unmodified enzymes were also assayed as control. However, no catalytic activity of the beads was observed, demonstrating that the functionalization of the enzyme with adamantane is required for binding to the beads. The specific activity of immobilized galactosidases on the beads was 7–8 U g^{-1} and 3–8 U g^{-1} for the enzymes from *E. coli* and from *B. bifidus*, respectively. In agreement with the remaining activity in the supernatant during the immobilization of the enzyme, the beads bearing TDI showed the highest specific activity, followed by XDI beads for both galactosidases. To show that the host–guest interaction between the enzyme and the support can be disrupted with a competitive host, the immobilized enzymes were immersed in a DMSO solution (1 : 1 H_2O/DMSO) of β-CD solution (50 mM) during 2 hours at room temperature. The supernatants were removed, the beads were washed several times with water, and the catalytic activity of the Ch-CD-beads was measured. No catalytic activity or presence of protein was detected in the beads, thus showing the reversible binding of the enzyme to β-cyclodextrin-grafted chitosan support.

The reusability of the catalytic materials prepared with different diisocyanates was assessed for 5 consecutive cycles by washing between each cycle with water until removing the *o*-nitrophenol stuck to the particles, so that it did not interfere with the activity assay. The immobilized β-Gal from *B. bifidum* showed a significant loss of catalytic activity after the first cycle, and around 75% decrease after the second cycle for the three tested type of beads. The marked loss of activity in the biomaterials with the enzyme β-Gal from *B. bifidum* is attributed to the low stability of the enzyme under the reuse conditions, independently of the spacer used. β-Gal from *E. coli* proved to have a better performance, whose catalytic activity remained high after five cycles, around 45% (Table S2, ESI†). Finally, the

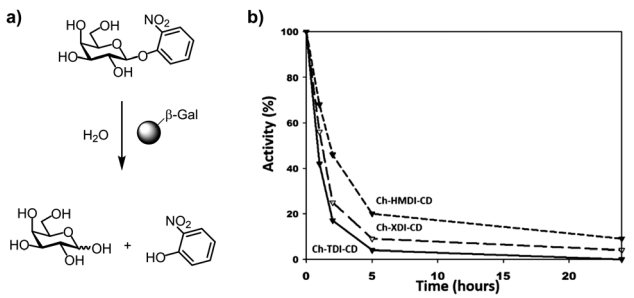

Fig. 6 (a) Enzymatic hydrolysis of *o*-nitrophenyl β-D-galactopyranoside by β-galactosidase. (b) Enzyme activity in the supernatant over the time of β-galactosidase from *E. coli* during the host–guest reversible immobilization process (the β-galactosidase from *B. bifidum* exhibited the same behaviour).

Table 1 Catalytic efficiency of β-galactopyranosides on β-cyclodextrin-grafted chitosan beads

Ch-CD bead·ada-β-Gal complexes	Specific activity (μmol min^{-1} g^{-1})
Ch-HMDI-CD·ada-β-Gal (*E. coli*)	7 ± 0.4
Ch-XDI-CD·ada-β-Gal (*E. coli*)	7.5 ± 0.5
Ch-TDI-CD·ada-β-Gal (*E. coli*)	8 ± 0.3
Ch-HMDI-CD·ada-β-Gal (*B. bifidum*)	3.6 ± 0.1
Ch-XDI-CD·ada-β-Gal (*B. bifidum*)	6.7 ± 0.3
Ch-TDI-CD·ada-β-Gal (*B. bifidum*)	8.8 ± 0.2

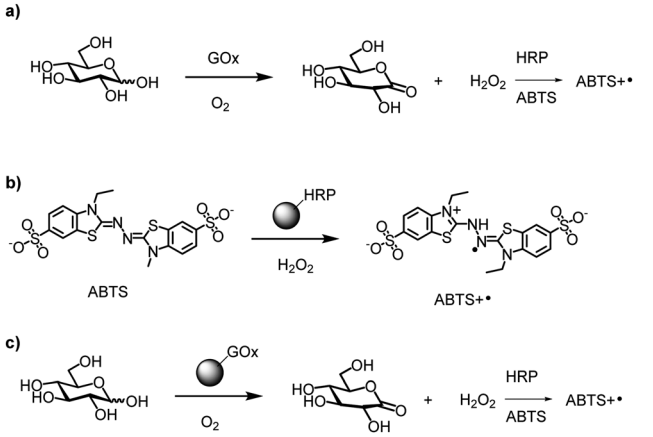

Fig. 7 (a)–(c) Bi-enzymatic cascade constituted by glucose oxidase from *A. niger* (GOx), and peroxidase from horseradish (HRP).

thermal stability of the immobilized β-galactosidase from *E. coli* and from *B. bifidum* retained more than 80% of the activity after 2 weeks of incubation (50 mM phosphate pH = 7) at −4 and 20 °C (Table S3, ESI†).

A similar procedure was applied to study the bi-enzymatic cascade constituted by glucose oxidase from *A. niger* (GOx), and peroxidase from horseradish (HRP). According to Fig. 7a, GOx catalyzes the oxidation of glucose to gluconolactone which results in the *in situ* production of H_2O_2. The latter is used by HRP to oxidize ABTS. The formation of ABTS oxidized product as a function of time is followed by reading by the absorbance at 405 nm. The modification of the enzymes with adamantane and subsequent immobilization on Ch-linker-CD beads were performed following the procedures used with the β-galactosidases.

Compared to unmodified enzymes, the modification with adamantane led to ada-HRP and ada-GOx with a loss of 50 and 40% activity, respectively (Table S3, ESI†). The catalytic activity of Ch-linker-CD·ada-HRP beads was determined measuring the rate formation of ABTS+• (Fig. 7b). For immobilized glucose oxidase (Ch-linker-CD·ada-GOx beads), aliquots of the reaction mixture were submitted to a couple reaction using soluble HRP and ABTS and following the rate of formation of ABTS+• (Fig. 7c). The results are summarized in Table 2. As in the case of the immobilized β-galactosidases, the highest specific activity was observed in the beads with TDI linker (entries 3 and 6, Table 2), followed by XDI (entries 2 and 5) and HMDI (entries 1 and 4). Therefore, the biocatalytic materials with the four enzymes studied show the same profile of increased residual activity when the spacer is more hydrophobic, more rigid, and more reactive. In this way, TDI, which has the isocyanate group directly linked to the aromatic ring, is the most reactive,[28] meaning that a greater number of spacers per mg of support are generated, and also a faster enzyme binding to this support with TDI as linker (Fig. 6).

We then investigated the bi-enzymatic cascade of the co-immobilized (Ch-TDI-CD·ada-HRP/ada-GOx) and of the independently immobilized enzymes (Ch-TDI-CD·ada-HRP + Ch-TDI-CD·ada-GOx). The co-immobilization of ada-GOx and ada-HRP on Ch-TDI-CD beads, to obtain the Ch-TDI-CD·ada-HRP/ada-GOx biocatalyst, was carried out mixing two separated solutions of ada-GOx (1 mg mL^{-1}) and ada-HRP (1 mg mL^{-1}) to obtain different mass ratios (GOx : HRP = 0.2 : 1, 0.5 : 1, 1 : 1, and 5 : 1). Then the resulting bi-enzymatic solution was added to Ch-TDI-CD support and left under stirring at room temperature for 24 h. The activity was determined by adding a solution containing glucose and ABTS and measuring the formation of ABTS+• (entries 7–10, Table 2). The results showed that the ratio ada-GOx/ada-HRP 0.2 : 1 (entry 7) had the highest activity and the increase of the relative proportion of ada-GOx respect to ada-HRP (entries 8–10) led to an important loss of activity. Since both enzymes compete for the cyclodextrin cavity during the immobilization process, higher GOx ratios will result in less HRP ratio in the beads. The decrease in activity observed in this case suggests that the reaction catalyzed by HRP is the rate-limiting step of the process.

Table 2 Specific activity of GOx and HRP immobilized on β-cyclodextrin-grafted chitosan beads

Entry	Ch-CD bead·ada-enzyme complexes	Specific activity (μmol min^{-1} g^{-1})
1	Ch-HMDI-CD·ada-HRP	0.29
2	Ch-XDI-CD·ada-HRP	0.39
3	Ch-TDI-CD·ada-HRP	0.67
4	Ch-HMDI-CD·ada-GOx	0.12
5	Ch-XDI-CD·ada-GOx	0.23
6	Ch-TDI-CD·ada-GOx	0.76
7	Ch-TDI-CD·ada-GOx/ada-HRP (0.2 : 1)	0.13
8	Ch-TDI-CD·ada-GOx/ada-HRP (0.5 : 1)	0.05
9	Ch-TDI-CD·ada-GOx/ada-HRP (1 : 1)	0.03
10	Ch-TDI-CD·ada-GOx/ada-HRP (5 : 1)	0.03
11	Ch-TDI-CD·ada-GOx + Ch-TDI-CD ·ada-HRP (0.2 : 1)	0.13
12	Ch-TDI-CD·ada-GOx + Ch-TDI-CD ·ada-HRP (0.5 : 1)	0.15
13	Ch-TDI-CD·ada-GOx + Ch-TDI-CD ·ada-HRP (1 : 1)	0.15
14	Ch-TDI-CD·ada-GOx + Ch-TDI-CD ·ada-HRP (5 : 1)	0.06

In the case of separately immobilized biocatalysts, different mass ratios of previously immobilized enzymes were mixed together and the activity was measured after adding the substrates. The increase of Ch-TDI-CD·ada-GOx with respect to Ch-TDI-CD·ada-HRP from 0.2 : 1 to 1 : 1 (entries from 11 to 13 in Table 2) did not result in a significant change in the activity, which was around 0.13–0.15 μmol min^{-1} g^{-1}. However, when a large excess of Ch-TDI-CD·ada-GOx was used compared to Ch-TDI-CD·ada-HRP (entry 14) catalytic activity decreased substantially, indicating again that the HRP-catalyzed oxidation of ABTS is the slowest reaction in the one-pot two-enzyme catalytic cascade.

To assess the operational stability of these biocatalysts, the possibility of their reuse was studied. For this purpose, the biocatalyst prepared separately with the two enzymes was used (Ch-TDI-CD·ada-GOx + Ch-TDI-CD·ada-HRP 0.5 : 1). The reusability of the biocatalytic material, showed > 85% activity after 5 five cycles (Table S4, ESI†). The recycling cycles of the reversibly immobilized catalysts were 5 because we started to observe that the morphology of the chitosan beads was lost.

Conclusions

Supramolecular interactions were exploited for eco-friendly immobilization on biomass-derived materials functionalized with β-cyclodextrin "in water". The entire immobilization process is easy, and the performance of the immobilized biocatalysts has been proved with three different enzymes. The immobilization yield was dependent on the linker used to attach the cyclodextrin to the chitosan beads; the best results were obtained with the most rigid linker derived from toluene-2,4-diisocyanate (TDI). For the case of the double sequential enzyme reaction catalyzed by immobilized GOx and HRP, the use of separately immobilized biocatalysts allows a better control of the reaction kinetic. A critical point is the stability of the non-covalent bond between the enzyme and the support. Although the association constant of the adamantane-β-cyclodextrin complex is very high, the enzyme can be desorbed from the support with the continue use limiting the reusability of the catalyst. Additionally, uncontrolled release could occur due to changes in the working medium. While immobilized GOx and HRP were reused several times maintaining a high activity, the immobilized β-galactosidases lost substantial activity during the recycling. This limitation, which is intrinsically associated with the non-covalent immobilization, could be overcome by future research using multi-point host–guest interactions between the enzyme and the support. For example, this could be addressed during the functionlization of the enzyme with adamantine, using reagents containing multiple adamantyl moieties and an adamatyl-enzyme flexible linker capable of forming inclusion complexes with the cyclodextrin on the surface of the support.[23,29]

The present technology based on green chemistry is an attractive proposal in the context of the circular economy model and can find applications in microfluidic reactors that use eco-friendly immobilization.

Experimental

Materials

All solvents and buffers used were of analytical grade from Merck (St. Louis, MO, USA). The peroxidase from horseradish (HRP) type I (250 U mg^{-1}), glucose oxidase from *A. niger* (GOx) (145 U mg^{-1}), and galactosidase from *E. coli* (250 U mg^{-1}) were provided from Sigma. Galactosidase from *B. bifidum* (Saphera, 1.506 U mL^{-1}) was supplied by Novozymes. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt. (ABTS), EDC (1-ethyl-3-(3-(dimethylaminopropyl)carbodiimide)), dimethyl sulfoxide (DMSO), 1-adamantanecarboxylic acid, NHS, HMDI (1,6-hexamethylene diisocyanate), XDI (xylylene diisocyanate), TDI (toluene-2,4-diisocyanate), *o*-nitro-phenyl-β-galactosidase (ONPG), and β-cyclodextrin (β-CD) were from Merck.

Preparation and characterization of β-cyclodextrin-grafted chitosan beads

Chitosan powder of high molecular weight (Ch) was dissolved in 1% (v/v) acetic acid solution and was pumped through a syringe into NaOH solution under stirring until the formation of Ch beads. Subsequently, the beads were washed with water until neutral.[22,23]

Ch beads were first functionalized with spacers (HMDI, XDI, TDI) and later with β-CD with respect to the methods described in previous works.[22] The β-cyclodextrin-grafted Ch beads were washed several times with water and lyophilized to be characterized by ^{13}C-, ^{15}N-solid state NMR and FT-IR spectra. FT-IR spectra were recorded on a One B PerkinElmer Spectrum spectrophotometer (PerkinElmer, Waltham, MA, USA). Spectra were recorded in the absorbance mode in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}.

The solid-state NMR measurements were performed at a temperature of 298 K on a Bruker AVANCE NEO™ spectrometer equipped with a wide bore superconductive magnet operating at 9.4 T (^{13}C and ^{15}N Larmor frequencies at 100.61 and 40.56 MHz, respectively). Samples of microspheres were packed in 4 mm ∅ zirconia rotors. The ^{13}C CP/MAS NMR measurements were carried out with 2 ms contact time, a repetition rate of 3 s, proton decoupling of 62.5 kHz and a spinning rate of 7 kHz. The spectra were externally reference to adamantane (34.48 ppm) secondary to TMS (0.00 ppm). In the case of ^{15}N, contact times of 3 ms, repetition rates of 3 s and proton decoupling (62.5 kHz) were used and spinning rates of 5 kHz. The spectra were externally referenced to ammonium chloride (−341.3 ppm) secondary to nitromethane (0.00 ppm).[30]

The microspheres morphology was analysed using scanning electron microscopy (SEM). Briefly, the microspheres were coated with approx. 5 nm Au/Pd and observed on a Philips (Eindhoven, Netherlands) XL30 scanning electron microscope at ambient temperature using the parameters indicated in each micrograph.

Preparation of β-cyclodextrin-grafted chitosan

β-Cyclodextrin was attached to chitosan beads in two reaction steps according to previously reported method.[31] Briefly, to a 2.5% (v/v) solution of diisocyanate HMDI, TDI or XDI in DMF (20 mL), dry chitosan beads (1 g) were added, stirring at room temperature for 5 min. Then, few drops of stannous 2-ethylhexanoate were added and the mixture was magnetically stirred for 1 h. After this time, the supernatant was discarded, and the beads washed with DMF. The chitosan beads were added to a solution of DMF (20 mL) containing few drops of 2-ethylhexanoate and β-cyclodextrin (0.2 g), stirring at room temperature for 24 h. The supernatant was decanted, and the beads were washed with deionized water and kept in suspension in a 1 : 1 H_2O/EtOH solution until used.

Measurement of galactosidase activity

The hydrolytic activity of β-galactosidase from *E. coli* and from *Bifidobacterium bifidum* (Saphera) or the corresponding immobilized enzyme was followed by formation of *o*-nitrophenol at 420 nm. Briefly, 13 mM of *o*-nitrophenyl β-galactopyranoside ONPG is prepared in a volume of 5 mL of phosphate buffer (0.05 M pH 6.6) in presence of $MgCl_2$ (1 mM). Then 5 μL of β-galactosidase from *E. coli* or 2.5 μL from *B. bifidum* (Saphera) previously prepared diluted in half with phosphate buffer or

50 mg of immobilized β-galactosidase-beads were added at 5 mL of ONPG. As a control, the same solution of ONPG is prepared without addition of the soluble or immobilized enzyme to follow its chemical hydrolysis under the same conditions. 0.5 mL of the reaction is stopped by addition of 0.5 mL of Na_2CO_3 solution and at different times the absorption (λ = 420 nm) was measured.

Measurement of peroxidase activity

Activity of the HRP soluble or immobilized on β-cyclodextrin-grafted chitosan beads was followed by a spectrofluorometric assay using ABTS as a substrate at 25 °C and pH 5.0. The reaction was initiated by adding 10 μL of HRP (0.1 mg mL^{-1}) to a solution of ABTS (9 mM at pH = 5.0, ε = 36.8 mM^{-1} cm^{-1}), and hydrogen peroxide (0.33%), measuring the formation of $ABTS^{+\bullet}$ at λ = 405 nm. In the case of immobilized HRP the reaction was carried out in the same way, but 15 mg of the immobilized enzyme-beads was added. The mixture was stirred at 25 °C and aliquots from the supernatant were taken at different time intervals to measure the formation of $ABTS^{+\bullet}$. The same protocol was carried out using the β-cyclodextrin-grafted chitosan beads, as control, with no green colour detected.

$$H_2O_2 + \text{ABTS peroxidase} > 2H_2O + ABTS^+$$

Measurement of glucose oxidase activity

Activity of glucose oxidase soluble or immobilized on β-cyclodextrin-grafted chitosan beads was measured by assay coupled to the HRP enzyme. Briefly, a volume of 1 mL of phosphate buffer (50 mM, pH 5), with ABTS (9 mM), and 0.02–0.2 mL of glucose (1 M), was added the enzyme GOx (0.005 mL) or 15 mg immobilized-GOx-beads is left time (3 min) to generate hydrogen peroxide *in situ* and later HRP soluble (0.005 mL) or immobilized (10 mg) is added to follow the reaction at 405 nm with time.

Preparation of adamantane-modified β-galactosidase from *E. coli* (ada-Gal)

0.9 mL of a solution of β-galactosidase from *E. coli* (1 mg mL^{-1}) is mixed with 4 mL of PBS at pH 6.6, and the initial activity is measured with ONPG at 420 nm. Subsequently, a DMSO solution (0.1 mL) containing 2.0 mg of NHS, 1.7 mg of EDC, and 1.0 mg of 1-adamantanecarboxylic acid were added. The mixture is left under gentle stirring 2 h at room temperature and overnight at 4 °C. After 24 hours, dialysis of the enzyme solution is performed against PBS buffer (50 mM, pH 6.6, 2 L) for 2 hours to remove excess reagents at 4 °C. The enzyme solution is then removed from the dialysis membrane, centrifugate and its activity is measured with ONPG at 420 nm using 5 μL of solution (ada-Gal).

Preparation of adamantane-modified β-galactosidase from *B. bifidum* (ada-Saphera)

0.8 mL of a Saphera enzyme solution commercial is mixed with 3 mL of PBS at pH 6.6, and the initial activity is measured with ONPG at 420 nm. Subsequently, a DMSO solution (0.2 mL) containing 2.0 mg of NHS, 1.7 mg of EDC, and 1.0 mg of 1-adamantanecarboxylic acid were added. The mixture is left under gentle stirring 2 h at room temperature and overnight at 4 °C. After 24 hours, dialysis of the enzyme solution is performed against PBS buffer (50 mM, pH 6.6, 2 L) for 2 hours to remove excess reagents at 4 °C. The enzyme solution is then removed from the dialysis membrane, centrifugate and its activity is measured with ONPG at 420 nm using 5 μL of solution (ada-Saphera).

Preparation of adamantane-modified horseradish peroxidase (ada-HRP)

0.5 mL of HRP enzyme solution (3.2 mg mL^{-1}) is mixed with 1.0 mL of PBS at pH 6.6, and the initial activity is measured with ABTS at 405 nm. Subsequently, a DMSO solution (0.1 mL) containing 2.0 mg of NHS, 1.7 mg of EDC, and 1.0 mg of 1-adamantanecarboxylic acid were added. The mixture is left under gentle stirring 2 h at room temperature and overnight at 4 °C. After 24 hours, dialysis of the enzyme solution is performed against PBS buffer (50 mM, pH 6.6, 2 L) for 2 hours to remove excess reagents at 4 °C. The enzyme solution is then removed from the dialysis membrane, and its activity is measured with ABTS at 405 nm using 10 μL of solution (ada-HRP).

Preparation of adamantane-modified glucose oxidase (ada-GOx)

0.5 mL of GOx from *A. niger* enzyme solution (3.2 mg mL^{-1}) is mixed with 1.0 mL of PBS at pH 6.6. Subsequently, a DMSO solution (0.1 mL) containing 2.0 mg of NHS, 1.7 mg of EDC, and 1.0 mg of 1-adamantanecarboxylic acid added. The mixture is left under gentle stirring overnight at 4 °C. After 24 hours, dialysis of the enzyme solution is performed against PBS buffer (50 mM, pH 6.6, 2 L) for 2 hours to remove excess reagents at room temperature. The enzyme solution is then removed from the dialysis membrane, and its activity is measured with ABTS, glucose and HRP enzyme at 405 nm using 10 μL (1 mg mL^{-1}) of ada-GOx.

Supramolecular immobilization of ada-HRP, ada-GOx, ada-Gal and ada-Saphera on β-cyclodextrin-grafted Ch beads

To a solution of 2 mL of ada-Gal, ada-Saphera, ada-HRP and ada-GOx prepared in the previous step were added to a 0.2 g of β-cyclodextrin-grafted Ch beads and left to stir for 24 h at room temperature. After filtration, the enzymatic activity of biomaterials and the supernatant (unbound enzyme) were measured. The co-immobilization of ada-GOx and ada-HRP on Ch-TDI-CD beads, to obtain the Ch-TDI-CD·ada-HRP/ada-GOx biocatalyst, was carried out mixing two separated solutions of ada-GOx (1 mg mL^{-1}) and ada-HRP (1 mg mL^{-1}) to obtain different mass ratios (GOx : HRP = 0.2 : 1, 0.5 : 1, 1 : 1, and 5 : 1 mL).

Stability of the soluble and immobilized enzymes

Wyle type enzyme (1 mg mL^{-1}) or immobilized enzyme suspension (10 mg mL^{-1} solution) in phosphate buffer at pH 7 was stored at different temperatures and its activity was measured over time as described above for each enzyme.

Reuse of biomaterials

β-Cyclodextrin-grafted chitosan beads with the HRP and GOx bound were used several times to catalyze glucose detection, and with the galactosidases the hydrolysis of ONPG. Before each reuse, the enzyme immobilized were washed with water to remove any retained substrates or products.

Release of HRP-ada and Gox-ada from β-cyclodextrin-grafted chitosan beads was performed using a solution of DMSO (1.1 H_2O-DMSO) containing an excess of free β-CD (50 mM) for 2 h at 25 °C. The surfaces were then rinsed using the same buffer. Finally, the enzymatic activity of the surface was evaluated. Equally, the protein concentration released in the solution was determined by the Bradford protein assay. All enzymatic experiments were carried out in triplicate.

Author contributions

Conceptualisation: A. F.-M. Experiment design and discussion: A. B., L. G. and A. F.-M. Functionalization of chitosan beads, enzyme immobilization and development of analytical methods: A. B. NMR experiments: L. G. Writing the manuscript: A. B. and A. F.-M.

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Funding for this project was provided by CSIC (grant 202480E048, 202480E094 and 202480141) and Ministerio de Ciencia e Innovación (grant PID2019-105337RB-C21).

Notes and references

1 C. Espro, E. Paone, F. Mauriello, R. Gotti, E. Uliassi, M. L. Bolognesi, D. Rodríguez-Padrón and R. Luque, *Chem. Soc. Rev.*, 2021, **50**, 11191–11207.
2 Y. Zhou, S. Wu and U. T. Bornscheuer, *Chem. Commun.*, 2021, **57**, 10661–10674.
3 L. Lin, X. Han, B. Han and S. Yang, *Chem. Soc. Rev.*, 2021, **50**, 11270–11292.
4 M. Shahid, R. Rajoka, L. Zhao, H. M. Mehwish and Y. W. S. Mahmood, *Appl. Microbiol. Biotechnol.*, 2019, **103**, 1557–1571.
5 M. N. Kantak and S. S. Bharate, *Carbohydr. Polym.*, 2022, **278**, 118999.
6 J. Revuelta, I. Fraile, D. T. Monterrey, N. Peña, R. Benito-Arenas, A. Bastida, A. Fernandez-Mayoralas and E. Garcia-Junceda, *Mater. Horiz.*, 2021, **8**, 2596–2614.
7 W. M. Argüelles-Monal, J. Lizardi-Mendoza, D. Fernández-Quiroz, M. C. T. Recillas-Mota and M. Montiel-Herrera, *Polymers*, 2018, **10**, 342.
8 T. Boominathan and A. Sivaramakrishna, *Top. Curr. Chem.*, 2021, **379**, 19.
9 O. Mahé, J.-F. Brière and I. Dez, *Eur. J. Org. Chem.*, 2015, 2559–2578.
10 P. K. Jaiswal, V. Sharma, M. Mathur and S. Chaudhary, *Org. Lett.*, 2018, **20**, 6059–6063.
11 Z. Wang, W. Liu, W. Liu, Y. Ma, Y. Li, B. Wang, X. Wei, Z. Liu and H. Song, *RSC Adv.*, 2021, **11**, 15785.
12 M. de, F. Matos de Freitas, L. C. Hortêncio, T. Lima de Albuquerque, M. V. Ponte Rocha and L. R. Barros Gonçalves, *Bioprocess Biosyst. Eng.*, 2020, **43**, 711–722.
13 Y. L. Nunes, F. L. de Menezes and I. G. de Sousa, *et al.*, *Int. J. Biol. Macromol.*, 2021, **181**, 1124–1170.
14 S. Wu, R. Snajdrova, J. C. Moore, K. Baldenius and U. T. Bornscheuer, *Angew. Chem., Int. Ed.*, 2021, **60**, 88–119.
15 J. Bié, B. Sepodes, P. C. B. Fernandes and M. H. L. Ribeiro, *Processes*, 2022, **10**, 494.
16 J. M. Bolivar, J. M. Woodley and R. Fernandez-Lafuente, *Chem. Soc. Rev.*, 2022, **51**, 6251–6290.
17 J. M. Bolivar and B. Nidetzky, *Chim. Oggi*, 2013, **31**, 50–54.
18 S. Pina, C. R. Lowe, A. Cecília and A. Roque, *Biotechnol. Adv.*, 2014, **32**, 366–381.
19 J. A. Finbloom and M. B. Francis, *Curr. Opin. Chem. Biol.*, 2018, **46**, 91–98.
20 Y.-H. Zhang, Y-H. Liu and Y. Lin, *Supramolecular Protein Chemistry: Assembly, Architecture and Application*, ed. P. B. Crowley, 2021, pp. 124–160.
21 M. V. Rekharsky and Y. Inoue, *Chem. Rev.*, 1998, **98**, 1875–1918.
22 R. Villalonga, S. Tachibana, R. Cao, P. D. Ortiz, L. Gomez and Y. Asano, *J. Exp. Nanosci.*, 2006, **1**, 249–260.
23 A. González-Campo, B. Eker, H. J. G. E. Gardeniers, J. Huskens and P. Jonkheijm, *Small*, 2012, **8**, 3531–3537.
24 A. Sanz de Leòn, N. Vargas-Alfredo, A. Gallardo, A. Fernàndez-Mayoralas, A. Bastida, A. Muñoz-Bonilla and J. Rodríguez-Hernández, *ACS Appl. Mater. Interfaces*, 2017, **9**, 4184–4191.
25 C. Li, X. Song, S. Hein and K. Wang, *Adsorption*, 2010, **16**, 85–91.
26 E. Tiburcio, E. García-Junceda, L. Garrido, A. Fernández-Mayoralas, J. Revuelta and A. Bastida, *Polymers*, 2021, **13**, 3326.
27 S. Hans and R. Kricheldorf, *Makromol. Chem.*, 1981, **182**, 1177–1196.
28 O. Welz, M. Pfeifle, P. M. Plehiers, R. Sure and P. Deglmann, *J. Phys. Chem. A*, 2022, **126**, 9333–9352.
29 N. Moridi, P. F.-X. Corvini and P. Shahgaldian, *Angew. Chem., Int. Ed.*, 2015, **54**, 14800–14804.
30 P. Bertani, J. Raya and B. Bechinger, *Solid State Nucl. Magn. Reson.*, 2014, **61**, 15–18.
31 S.-H. Chiu, T.-W. Chung, R. Giridhar and W.-T. Wu, *Food Res. Int.*, 2004, **37**, 217–223.