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Efficient Magnetic Recycling of Covalently Attached Enzymes on Carbon-Coated Metallic Nanomagnets

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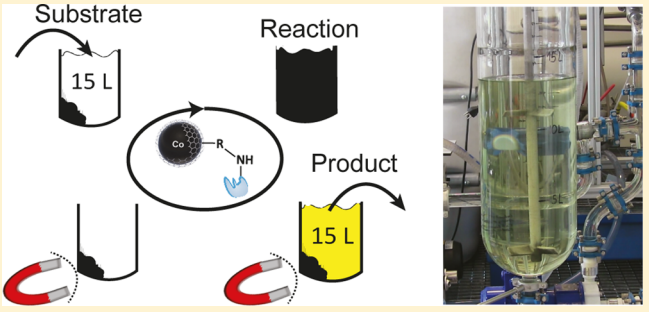
Supporting Information

ABSTRACT: In the pursuit of robust and reusable biocatalysts for industrial synthetic chemistry, nanobiotechnology is currently taking a significant part. Recently, enzymes have been immobilized on different nanoscaffold supports. Carbon coated metallic nanoparticles were found to be a practically useful support for enzyme immobilization due to their large surface area, high magnetic saturation, and manipulatable surface chemistry. In this study carbon coated cobalt nanoparticles were chemically functionalized (diazonium chemistry), activated for bioconjugation (*N,N*-disuccinimidyl carbonate), and subsequently used in enzyme immobilization. Three enzymes, β-glucosidase, α-chymotrypsin, and lipase B were successfully covalently immobilized on the magnetic nonsupport. The enzyme−particle conjugates formed retained their activity and stability after immobilization and were efficiently recycled from milliliter to liter scales in short recycle times.



INTRODUCTION

Recent advances in biotechnology have opened the way for the widespread application of biocatalysis in industrial organic synthesis by offering biodegradable catalysts (enzymes) with a high stereo-, chemo-, and regioselectivity as well as mild operating conditions.[1] Enzyme separation from product after reaction and efficient recovery and reuse of costly enzymes especially in industrial (large-scale) processes have led to the development of enzyme immobilization via adsorption, entrapment, cross-linking, and covalent linkage on solid supports.[2] Throughout the years, substantial research has been completed in order to optimize the carrier material for having high surface area/volume ratio, enzyme loading, and substrate diffusion.[3] The most important property of enzyme carriers is their mechanical and chemical stability over a vast range of pH values.[1] The latest achievements in nanotechnology show a possibility to develop a revolutionary class of biocatalyst nanostructured materials (nanoparticles, nanofibers, mesoporous materials, and single enzyme nanoparticles) for enzyme immobilization. When enzymatic carriers are on the submicrometer scale, the limitation is the difficult separation of the catalyst from solution. Magnetically driven separations are much easier and faster than cross-flow filtration and centrifugation. Magnetic nanoparticles with good stability (core/shell particles) and high magnetic saturation (M_s) are of great interest in biocatalysis as enzymatic carriers because they combine the advantages of easy and fast separation with high dispersion and reactivity.[4] Iron oxide nanoparticles (maghemite, γ-Fe_2O_3, or magnetite, Fe_3O_4), with a magnetic saturation of 30−85 (emu/g), have been shown to be a possible platform for enzyme immobilization.[5−13] The M_s of the iron oxide nanoparticles however leads to a slow separation from liquid media (min to h) even after applying strong magnetic fields (see SI).[14,15] Further, the iron oxide materials have a limited chemical stability and product leaching;[16,17] in addition, loss of immobilized enzyme is a problem when scaling the idea of magnetic separation to larger volumes. Carbon coated metal nanoparticles (Co/C and Fe_3C/C), which combine the beneficial magnetic properties of the core (high magnetic saturation of >158 emu/g) and the possibility of covalent surface chemistry, have been reported in literature (average size of 30 nm)[18] and are commercially available. The advantages of the carbon coated metal nanomagnets compared to iron oxides (e.g., SPIONS) have previously been reported for applications in semiheterogeneous catalysis,[19,20] water,[21] and blood[14,22] purification. For application in biocatalysis, the possibility of linking the enzyme to the support via a reliable organic chemistry based procedure is especially attractive as it promises

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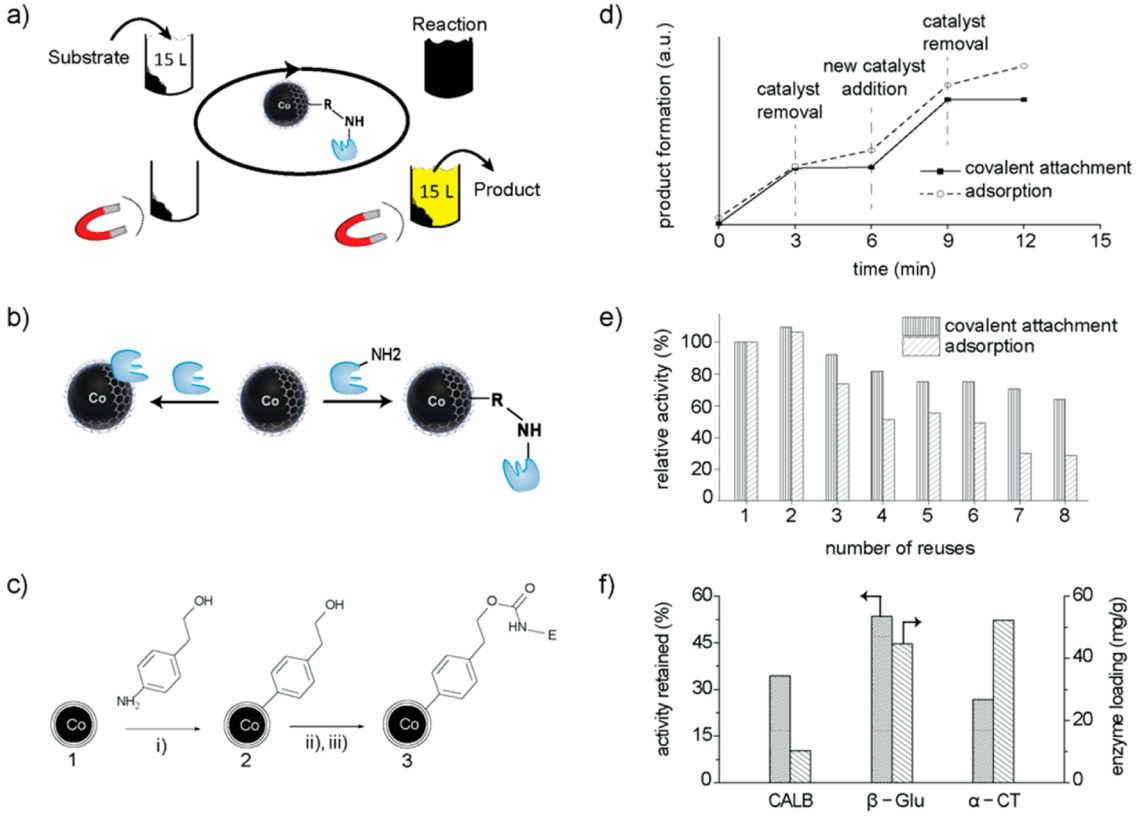

Figure 1. (a) Biocatalyst immobilized onto the particle surface (center) is quasi-homogeneously mixed with the substrate during the reaction mixture. It can be rapidly recovered from the product by magnetic force after reaction and directly reused without further workup. (b) Methods for enzyme immobilization employed: adsorption (to the left) and covalent attachment (to the right). (c) Chemical functionalization and activation of the particle surface for the immobilization of enzymes, E = enzyme: (i) HCl, $NaNO_2$, H_2O, RT, 30 min; (ii) DSC, DMAP, dry DMF, RT, 24 h; (iii) enzyme, H_2O, RT, 5 h; (d) Tracking of product formation (nitrophenol, optical absorption at 405 nm) after repeated addition and magnetic removal of magnetic particles with adsorbed and chemically attached β-Glu. (e) Recyclability experiments of the same product; activity in both cases is relative to the one of the first cycle. (f) Comparison between the enzymatic activities and protein loadings of the three chemically immobilized enzymes.

good catalyst recycling and low protein byproducts in the final product.

In this study, we report a successful covalent immobilization of three widely used enzymes on the surface of carbon coated metallic nanoparticles: β-glucosidase (β-Glu) from almonds (E.C. 3.2.1.21), α-chymotrypsin (α-CT) from bovine pancreas (E.C. 3.4.21.1), and *Candida antarctica* Lipase B (CALB) (E.C. 3.1.1.3). We show that these enzymes retain a large fraction of their activity when immobilized on the nanosupport, and can be easily separated (s to min) from reaction media and reused with consistent results (Figure 1a). This was shown not only on a small (mL) but also on larger scale utilizing a 20 L glass reactor modified with a magnetic filter.

RESULTS AND DISCUSSION

Prior to covalently attaching the enzymes to the surface of the magnetic beads, enzymes were merely adsorbed to the hydrophobic surface (garphene-like carbon) of the beads (Figure 1b). The immobilization of proteins on hydrophobic surfaces is a common technique in protein immobilization and is best known from the preparation of ELISA (enzyme-linked immunosorbent assay) plates.[23] Indeed, when the particles were immersed in a glucosidase solution they were rapidly covered with a monolayer of the enzyme (30 mg enzyme per 1 g particles). After washing away any nonbound material, the enzyme adsorbed onto the particle surface retained about 50% of its original activity (see Scheme S1 in SI for method of activity comparison). However, during several reuse cycles the activity of the particles decreased rapidly (Figure 1e). The following experiment was conducted to elucidate why the activity of the particles decreased: enzyme loaded particles were added to a substrate solution and the reaction was followed by measuring the product formed (UV−vis) for 3 min, when the particles were removed by magnetic attraction. Any product formation after removal of the particles could then be ascribed to the nonremoved enzyme (i.e., nonbound enzyme). We found this method to be most sensitive and it quickly showed the degree of enzyme loss from the particle surface (Figure 1d, dotted line).

Several methods were tried to chemically desorb previously adsorbed β-Glu on the Co/C. The methods included single washings of the Co/C-β-Glu (adsorbed) with aqueous solutions of acetonitrile (10%, 30%, 50%, 70%, and 90%), phenol (5%), or SDS (5%). Elemental microanalysis was utilized to follow the course of any protein desorption, as this is a very precise and quantitative measurement technique. In all described cases with our washing strategy no substantial amount of protein could be eluted in a short time (20 min of shaking at 600 rpm, see Table 1). In the literature there is already data on carbon nanotubes (CNTs) and their interaction with proteins. Although geometrically different, both CNTs and our Co/C nanoparticles share the same surface structure, which

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Table 1. C, H, N Weight Change of the Adsorbed Co/C−β-Glu after Being Washed with Different Solvents Relative to the Nonwashed Adsorbed Enzyme Mass % Determined by Elemental Microanalysis Measurement (Co/C−β-Glu (adsorbed): C: 8.8%, H: 0.41%, N: 0.74%)

Sample	C (%)	H (%)	N (%)
Acetonitrile (10%)	+0.3	+0.04	+0.01
Acetonitrile (30%)	+0.2	+0.01	+0.01
Acetonitrile (50%)	+0.2	+0.03	+0.01
Acetonitrile (70%)	+0.1	+0.04	+0
Acetonitrile (90%)	−0.2	+0	−0.01
Phenol in H_2O (5%)	+0.05	+0.06	+0
SDS in H_2O (5%)	+1.15	+0.25	−0.01

is a graphene-like layer. There are four types of forces that can contribute to the noncovalent binding of proteins on carbon: van der Waals interactions ($\pi-\pi$ stacking), hydrophobic interactions, amphiphilicity, and weaker but still present electrostatic interactions.[24] These forces are very strong to even hold most of the enzyme molecules attached to such a surface without the need of a covalent binding.[25] However, in order to achieve a 100% effective biocatalytic switch we had to be sure that not even a single molecule desorbs from the particle surface during prolonged enzymatic action and following prolonged product storage, which was not the case with the adsorbed β-Glu (Figure 1d). Slow (course of hours/days) desorption of enzyme molecules from a surface can happen due to the transient complex turning where, due to structural changes of the biomolecules, some enzymes which are adsorbed loosely are squeezed out spontaneously and released over time.[26,27] This is consistent with the loss of enzymatic activity after prolonged storage of the enzyme adsorbed to the magnetic substrate (Figure 2). Further, repetitive retractions with the aid of a magnet could also have a mechanical impact releasing free enzyme in solution after multiple particle collisions.

To reduce the enzyme loss to an absolute minimum, the active protein was covalently attached to the surface of the particles by diazonium and carbamate chemistry (Figure 1c). Diazonium chemistry is one of the few chemistries known for the functionalization of the rather inert carbon surfaces and its application with 2-4(-aminophenyl)ethanol enables the introduction of an alcohol group on the particle surface which is compatible with *N,N*-disuccinimidyl carbonate (DSC) activation and peptide immobilization. The individual steps of the immobilization were followed by FTIR spectroscopy (Figure 3)

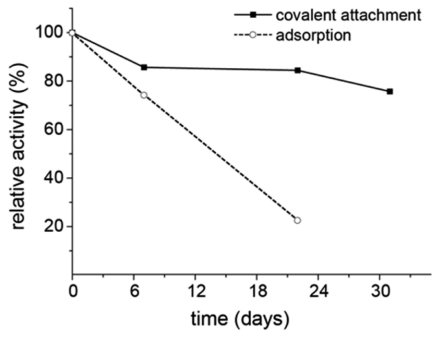

Figure 2. Enzymatic activity changes of the immobilized β-Glu over a prolonged storage period at +4 °C. The covalently attached enzyme (solid line, squares) showed 76% of activity retained after one month, whereas the adsorbed enzyme (dashed line, circles) showed 23% after 22 days of storage. The enzymatic activities are relative to the activities of the immobilized enzymes measured immediately after immobilization (100%).

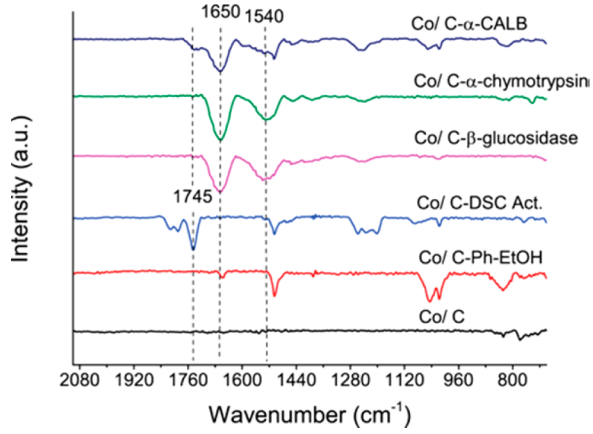

Figure 3. Fourier transform IR (FTIR) spectra of the consecutive steps leading to enzyme immobilization (from bottom to top).

and CHN microanalysis (see SI). The IR peak at 1745 cm^{-1} reflects the presence of the NHS-carbonate (asymmetric stretch of the NHS-carbonyl)[28] during the activation step, which then disappears after protein addition. The final particles displayed IR absorption peaks at 1650 and 1540 cm^{-1}, corresponding to amide 1 and 2 vibrations and bends characteristic for proteins.[29] To quantify the amount of bound enzyme, CHN microanalysis was conducted and yielded a protein loading of 4.5 wt % (Figure 1f). The NHS-ester formation was additionally confirmed by X-ray photoelectron spectroscopy analysis (see SI). In the case of Co/C-Ph-EtOH particles Co, C, and O could be detected but no N. Co was mainly in a metallic state. Co, C, O, and N could be clearly detected as expected from the structure of the Co/C-DSC activated particles where Co is in a metallic state. The O to N ratio was 6.9, which is slightly higher than expected (5). This indicates an additional O source such as Co−O or C−O components or an incomplete DSC functionalization.

Similar to the adsorbed enzyme, the chemically attached β-Glu had an initial activity of ∼50% when compared to the free enzyme activity under the same conditions (Figure 1f). However, in comparison to the enzyme adsorbed on the particle surface, the chemically attached enzyme completely stayed with the magnetic particles. Therefore, a fully magnetic activity switch (Figure 1d, solid line) could be performed. As can be seen from the figure there is no increase in product formation in the supernatant 3 min after the magnetic particle removal. After addition of a new magnetic catalyst to the same substrate solution, the reaction could be turned on again, to be turned off by a second magnetic separation step.

The reliable nature of the bond was also supported by the reusability experiment (Figure 1e) where new substrate solutions (8 times in a row) were added to the same catalyst, each time retracting the particles by magnet and discarding the last product solution. The slight gradual decrease in activity (ca. 5% per run) can be attributed to enzyme deactivation during prolonged, repeated use. Also, the maintained activity after storage is additional evidence of the covalent immobilization (Figure 2).

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To give an insight into the binding on the protein level, LC/MS/MS peptide identification analysis from proteolytic digests of β-Glu both free in solution and covalently immobilized on the Co/C magnetic beads was performed (see SI). In the MS spectrum of the covalently immobilized enzyme, the sequence at ~1000 m/z was poorly covered, which was well covered in the MS spectrum of the free sample (see SI). From this data it is assumed that the enzyme is mostly bound via this sequence ((K)EDIDAVFR(A)). Further supporting this hypothesis is the fact, that the most abundant peptide in the covalently bound enzyme digest (~1750 m/z; (R)GPSIWDTFTHKHPEK(I)) is on the opposite side of the enzyme (i.e., attack by protease is sterically least hindered).

Since the high magnetic saturation is one of the key features of the metallic nanoparticles, vibrating sample magnetometry (VSM) hysteresis data was obtained for both the enzyme–particle conjugates and their precursors (see SI). There was almost no decrease in M_s of **2** (<1% loss) and very little decrease of **3** (5–8% loss) which was expected due to the presence of the biopolymer around the particles. On the other hand, no change in the particle surface morphology was detected by means of scanning electron microscopy (SEM) (see SI). Co/C nanoparticles either naked or immobilized tend to form dynamic aggregates once dispersed in solution as it can be seen from both SEM and STEM pictures. Due to mixing effects, particles bud off and reagglomerate with other clusters. As a result, the active (exposed) surface area is more or less the same at any instant of time.

In order to show that the presented material and concept can be scaled up, a pilot scale experiment was conducted (Figure 4). This experiment showed that the β-Glu covalently

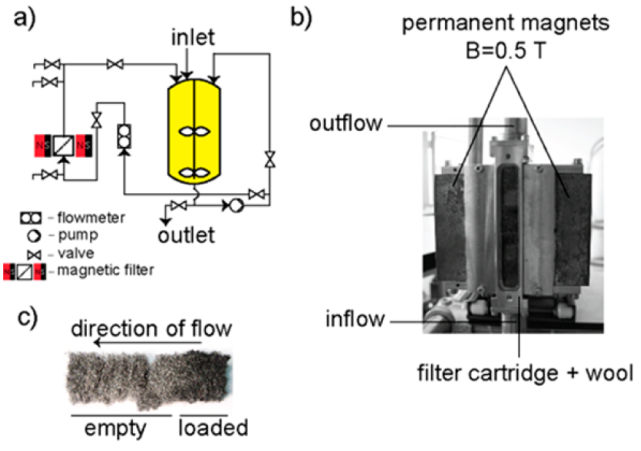

Figure 4. (a) Large scale setup (20 L glass tank reactor modified with a magnetic filter) used in enzymatic catalysis is schematically displayed. (b) Magnetic filter used in the separation. (c) Metallic wool partly loaded with particles (on the right) after magnetic separation and removal from the cartridge.

immobilized on the magnetic nanoparticles could be rapidly and efficiently separated and recycled from 15 L suspensions (Figure 5; Movie1 in SI). For this purpose a glass tank reactor equipped with a pump and stirrer and modified with the addition of a magnetic filter was used.[30] The reaction started with the addition of the catalyst (0.6 g β-Glu loaded nanomagnets) to the substrate (2 g) solution under continuous stirring and continued for 18 min. In the first magnetic separation experiment the particles were separated from the

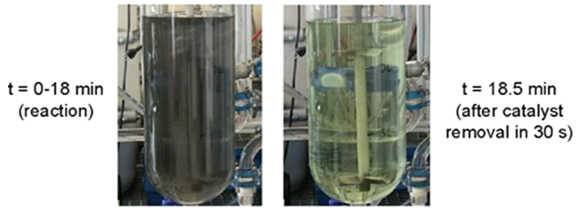

Figure 5. β-Glu coated nanomagnets in 15 L of substrate solution (left) facilitated the catalytic generation of the yellow nitrophenol product and could be magnetically removed from the product mixture (right) within merely 30 s.

volume by a small magnet placed on the wall of the reactor (nonoptimized separation). Under these conditions, the particles could be separated from the reaction mixture in 18 min to yield a yellow product solution. The particles were then reused by the addition of a new substrate solution (old was discarded) and the reaction took place again. The second time the particle suspension was pumped (>1.5 m^3/h) through a magnetic filter unit, which was composed of a cartridge filled with stainless steel wool and four permanent magnets, which created a magnetic field (B) of 0.5 T (see SI). Under these optimized separation conditions, the particles could be removed from the reaction mixture (15 L) within only 30 s.

To address the issue of possible cobalt toxicity an experiment was performed to investigate the cobalt leaching from the Co/C-β-Glu particles with the help of atomic absorption spectroscopy (AAS). The cobalt concentration was determined to be below the detection limit of the instrument (<1 mg/L) when 1 mg of particles was shaken in reaction buffer (20 mL) for 30 min. This is well below any toxicologically relevant level.[31,32]

Finally, our catalyst was compared to a commercially available gold standard in the area of protein immobilization on magnetic carriers. *N*-Ethyl-*N*′-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC) activation capable Dynabeads, MyOne Carboxylic Acid (Life technologies), were purchased and their enzymatic activity and separation speed were measured and compared to the same results obtained with the Co/C-β-Glu. Both activation and incubation steps were performed exactly as described in the coupling protocol provided by the supplier. The enzymatic activity was measured in an identical way as with the Co/C-β-Glu. Comparison between the specific enzymatic activities of both free and immobilized enzymes is given in Table 2. As shown in the table, the β-Glu immobilized on the Dynabeads showed activity of about 500 U/g, which is 100 U/g less than Co/C-β-Glu. Magnetic separation speed was compared by placing a small magnet in between suspensions of both immobilized catalysts (see SI). As shown on Figure S4, when the magnet was placed

Table 2. Quantitative Comparison in Enzyme Loading, Activity, Cost, and Magnetization of Co/C and Dynabeads (DB) as Supports for β-Glu Immobilization

sample	enzyme loading (mg protein/g particles)	activity per carrier (U/g nanoparticles)	cost per activity (USD/U activity)	magnetic saturation (emu/g)
Co/C-β-Glu	45	585	0.3	133
DB-β-Glu	30	483	18	25.1

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at zero distance from the sample vials after 1 min of separation the Co/C suspension was about 85% clear. At the same time point hardly any clearance could be observed with the Dynabeads. From these experiments it can be concluded that much faster separation is achieved when the Co/C particles are employed, which is especially important in large volumes. Even with the aid of a bigger magnet ($4 \times 4 \times 2$ cm^3) it took 3−4 min to obtain a sort of clear supernatant in the case of the Dynabeads sample. The slower separation is a result of the lower magnetic saturation values (M_s) of the Dynabeads when compared to the magnetic saturation values of the Co/C (Table 2, see SI). It could be concluded that the low magnetic saturation (M_s) of the Dynabeads limits their usage mainly to small volumes, whereas the Co/C beads can also be separated from larger (multiliter) volumes. In addition, the Dynabeads are more expensive than the commercially available Co/C particles. Only 20 mg of the Dynabeads MyOne Carboxylic Acid cost ~150 USD (Life Technologies). On the other hand, functionalized Co/C nanoparticles are currently available at a price of 410 USD per 2.5 g (e.g., Sigma-Aldrich Product 742406). Calculated per achievable unit of enzymatic activity, the carbon coated carrier is more than 50 times more cost-effective.

To show the potential usage as a universal enzyme carrier the magnetic nanoparticles also served as a support for other enzymes; α-CT and CALB were covalently immobilized utilizing the same method and their activities were assayed and directly compared to the free enzyme in solution (Figure 1f). The enzymatic assay of the α-CT is based on ester bond hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester and *N*-benzoyl-L-tyrosine detection at 256 nm.[33] In the case of CALB the hydrolysis of tributyrin was followed titrimetrically. While the relative activities remained similar to previously reported values on magnetic iron oxide nanoparticles (Table 3), and are in line with the typical loss of enzyme activity when immobilized,[1,34] the highly increased magnetic properties and reliable magnetic attachment facilitated a highly increased ease of recyclability.

Table 3. Comparison of Retained Enzymatic Activities and M_s of the Particle−Enzyme Conjugates Compared to Literature Data on Magnetic Immobilized Enzymes

magnetic nanoparticles	enzyme immobilized	M_s (emu/g)	retained activity (%)	ref.
Fe_3O_4/SiO_2	lipase	30	18−24	9
Fe_3O_4/SiO_2	α-CT	30	6−18	9
Fe_3O_4/CS	lipase	36	56	12
Fe_3O_4	YADH[a]	63	62	5
Fe_3O_4/SiO_2	β-lactamase	76	54	7
Fe_3O_4/APTES	GOD[a]	85	15−23	10
Fe_3O_4	ALP[a]	82	20−43	11
Fe_3O_4	GOD[a]	-	30	8
γ-Fe_3O_4	lipase	61	0.3−0.6	6
Fe_3O_4	b-DI[a]	38	22−43	13
Co/C	β-Glu	142	55	[b]
Co/C	lipase	142	36	[b]
Co/C	α-CT	142	23	[b]
DB-COOH[a]	β-Glu	26.2	47	[b]

[a]DB-COOH stands for carboxylic acid functionalized dynabeads. GOD: glucose oxidase; ALP: alkaline phosphatase; YADH: yeast alcohol dehydrogenase; b-DI: biotinylated diaphorase. [b]This work.

CONCLUSION

Chemical functionalization of carbon surfaces and protein coupling chemistry using activated carboxylic acids enables the immobilization of enzymes on highly magnetic (metallic) nanosupports. In terms of activity, the covalent immobilization yields good storage stability and recyclability of the conjugates. In terms of applicability the improved magnetic properties allow the usage of magnetically immobilized enzymes to multiliter volumes. With this, the rapidly growing field of chemical biocatalysis can profit from magnetic separation technology, which is already well established in the fields of analytical immunoprecipitation and cell separation on the milliliter scale utilizing metal oxide based particles.

MATERIALS AND METHODS

Chemical Functionalization of the Co/C Nanoparticles. *Diazonium Chemistry.* The Co/C nanoparticles (5 g, TurboBeads, Zurich) were suspended in dH_2O (250 mL) by the use of an ultrasonic bath (Sonorex RK 106, Bandelin) for 15 min. A cooled (ice bath) solution of 2-(4-aminophenyl)-ethanol (1.5 g, 11 mmol, Sigma-Aldrich) in 50 mL dH_2O and concentrated HCl (5 mL, Sigma-Aldrich) was prepared and added to the particle suspension followed by another ultrasonic bath cycle of 30 min. In the first 5 min a cool solution of sodium nitrite (2 g, 29 mmol, Fluka) in 15 mL deionized H_2O was added dropwise to the final suspension. The Co/C-Ph-EtOH nanoparticles were recovered from the reaction mixture with the aid of a neodymium based magnet (Q-30-30-15-N, Webcraft GmbH, side length 30 mm) and washed 4× with water, 4× with ethanol, 2× with ethyl acetate, and 4× with acetone. Each washing step consisted of suspending the particles in the solvent, ultrasonicating for 4 min, and retracting the particles from the solvent with the aid of the magnet. After washing the particles were dried in a vacuum oven at 60 °C.

DSC Activation. Under N_2 flow the Co/C-Ph-EtOH nanoparticles produced via diazonium chemistry (300 mg) were added to a previously evacuated Schlenk flask together with *N,N′*-disuccinimidyl carbonate (200 mg, 0.78 mmol, Fluka) and 4-dimethylaminopyridine (50 mg, 0.41 mmol, Acros) by quickly opening and closing the septum. Dimethylformamide (30 mL, dry) was then injected through the septum and the reaction mixture was suspended by the use of an ultrasonic bath (20 min) and shaken overnight on an orbital shaker (VXR Basic, IKA). The nanoparticles were recovered from the reaction mixture with the aid of a magnet and washed 2× with dimethylformamide (DMF) and 2× with acetone. Each washing step consisted of suspending the particles in the solvent, ultrasonicating for 4 min, and retracting the particles from the solvent with the aid of the magnet.

FTIR Spectroscopy. Samples were prepared in pure KBr (Sigma-Aldrich) (5 wt % particles) and measured by a Tensor 27 Spectrometer (Bruker Optics, equipped with a diffuse reflectance accessory, DiffusIR, Pike technologies).

Enzyme Immobilization. *Covalent Binding.* The DSC-activated particles (300 mg) immediately after washing were suspended in acetone (300 mL), out of which 50 mL were taken for analysis. The remaining particles (250 mg) were recovered by magnet, the acetone was discarded, and respective enzyme solution (80 mL) was added. The enzyme solutions contained either lyophilized powder in the cases of β-Glu from almonds (50 mg, Sigma-Aldrich) and α-chymotrypsin from bovine pancreas (25 mg, Sigma-Aldrich) or enzyme in liquid

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storage solution in the case of the *Candida antarctica* lipase B (250 μL, Almac Sciences) dissolved in 80 mL of dH_2O. The final suspensions were ultrasonicated for 5 min and left shaking for 5 h. The particles were then washed 5× with dH_2O. Each washing step consisted of suspending the particles in water, ultrasonicating for 1 min, and retracting the particles from the solution with the aid of a magnet. The immobilized enzymes were stored at +4 °C in dH_2O containing 0.02% NaN_3 (Brunschwig).

Adsorption. The procedure is the same as for the covalent immobilization with a difference in the enzymatic support (nonfunctionalized Co/C instead of the DSC-activated particles were used).

Enzymatic Activity Assays. *α-Chymotrypsin Protocol.* The increase in absorbance (production of *N*-benzoyl-L-tyrosine) over time at a wavelength of 256 nm was measured on a spectrophotometer (Nanodrop 2000c, Thermo Scientific) in a quartz cuvette at 25 °C. The assay mixture of the free enzyme contained: Tris/HCl buffer pH 7.8 (1.42 mL, 80 mM, Fluka), *N*-benzoyl-L-tyrosine ethyl ester substrate (1.4 mL, 1.18 mM, Sigma-Aldrich), $CaCl_2 \times 2H_2O$ (80 μL, 2 M, Fluka) and either HCl (100 μL, 1 mM, Sigma) in the case of the blank or enzyme solution (100 μL) in the case of the sample to a final volume of 3 mL. Immediately after the enzyme addition, 1.5 mL of the mixture were transferred to a cuvette and absorbance was monitored each minute for 8 min. The assay mixture of the immobilized enzyme contained five times the components of the free enzyme mixture to a final volume of 15 mL. Reaction started with the addition of the immobilized enzyme suspension (500 μL) and was measured every second minute for 14 min. Each time a sample was taken out (1.5 mL) the particles were retracted with the aid of a magnetic separator and the clear solution was measured in a cuvette.

β-Glucosidase Protocol. The increase in absorbance (production of *p*-nitrophenol) over time at a wavelength of 405 nm was measured with a microtiter plate reader (Infinite f200 Tecan) in a transparent flat bottom 96-well plate (TPP) at 25 °C. The assay mixture of both free and immobilized enzyme contained 4-nitrophenyl β-D-glucopyranoside (11 mg, 0.037 mmol, Sigma-Aldrich) dissolved in PBS buffer (1.404 mL, Gibco, adjusted to pH 6.5 with 0.1 M HCl) to which enzyme solution was added (48 μL). Samples (242 μL) were added to a stopping $NaHCO_3$ (Fluka) buffer solution (62 μL, 0.1 M, pH 11) and transferred to the microplate for measurement. In the case of the immobilized enzyme, particles were first retracted with the aid of a magnetic separator and the clear supernatant was put into the stopping buffer. Samples were taken every minute for 4 min in total.

CALB Protocol (Tributyrin Hydrolysis Assay). Lipase catalyzed hydrolysis of tributyrin (Sigma-Aldrich) was followed titrametrically with the aid of a pH meter (Metler Toledo) in a thermoregulated (28 °C) reaction vessel. The vessel contained potassium phosphate buffer pH 7.0 (60 mL, 5 mM) and tributyrin (5 mL) to which enzyme was added (100 μL of each free and immobilized suspension). Reaction took place for 2 h and a buret titration with NaOH (0.1 M) followed to get to pH 7.

Enzymatic Activity Calculation. The specific enzymatic activities (U/mg) of both free and immobilized β-Glu and α-CT were calculated with the given formula: Specific Activity = $(\Delta A \times V_t \times D_f)/(\varepsilon \times l \times V_s \times C)$ where $\Delta A = (\Delta A$ Test $- \Delta A$ Blank)/min at the desired wavelength (256 or 405 nm); V_t = total volume of the reaction mixture; D_f = dilution factor; ε = extinction coefficient; l = path length; V_s = volume of the sample; C = protein concentration.

The extinction coefficient in the case of the *p*-nitrophenol (β-Glu assay) was calculated under our assay conditions: ε = 13394.43 ($M^{-1} \times cm^{-1}$) and in the case of the *N*-benzoyl-L-tyrosine (α-CT assay) is 0.964 ($mM^{-1} \times cm^{-1}$) (Sigma-Aldrich). The path length is l = 1 cm.

The specific enzymatic activity (U/mg) of both free and immobilized CALB was estimated according to the titrant consumption over time. One enzymatic unit (U) corresponds to a consumption rate of 1 μmol NaOH per minute. When divided by the total enzyme mass (mg) in the reaction mix we obtain the specific enzymatic activity.

Protein Concentration Measurement. The amount of protein immobilized on the particles was estimated from the C, H, N percentage mass increase after immobilization obtained by elemental microanalysis measurement (Vario Micro Cube, Elementar) and from knowing the elemental content of the enzymes (% N and % C).

Large Scale Experiment. The large scale experiments of the covalently immobilized β-Glu were performed in a 20 L reactor tank (Büchi Glas Uster) equipped with a stirrer, flow pump, and flow meter and modified with a magnetic filter. The substrate solutions (2×) were prepared by dissolving 4-nitrophenyl β-D-glucopyranoside (2 g, 6.64 mmol) in PBS buffer pH 6.5 (15 L). After the tank was filled with substrate solution the enzyme−particle conjugates were added (600 mg) and the reaction was left to take place (18 min) under continuous stirring. The particles were separated with aid of a small magnet placed on the wall of the reaction vessel (15 min), the yellow product was discarded, and the second substrate solution was added. The magnet was removed, particles suspended, and the reaction was left to take place once again (18 min). The solution was then pumped through the built-in magnetic filter.

Co/C-Enzyme Conjugate Characterization. *SEM/STEM Analysis.* The increase in mass of the nanoparticle−enzyme conjugates was shown by elemental microanalysis (see SI). Samples were prepared in gelatin capsules and VSM hysteresis data was obtained (MicroMag 3900 VSM; see SI) and their morphology was investigated by means of scanning electron microscopy (SEM) and scanning transmission electron microscopy (STEM) (FEI Nova NanoSEM 450 and FEI Magellan 400 FEG, Figure S1). For SEM, the samples were sputter-coated with a 3−4 nm platinum layer and pictured at 5 kV. For STEM, the nanoparticles were loaded onto copper/carbon grids and the microscope was operated at 30 kV.

XPS Analysis. Sample pretreatment: a spatula tip of the powder was put on top of double sided sticky C-tape. No further sample treatment was performed. XPS analysis was performed using a PhI5000 VersaProbe spectrometer (ULVAC-PHI, Inc.) equipped with a 180° spherical capacitor energy analyzer and a multichannel detection system with 16 channels. Spectra were acquired at a base pressure of 5×10^{-8} Pa using a focused scanning monochromatic Al Ka source (1486.6 eV) with a spot size of 200 μm. The instrument was run in the FAT analyzer mode with electrons emitted at 45° to the surface normal. Pass energy used for survey scans was 187.85 and 46.95 eV for detail spectra. Charge neutralization utilizing both a cool cathode electron flood source (1.2 eV) and very low energy Ar^+-ions (10 eV) was applied throughout the analysis.

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Peptide Analysis. 50 μL sample (27.4 pmol/μL) from both native and immobilized β-Glu in water plus 50 μL buffer (10 mM Tris/2 mM $CaCl_2$, pH 8.2) plus 5 μL trypsin (100 ng/μL in 10 mM HCl) were incubated overnight at 37 °C. Supernatant was collected and the beads were extracted with 50 μL 0.1% TFA/50% acetonitrile. All supernatants were combined, dried, dissolved in 100 μL 0.1% formic acid, and transferred to autosampler vials for LC/MS/MS. The instrument used is a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific inc.) equipped with a nanoAcquity UPLC System (Waters Corp.). 5 μL of each sample were injected for analysis. Database searches were performed by using the Mascot (NCBI_nr, all species) search program.

Desorption Experiment. Solutions of acetonitrile were prepared by mixing acetonitrile (HPLC grade, Sigma-Aldrich) with water to obtain the following concentrations: 10%, 30%, 50%, 70%, and 90%. 20 mg of adsorbed protein particles were dispersed in 20 mL of the acetonitrile solutions by ultrasonication for 3 min and then shaken at 600 rpm for 20 min. The solvent was discarded with the aid of a magnet and particles were dried in a vacuum oven overnight. Sodium dodecyl sulfate (SDS) was prepared by dissolving 1 g of SDS powder (Fisher Scientific UK, electrophoresis grade) into 20 mL dH_20 and cooking it in the microwave for couple of seconds until it dissolves completely. 10 mg of Co/C-β-Glu (adsorbed) were dispersed in 2 mL of the 5% SDS by ultrasonicating for 3 min and then incubating in a thermomixer (Eppendorf, comfort) for 10 min at 95 °C. Five washing cycles were performed with water where the particles were retracted by magnet each time. Particles were dried in a vacuum oven. Phenol solution was prepared by dissolving 1 g of phenol powder (Sigma-Aldrich, puriss) into 20 dH_2O. 10 mg of Co/C-β-Glu (adsorbed) were dispersed in 2 mL of the 5% phenol solution by ultrasonicating for 3 min and shaking at 600 rpm for 20 min. The solvent was discarded with the aid of a magnet; particles were washed 5 times with water and dried in a vacuum oven overnight.

Cobalt Leaching. Atomic absorption spectroscopy analysis (Varian, Spectraa 220 FS) was performed to determine Co concentration from solution. 1 mg of the β-Glu immobilized Co/C nanoparticles were incubated for 30 min in 20 mL of the enzymatic reaction buffer (PBS pH 6.5), particles were retracted with the aid of a magnet, and the clear supernatant was analyzed. Standard curve was prepared by using Co standard for AAS (Fluka).

Magnetic Separation with a Small Magnet. Two dispersions both containing dispersed nanoparticles in water either Co/C or Dynabeads at a concentration of 1 mg/mL were prepared in a final volume of 20 mL. A small cubical a neodymium based permanent magnet with a side length of 1.1 cm was placed between the vials. Images were taken at different time points. The separation speed was correlated to increase in relative transmission through the vial.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Elemental microanalysis (Table S1); VSM (Table S2); XPS analysis (Table S3); SEM/STEM pictures (Figure S1); LC/MS/MS analysis (Figure S2); Swiss-PdbViewer image (Figure S3); magnetic separation comparison (Figure S4); large-scale experiment movie (Movie1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Two authors (W.J.S. and R.N.G.) declare financial interests, as they are shareholders of TurboBeads LLC, a company active in magnetic nanoparticles.

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