ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/6606806

Electrode Surface Confinement of Self-Assembled Enzyme Aggregates Using Magnetic Nanoparticles and Its Application in Bioelectrocatalysis

ARTICLE *in* ANALYTICAL CHEMISTRY · FEBRUARY 2007

Impact Factor: 5.64 · DOI: 10.1021/ac061367a · Source: PubMed

CITATIONS
22

READS
57

5 AUTHORS, INCLUDING:

François Mavré
Paris Diderot University
34 PUBLICATIONS **607** CITATIONS
SEE PROFILE

Souad Merah
Paris Diderot University
128 PUBLICATIONS **1,741** CITATIONS
SEE PROFILE

Damien Marchal
Paris Diderot University
36 PUBLICATIONS **458** CITATIONS
SEE PROFILE

Benoît Limoges
Paris Diderot University
118 PUBLICATIONS **2,326** CITATIONS
SEE PROFILE

Available from: Benoît Limoges
Retrieved on: 30 January 2016

Anal. Chem. **2007,** *79,* 187−194

Electrode Surface Confinement of Self-Assembled Enzyme Aggregates Using Magnetic Nanoparticles and Its Application in Bioelectrocatalysis

François Mavré,[†] Mélanie Bontemps,[†] Souad Ammar-Merah,[‡] Damien Marchal,[†] and Benoît Limoges*,[†]

Laboratoire d' Electrochimie Moléculaire, UMR CNRS 7591, and Interfaces, Traitement, Organisation et Dynamiques des Systèmes (ITODYS), UMR CNRS 7086, Université de Paris 7, Denis Diderot, 2 place Jussieu, 75251 Paris Cedex 05, France

Self-assembled enzyme aggregates, prepared from magnetic iron oxide nanoparticles, avidin, and a biotinylated redox enzyme, were shown particularly useful for the simple, fast, and efficient construction of highly enzyme-loaded electrodes with the help of a magnet. The approach was illustrated in the case of the bioelectrocatalytic oxidation of NADH by a diaphorase oxidoreductase in the presence of a ferrocene mediator. Two different self-assembling procedures were tested, taking advantage of the spontaneous aggregation of the nanoparticles in the presence of avidin and also of the multivalency binding of biotinylated diaphorase toward avidin. Activities of the bound and unbound diaphorase were systematically controlled allowing determination of the number of active biotinylated diaphorase per nanoparticle incorporated within each magnetic enzyme aggregate. An active enzyme loading capacity of up to 2.35 nmol mg^{-1} was found for the best nanostructured enzyme assembly, which is 200 times better than for commercialized magnetic micrometer-sized beads coated with streptavidin and saturated with diaphorase. With the help of a permanent magnet, the magnetic enzyme aggregates were finally magnetically collected as a film on the surface of a small screen-printed carbon electrode and the catalytic currents recorded by cyclic voltammetry. From the analysis of the steady-state catalytic current responses and the kinetic rate constants of biotinylated diaphorase, it was possible to determine the enzyme concentration within the magnetic films. Owing to the high enzyme loading in the aggregates of nanoparticles (i.e., 130 μM), the catalytic current responses were definitely higher than the ones measured at an electrode coated with a closed-packed monolayer of diaphorase or at an electrode covered with a film of magnetic micrometer-sized streptavidin beads saturated with diaphorase.

Deposition of organized enzyme films on electrode surfaces, with thickness ranging from nano- to micrometer length, continue to attract increasing attention because of their broad biotechnological applications including biosensors[1−4] and bioprocesses.[5,6] With the aim to achieve nanostructured enzyme multilayer films with a high protein density, various immobilization strategies have been realized such as step-by-step attachment of enzyme layers through biospecific interactions,[7−11] covalent attachment,[12] or alternate layer assembly of oppositely charged macromolecules.[13−15] Although these strategies offer the possibility to build thin ordered enzymatic films of high catalytic activity, the step-by-step procedure is a slow and tedious process, and it cannot be easily transferred to industrial scale for the mass production of low-cost biosensors.

To circumvent these drawbacks, a possible route consists in prearranging, in the bulk of a liquid phase, a three-dimensional nanostructured assembly of the enzyme and next to collect it as a regular film on the surface of an electrode. Such an idea can be put into practice with the help of magnetic nanoparticles that can be used as a unique building block in the design of an enzyme-based supramolecular network, assembled, for example, through multivalent and three-dimensional interactions,[16] to finally produce a magnetic solid phase that can be easily deposited as a film on the surface of an electrode by means of an external magnetic field.

* To whom correspondence should be addressed. E-mail: limoges@paris7.jussieu.fr. Telephone: (33) 1 44 27 28 01. Fax: (33) 1 44 27 76 25.

[†] UMR CNRS 7591.

[‡] UMR CNRS 7086.

(1) Davis, F.; Higson, S. P. J. *Biosens. Bioelectron.* **2005**, *21*, 1.
(2) Willner, I.; Katz, E. *Angew. Chem., Int. Ed.* **2000**, *39*, 1181.
(3) Campas, M.; O'Sullivan, C. *Anal. Lett.* **2003**, *36*, 2551.
(4) Cosnier, S. *Biosens. Bioelectron.* **1999**, *14*, 443.
(5) Zhang, X.; Sun, Y.; Shen, J. In *Protein Architecture. Interfacing Molecular Assemblies and Immobilization Biotechnology*; Lvov, Y., Moehwald, H., Eds.; Marcel Dekker Inc.: New York, 2000; pp 229−249.
(6) Ai, H.; Jones, S. A.; Lvov, Y. M. *Cell Biochem. Biophys.* **2003**, *39*, 23.
(7) Anicet, N.; Bourdillon, C.; Moiroux, J.; Savéant, J-M. *J. Phys. Chem. B* **1998**, *102*, 9844.
(8) Anzai, J.; Takeshita, H.; Kobayashi, Y.; Osa, T.; Hoshi, T. *Anal. Chem.* **1998**, *70*, 811.
(9) Bourdillon, C.; Demaille, C.; Moiroux, J.; Savéant, J.-M. *Acc. Chem. Res.* **1996**, *29*, 529.
(10) Bourdillon, C.; Demaille, C.; Moiroux, J.; Savéant, J.-M. *J. Am. Chem. Soc.* **1995**, *117*, 11499.
(11) Limoges, B.; Savéant, J.-M.; Yazidi, D. *Aust. J. Chem.* **2006**, *59*, 257.
(12) Zhang, S.; Yang, W.; Niu, Y.; Sun, C. *Anal. Chim. Acta* **2004**, *253*, 209.
(13) Pishko, M. V.; Katakis, I.; Lindquist, S-E.; Ye, L.; Gregg, B. A.; Heller, A. *Angew. Chem., Int. Ed.* **1990**, *29*, 82.
(14) Lvov, Y.; Caruso, F. *Anal. Chem.* **2001**, *73*, 4212.
(15) Calvo, E. J.; Danilowicz, C. B.; Wolosiuk, A. *Phys. Chem. Chem. Phys.* **2005**, *7*, 1800.
(16) Perez, J. M.; Simeone, F. J.; Saeki, Y.; Josephson, L.; Weissleder, R. *J. Am. Chem. Soc.* **2003**, *125*, 10192.

10.1021/ac061367a CCC: $37.00 © 2007 American Chemical Society
Published on Web 12/01/2006

The perceived advantage of using particles of nanometric size instead of micro- or submicrometric size is their larger surface area for the attachment of the enzymes, enabling the preparation of nanostructured biomaterial with a high enzyme loading per unit of mass and subsequently of enzyme electrodes with improved analytical performances. The others advantages are (i) a simple and fast enzyme immobilization that can be implemented just before doing a biosensing experiment, (ii) an easy electrode surface regeneration by magnetic removing or washing of the magnetic biomaterial, and (iii) a straightforward adjustment of the amount of enzyme magnetically deposited on the electrode surface. These attractive incomes have been recently emphasized with mesoporous carbon foam incorporating magnetic nanoparticles and glucose oxidase cross-linked with glutaraldehyde.[17] However, the specific activity of glucose oxidase immobilized within such a mesoporous structure was significantly decreased (~70% loss of activity), probably because of enzyme inactivation during its chemical cross-linking or because of substrate diffusion constraints throughout the small pores of the mesoporous carbon foam. Similar important loss of enzyme activity was reported for other enzymes covalently attached to the surface of magnetic nanoparticles.[18,19]

In the present work, we have devised a noncovalent approach to self-assemble a magnetic enzyme nanostructure from a colloidal solution of iron oxide magnetic nanoparticles. The method used to prepare the magnetic enzyme solid phase is very simple and also versatile, and it was shown to be very useful for the construction of magnetically controllable enzyme electrodes with high enzyme loading. For such a purpose, we have taken advantage of the high-affinity recognition between avidin and biotin to mediate the self-assembly/aggregation of magnetic nanoparticles with a redox enzyme. The enzyme we have selected was the diaphorase from *Bacillus stearothermophilus* (DI), an oxidoreductase that was previously thoroughly kinetically characterized by us in cyclic voltammetry, both in homogeneous solution and as a monolayer immobilized on a carbon electrode.[20] DI has the peculiarity to very efficiently catalyze the hydride transfer from NADH to a variety of redox mediators, and because of its remarkable thermal stability (up to 70 °C), insensitivity to dioxygen, and high rate with NADH, it is a good candidate for the development of numerous kinds of NADH-based electrochemical biosensors.

EXPERIMENTAL SECTION

Reagents. Avidin (Sigma), bovine serum albumin (BSA) (Sigma), neutravidin (Pierce), 2,6-dichloroindophenol (DCIP) (Aldrich), and NADH (Sigma) were used as received. Ferrocenylmethanol (FcMeOH; Aldrich) was recrystallized twice from toluene and cyclohexane. Lyophilized diaphorase (DI) from *Bacillus stearothermophilus* (EC 1.6.99) was purchased from Unitika. DI was biotinylated and kinetically characterized as previously described.[20] The biotinylated DI (b-DI) concentration was determined spectrophotometrically at 460 nm using for its flavin mononucleotide prosthetic group an extinction coefficient of 12 000 M^{-1} cm^{-1}.[21] Streptavidin-coated magnetic microparticles, with a mean particle diameter of 1 μm and a binding capacity of 350 pmol of free biotin or 10 pmol of 1.5-kb biotin-labeled dsDNA fragment/mg of particles, were purchased from Roche. A phosphate buffer (PB) of pH 7.4 was prepared from 2.7 g L^{-1} NaH_2PO_4 and 11.05 g L^{-1} Na_2HPO_4. The 0.1 M Tris buffer of pH 8.5 was prepared from 4.42 g L^{-1} Trizma base (Sigma) and 8.72 g L^{-1} Trizma HCl (Sigma). All aqueous solutions were prepared with water purified by a Milli-Q water purification system from Millipore.

The concentration of avidin was determined by UV−visible spectrophotometry in the presence of 2-(4-hydroxyphenylazo)-benzoic acid (HABA) and the biotin contents of b-DI by competitive displacement of HABA bonded to avidin.[22]

Synthesis and Characterization of Iron Oxide Nanoparticles. Iron oxide nanoparticles were synthesized by an alternative sol−gel method.[23] All chemicals were reagent grade and were used without further purification. Precursor salts, anhydrous Fe(CH_3CO_2) (Acros), anhydrous sodium acetate (Prolabo), and distilled water in an atomic ratio of 1:3:18 were added to 125 mL of diethylene glycol (Acros Organics). The mixture was then heated under reflux (155 °C) for 3 h. The precipitated black powder was recovered by centrifugation and rinsed with ethanol. Finally, the particles were dried overnight in air at 50 °C. X-ray diffraction, magnetic measurement with a superconducting quantum interference device (SQUID), transmission electronic microscopy (TEM), and thermal gravimetric analysis (TGA) were performed to characterize the produced powder. Superparamagnetic particles of magnetite (Fe_3O_4) with a room-temperature saturation magnetization of 38 emu g^{-1} were finally obtained. The consistency of particle size inferred from XRD analysis and TEM observation indicates that the nanoparticles are single crystals. Sampling of 250 particles from different TEM micrographs and modeling the size distribution with a log-normal law shows that the particles are almost uniform with an average diameter of 7 nm and a standard deviation of less than 20%.

From the TGA measurement, a relative mass of 16.2% was lost from the particle powder. This mass was attributed to residual diethylene glycol coming from the synthesis of nanoparticles. Such an organic contamination was frequently observed on oxide particles prepared in a polyol medium, and it was usually assigned to polyol species adsorbed at the surface of the particles.[24] Taking into account this mass and the densities of magnetite and diethylene glycol, a particle density of 4.5 g cm^{-3} was calculated.

Preparation and Characterization of the Magnetic Enzyme Aggregates of Nanoparticles. The magnetic enzyme aggregates of nanoparticles were prepared from two procedures, noted A and B, and tested with different initial stoichiometric ratios of particles, avidin, and enzyme (P/Av/Enz ratio).

(17) Lee, J.; Lee, D.; Oh, E.; Kim, J.; Kim, Y.-P.; Jin, S.; Kiml, H.-S.; Hwang, Y.; Kwak, J. H.; Park, J. G.; Shin, C. H.; Kim, J.; Hyeon, *T. Angew. Chem., Int. Ed.* **2005**, *44*, 7427.

(18) Dyal, A.; Loos, K.; Noto, M.; Chang, S. W.; Spagnoli, C.; Shafi, K. V. P. M.; Ulman, A.; Cowman, M.; Gross, R. A. *J. Am. Chem. Soc.* **2003**, *125*, 1684.

(19) Liao, M.-H.; Chen, D.-H. *J. Mol. Catal. B* **2002**, *18*, 81.

(20) Limoges, B.; Marchal, D.; Mavré, F.; Savéant, J.-M. *J. Am. Chem. Soc.* **2006**, *128*, 2084.

(21) Matsue, T.; Yamada, H.; Chang, H-C.; Uchida, I.; Nagata, K.; Tomoita, K. *Biochim. Biophys. Acta* **1990**, *1038*, 29.

(22) Green, N. M. In *Methods in Enzymology*; McCornick, D. B.. Wright, L. D.. Eds.; Academic Press: New York, 1970; Vol. 18A, p 418.

(23) Poul, L.; Ammar, S.; Jouini, N.; Fiévet, F.; Villain, F. *J. Sol-Gel Sci. Technol.* **2003**, *26*, 261.

(24) Ammar, S.; Helfen, A.; Jouini, N.; Fiévet, F.; Rosenman, I.; Villain, F.; Molinié, P.; Danot, M. *J. Mater. Chem.* **2001**, *11*, 186.

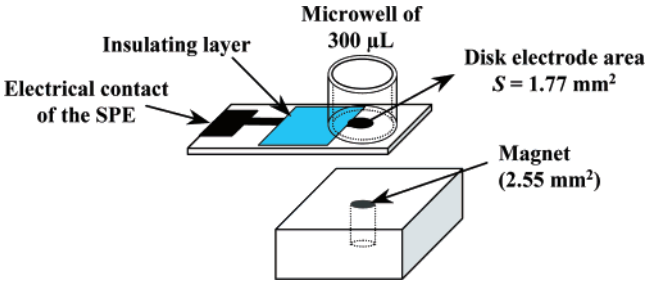
Procedure A: 2 mg of iron oxide nanoparticles (i.e., 1.8×10^{15} nanoparticles or 3×10^{-9} mol estimated from a particle size of 7 nm and a particle density of 4.5 g cm^{-3}) was dispersed in 1 mL of PB and sonicated for 10 min. Next, 403 μL of a 1 mg mL^{-1} avidin solution in PB was added (leading to a P/Av stoichiometric ratio of 1:2) and the volume adjusted with PB to 2.5 mL. After ~15-min incubation, the formation of nanoparticles/avidin aggregates was visible and their separation was facilitated by the application of a permanent magnet at the bottom of the tube. The supernatant was then carefully removed, and the magnetic aggregates were washed and magnetically separated two times with 1 mL of PB. The aggregates were next homogeneously redispersed in 1 mL of PB, and 30 μL of this solution was mixed with 12.5 μL of a 37 μM b-DI solution in PB for 30 min, allowing us to define a P/Av/Enz ratio of 1:2:5. After magnetic separation, the supernatant (~1 mL) was removed and set aside for quantification of the activity of unbound b-DI (vide infra). The enzyme aggregates were finally redispersed in 50 μL of PB and stored at 4 °C until used. Two supplementary P/Av/Enz ratios of 1:2:0.8 and 1:2:1.7 were prepared by adjusting the amount of b-DI.

Procedure B: 10 μL of a 1 mg mL^{-1} avidin solution in PB and 10 μL of a 37 μM solution of b-DI in PB were mixed in a microtube and let to react for 5 min. Separately, 2 mg of iron oxide nanoparticles was dispersed in 2 mL of PB and the resultant mixture sonicated for 10 min. Next, 50 μL of this colloidal solution was added to the solution containing the premixed avidin and b-DI, and the reaction volume was adjusted to 1 mL by addition of PB. The final mixture leads to a P/Av/Enz ratio of 1:2.4:4.8. After magnetic separation of the enzyme aggregates, the supernatant (~1 mL) was removed and its remaining DI activity determined spectrophotometrically. The enzyme aggregates were redispersed in 50 μL of PB and stored at 4 °C until used. Two supplementary P/Av/Enz ratios of 1:2.4:2.4 and 1:7.2:7.2 were also prepared from procedure B by just changing the initial volumes of the mother solutions of avidin and b-DI.

The amount of active b-DI immobilized within the magnetic enzyme aggregates was obtained by measuring spectrophotometrically the activity of b-DI in the mother solution, in the supernatants, and in the solution of redispersed magnetic aggregates. The spectrophotometric enzyme activity assay of DI was determined in a standard quartz cell (1-cm path length) by following at 602 nm the initial rates of DCIP (40 μM) oxidation in the presence of excess of NADH (125 μM) and at pH 8.5 (0.1 M Tris buffer containing 0.1% BSA). A UV−visible Hewlett-Packard 8452 diode array spectrophotometer interfaced with a PC computer was used, and the temperature of the quartz cell maintained at 20 °C. The unknown concentrations of b-DI contained both in the supernatants and in the solution of redispersed magnetic aggregates were determined from a standard calibration plot of b-DI ranging from 10^{-10} to 2.5×10^{-9} M.

Immobilization of a Monolayer of Biotinylated Diaphorase on a Carbon Electrode. All experiments were performed at room temperature. A drop of 8 μL containing 1 mg mL^{-1} neutravidin in PB was locally deposited on the sensing area of a freshly polished glassy carbon electrode (3-mm diameter) and incubated for 2 h in a water-saturated atmosphere. After a thorough rinsing with PB, the neutravidin-coated electrode was immersed for 15 min in a PB solution containing 0.1% BSA, rinsed with PB, and next dipped for at least 2 h in a Tris buffer containing 10^{-7} M biotinylated diaphorase. Once prepared, the enzyme electrode was rinsed and stored in Tris buffer at 4 °C until used.

Chart 1. Schematic Design of the Electrochemical Microwell Aligned with the Magnet Holding Block[a]



[a] The magnetic particles dispersed in the liquid of the microwell were collected by precisely bringing the magnet holding block in contact with the underneath of the working disk electrode.

Immobilization of b-DI on the Surface of Micrometer-Sized Streptavidin Magnetic Beads. The commercially available streptavidin-coated magnetic microbeads (Roche) were composed of polydisperse polystyrene core−shell beads (average diameter of 1 μm) incorporating small superparamagnetic particles. They were supplied in a solution at 10 mg mL^{-1}. According to the manufacturer, the microbeads were first carefully washed. This was accomplished by mixing 20 μL of the commercial suspension of microbeads in 1 mL of Tris buffer, followed by two consecutive magnetic separations and resuspension in 2 × 1 mL Tris buffer. Then, the cleaned streptavidin-coated magnetic microbeads were redispersed in 1 mL of Tris buffer and reacted with 2.7 μL of a 37 μM b-DI solution (i.e., ~500 pmol of b-DI/mg of magnetic microbeads). The mixture was incubated for 30 min, and the magnetic microbeads were separated with a magnet, rinsed with 4 × 1 mL Tris buffer, and finally redispersed in 1 mL of Tris buffer. The suspension was stored at 4 °C until used.

Electrochemical Experiments. Cyclic voltammetry measurements were performed with an Autolab potentiostat, PGSTAT 12 (Ecochemie) interfaced to a PC computer (GPES software). Homemade disposable electrochemical microwells (Chart 1), having a maximal volume of 300 μL, were constructed as previously noted[25] from polystyrene cylinder and screen-printed electrodes (SPE; 1.5-mm diameter). The SPE were screen-printed from a commercial carbon-based ink (Electrodag PF-407C, Acheson Colloid). The measurements were carried out at room temperatutre, using a small silver/silver chloride reference electrode and a platinum wire counter electrode. In the case of the b-DI-modified glassy carbon electrode, the experiments were performed in a classical water-jacketed electrochemical cell (maintained at 20 ± 0.5 °C) equipped with a saturated calomel reference electrode (SCE) and a platinum counter electrode.

All of the electrochemical experiments were carried out at pH 8.5 in a 0.1 M Tris buffer solution, since it is the optimal pH of diaphorase catalysis. The voltammetric curves presented in this work were systematically referred to the normal hydrogen electrode.

The bioelectrocatalytic detection of b-DI immobilized on the magnetic microbeads and enzyme aggregates of nanoparticles was

(25) Dequaire, M.; Degrand, C.; Limoges, B. *Anal. Chem.* **1999**, *71*, 2571.

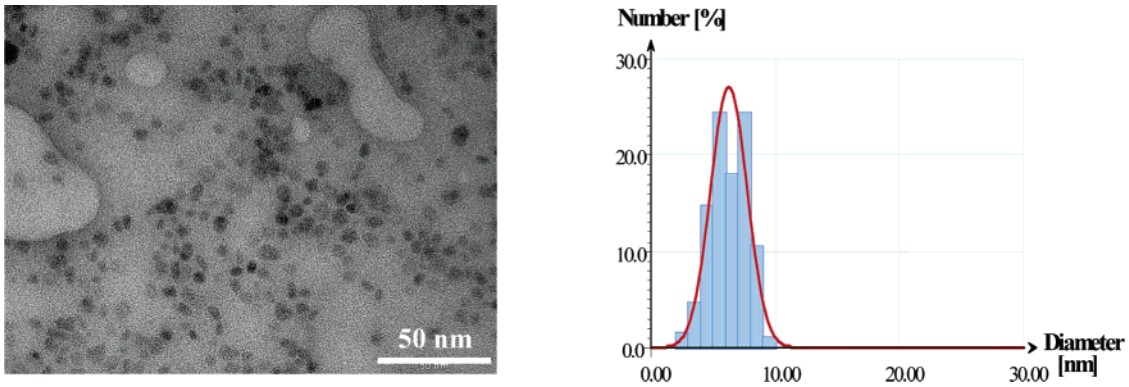


Figure 1. Transmission electron micrograph of the iron oxide nanoparticles used in this work and the histogram of their size distribution.

done in the electrochemical microwell as follows. In the case of micrometer-sized magnetic beads, 50 μL of a suspension of b-DI-bounded streptavidin magnetic microbeads followed by 5 μL of a 1 mM FcMeOH and 5 μL of a 0.1 M NADH was added to 190 μL of Tris buffer. After mixing the solution, the electrochemical microwell was positioned over the magnet holding block, in such a way that the magnet cylinder was just beneath the working disk electrode area. The overall magnetic particles were magnetically collected over the electrode surface in less than 5 min, forming thus a thin film, and a voltammetric curve was then recorded. Similar experiments were carried out with the magnetic enzyme aggregates of nanoparticles, with the exception that the aggregates were first redispersed in 50 μL of Tris buffer and next diluted in the electrochemical microwell by injecting 5 μL of this solution to 230 μL of Tris buffer, complemented with 5 μL of a 1 mM FcMeOH and 10 μL of a 0.1 M NADH. After the electrochemical microwell was positioned over the magnet holding block, a waiting time of at less 15 min was required to collect the overall magnetic enzyme aggregates over the electrode surface.

RESULTS AND DISCUSSION

Superparamagnetic iron oxide nanoparticles with an average diameter size of 7 nm were utilized (Figure 1).

These particles are single-domain magnetic dipoles that, in the absence of an applied magnetic field, show no preferred directional ordering, since the magnetic dipole−dipole interactions are weak, and thermal forces dominate dispersion of these particles. When a sufficiently high magnetic field gradient is applied, the particles show a preferential ordering in the direction of this field, which also exerts a force on the particles, proportional to the magnetic field, the particle magnetic susceptibility and the volume of the particle core. It is this magnetic force that is exploited in the capture of the particles. However, because of their very small size, the magnetic field gradient generated by permanent magnets (e.g., the ones that are typically employed with micrometer-sized magnetic particles) is generally not sufficiently strong to capture magnetic nanoparticles dispersed in water as a colloidal suspension. To overcome this problem, a route consists in directing the self-assembly of magnetic nanoparticles into supramolecular nanostructured aggregates with enhanced magnetic properties. We hypothesized that it could be achieved by using the avidin/biotin binding, as a three-dimensional cross-linker, to direct the self-assembly between the magnetic nanoparticles and the biotinylated enzyme diaphorase.

The nanostructured magnetic enzyme aggregates were prepared from two different procedures (Scheme 1), noted A and B, each one taking advantage of the multivalent binding capacity of avidin toward the biotin residues attached to the enzyme, and also of the nonspecific adsorption of avidin to the surface of the iron oxide magnetic nanoparticles.

The first step in method A was inspired from the conventional method used to prepare nanoparticle gold-labeled proteins.[26,27] It basically consists in stabilizing a colloidal solution by spontaneous adsorption of a protein on the surface of the particles. We have supposed that avidin should efficiently stabilize a suspension of iron oxide nanoparticles since at neutral pH avidin is positively charged (avidin isoelectric point, 10.2) whereas the surface of iron oxide nanoparticles is negatively charged. However, contrary to what was believed, the addition of avidin (6.25 nmol) to a colloidal suspension of iron oxide nanoparticles (2 mg in a total volume of 2.5 mL) does not lead to a stabilized colloidal suspension since, after a few minutes, eyes-detectable aggregates became visible into the solution, with a propensity to slowly flocculate with time. Moreover, these aggregates were easily separated from the solution using a simple magnet. The amount of unbound avidin remaining in the supernatant was then quantified by UV−visible spectrophotometry in the presence of HABA, and a value of 0.52 nmol was found. By difference with the starting amount of avidin, it allows us to determine that more than 90% of added avidin was bound to the magnetic aggregates, leading thus to ~3 nmol of avidin/mg of nanoparticles (i.e., ~2 avidin/particle). This quantity was corroborated by TGA experiments from which a mass equivalent to 2 avidin/particle was found. The biotin binding capacity per mass unit of nanoparticles was also measured spectrophotometrically by competitive displacement of HABA bound to avidin,[22] and a value of ~5 nmol mg^{-1} was found (i.e., ~3 biotin/particle). This value means that only 40% of the binding sites of avidin (considering 4 biotin sites/avidin) were accessible to biotin. Such a binding capacity is nevertheless 9-fold higher than the binding capacity of the commercially available streptavidin-coated magnetic microbeads used in this work (i.e., 0.36 nmol mg^{-1}). In an attempt to identify the origin of aggregate formation, avidin was replaced by BSA, a globular protein that is known to adsorb on many type of solid surfaces. In contrast to

(26) Beesley, J. E. *Proc. RMS* **1985**, *20*, 187.
(27) Leuvening, J. H. W.; Thal, P. S. H. M.; White, D. D.; Schurs, A. H. W. M. *J. Immunol. Methods* **1983**, *62*, 163.

Scheme 1 Procedures A and B Used To Prepare the Nanostructured Magnetic Enzyme Aggregates

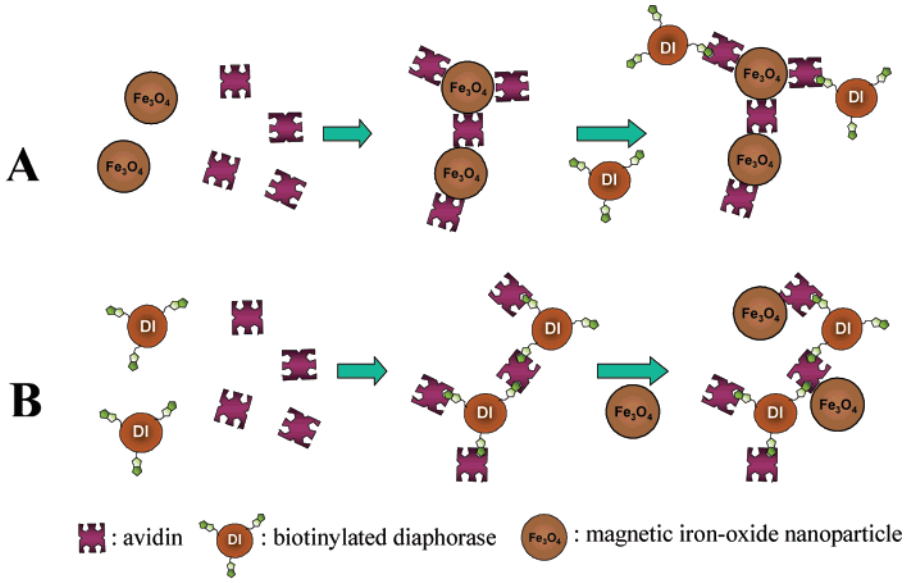


Table 1. Amount of Active b-DI Recovered in the Supernatant and in the Enzyme Aggregates

P/Av/Enz ratios (procedure)	active b-DI in supernatants and washing solutions (%)	active b-DI in the aggregates (%)	total recovery of b-DI activity (%)	number of b-DI bound per particle: determined indirectly from unbound b-DI	number of b-DI bound per particle: determined directly from bound b-DI	binding capacity of active b-DI (pmol mg^{-1})
1:2:0.8 (A)	61	12.5	73.5	0.33	0.12	215
1:2:1.7 (A)	72	10	82	0.48	0.18	325
1:2:5 (A)	84	4.5	88.5	0.81	0.24	430
1:2.4:2.4 (B)	11	27	38	2.14	0.65	1130
1:2.4:4.8 (B)	12	26	38	4.22	1.25	2175
1:7.2:7.2 (B)	15	19	34	6.12	1.37	2345

avidin, no aggregates were formed and the colloidal suspension was stable even under the field of a magnet.

From these overall results, it is clear that the aggregation of nanoparticules is self-induced by avidin. It can be explained by both the adsorption of avidin on the surface of the nanoparticles and a cross-linking effect of avidin. The latter could arise from nonspecific interactions between different avidins themselves adsorbed on different iron oxide particles. Such a hypothesis is supported by the fact that the surrounding carbohydrate shell of avidin has been identified as a source of significant nonspecific interaction with glycosylated proteins.[28] Another plausible explanation is that the same avidin might connect two or more nanoparticles, and reciprocally, the same nanoparticle can be covered by more than one avidin. Whatever the mechanism involved during spontaneous aggregation between avidin and iron oxide nanoparticles, we have turned this unexpected phenomenon to our advantage for efficient magnetic separation of the bioaggregates.

We have pursued the assemblage with the binding of b-DI to the avidin aggregates as depicted in procedure A of Scheme 1. The magnetic avidin aggregates initially prepared from the 1:2 particle/avidin stoichiometric ratio, were then mixed with different proportions of b-DI (stoichiometric ratios ranging from 0.8 to 5). After the binding reaction, the supernatants as well as the washing solutions containing unbound b-DI were separated from the magnetic aggregates, and activities of bound and unbound b-DI were determined spectrophotometrically in the presence of the NADH substrate and DCIP cosubstrate. We have assumed that the reactivity of b-DI bounded to the aggregates was the same as b-DI free to diffuse in homogeneous solution. The results are reported in Table 1 for the different P/Av/Enz stoichiometric ratios finally obtained. It appears by difference with the unbound b-DI recovered in the supernatant (the complementary to 100%) that between 16 and 39% of the initially added b-DI was linked to the aggregates. These values were however systematically higher than the percentage recoveries found from direct activity assay of b-DI linked to the aggregates (values ranging from 4.5 to 12.5%), leading to a total recovery of b-DI activities significantly lower than 100%. These data demonstrate a loss of b-DI activity within the enzyme aggregates. Considering the starting amount of b-DI used to prepare the different enzymes aggregates, the number of bound b-DI per nanoparticle was calculated and found to range from 0.33 to 0.81 b-DI/nanoparticle. In line with the direct activity assay of b-DI linked to the aggregates, a corresponding number of active bound b-DI per nanoparticle was also calculated, leading to values ranging from 0.12 to 0.24 b-DI/nanoparticle. It comes from these latter data and the total number of bound b-DI per

(28) Hiller, Y.; Gershoni, J. M.; Bayer, E. A.; Wilchek, M. *Biochem. J.* **1987**, *248*, 167.

nanoparticle that only ~30−40% of the bound b-DI remains fully active within the magnetic aggregates. This loss of b-DI activity compared with that of b-DI in homogeneous solution suggests steric hindrance and diffusional constraints within the aggregates. It is consistent with our previous kinetic study of diaphorase immobilized as a monolayer on an avidin-modified electrode, for which a significant decrease of enzyme reactivity toward NADH was noticed.[20] Taking into account the activity of b-DI measured within the enzyme aggregates, a maximal binding capacity of 430 pmol active b-DI/mg of nanoparticles was estimated (Table 1).

In procedure B (Scheme 1), we have investigated the possibility of prearranging in a first step a self-assembled network of avidin and b-DI, followed in a second step by its labeling with magnetic nanoparticles. After mixing b-DI with avidin, there was no discernible precipitation or flocculation of aggregate in the solution. It was only after the addition of the magnetic nanoparticles that visible aggregates were formed, suggesting as previously a cross-linking of iron oxide nanoparticles by avidin contained in the self-assembled network of avidin/b-DI. Moreover, as with the ones obtained in procedure A, the magnetic enzyme aggregates were easily separated from the solution using a magnet. Measurement of the enzyme activities in the supernatant and in the enzyme aggregate solutions for each of the different aggregates prepared by procedure B (Table 1) clearly shows that a systematic higher amount of active b-DI than in procedure A can be linked to the magnetic nanoparticles. The activity of b-DI incorporated within the aggregates was however substantially decreased since an activity recovery of only ~20−30% was found. This value is slightly lower than the one found for the enzyme aggregates prepared with procedure A (~30−40%). It suggests again a decrease of substrate accessibility to the enzyme active site, probably more important within highly b-DI-loaded aggregates. Finally, examination of the results obtained with increasing proportion of b-DI in the starting solution shows that there is an enzyme-loading limit (Table 1), reaching a maximal value of ~1.5 active enzymes/nanoparticle. A maximal binding capacity of more than 2 nmol of active b-DI/mg of nanoparticle was finally calculated.

The beneficial effect of localizing the magnetic enzyme aggregates on the electrode surface for enzyme-mediated electrocatalysis is illustrated by the series of cyclic voltammetric curves shown in Figure 2. In the absence of NADH, the cyclic voltammetric curve shows only the reversible wave of the FcMeOH mediator (Figure 2a), which remains unchanged whether the magnetic enzyme aggregates were magnetically localized or not over the electrode surface. Such behavior denotes that the enzymatic film of aggregates deposited onto the electrode does not hamper FcMeOH to access the electrode surface, but also that the mediator is relatively free to diffuse throughout the permeable and porous enzymatic film (no film diffusion limitation and no film partitioning). In the presence of homogeneously dispersed b-DI aggregates in solution and large excess of NADH, a small steady-state catalytic current was recorded (Figure 2b). The latter was considerably enhanced (by a factor 22) after the magnetic b-DI aggregates of nanoparticles were brought on top of the electrode surface with a magnet (Figure 2c). The high amplification of the catalytic current reflects a considerable increase of the enzyme concentration near the working electrode

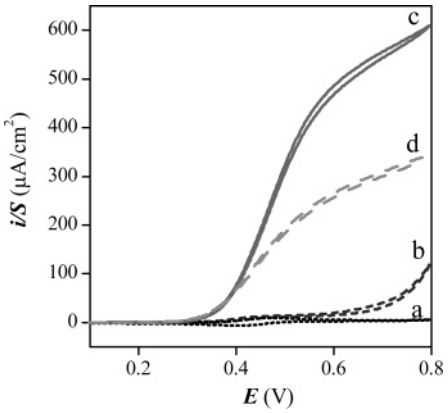


Figure 2. Cyclic voltammetric curves recorded in an electrochemical microwell (total volume of 250 μL, Tris buffer of pH 8.5) containing (a−d) 20 μM FcMeOH, (b−d) 4 mM NADH, and (b, c) 5-μL solution of enzyme aggregates prepared from procedure B and the 1:2.4:4.8 P/Av/Enz ratio or (d) 5-μL solution of enzyme aggregates prepared from procedure A and the 1:2:5 P/Av/Enz ratio. The curves were recorded (b) before and (c, d) after the enzyme aggregates were magnetically localized onto the electrode surface. Scan rate, 100 mV s^{-1}.

surface, an amplification that also depends on the aggregates' enzyme loading since a lower steady-state current response was recorded with the enzyme aggregates prepared from procedure A (Figure 2d, 0.24 active b-DI/particle) compared with that prepared from procedure B (Figure 2c, 1.25 active b-DI/particle). These results underlines the importance of preparing a magnetic biomaterial with a high b-DI binding capacity, but it also illustrates that the catalytic response is not simply proportional to the number of active b-DI per nanoparticle. The plateau currents of the voltammetric curves c and d in Figure 2 were in fact the maximum value that could be obtained, since further increase of the amount of magnetic enzyme aggregates did not lead to higher plateau current densities. This observation points toward the formation of an enzymatic film sufficiently thick to consider that the catalytic enzyme reaction layer is entirely confined within the enzymatic films close to the electrode surface and, hence, that the magnetic film can be considered as of semi-infinite film thickness. Under this limiting case condition and assuming that the enzyme is homogeneously distributed inside the film of magnetic enzyme aggregates and also that the film is highly porous and permeable (no substrate and cosubstrate film diffusion limitation and film partitioning), the expression of steady-state catalytic current, i_{pl}, should be identical to that previously established for the ping-pong mechanism of diaphorase in homogeneous solution:[20]

$$\frac{i_{pl}}{FSC_P^0} = 2\sqrt{\frac{k_2 C_E^0 D_P}{k_2 C_P^0\left(\frac{1}{k_{cat}} + \frac{1}{k_1 C_S^0}\right)}\left[1 - \frac{\ln\left(1 + k_2 C_P^0\left(\frac{1}{k_{cat}} + \frac{1}{k_1 C_S^0}\right)\right)}{k_2 C_P^0\left(\frac{1}{k_{cat}} + \frac{1}{k_1 C_S^0}\right)}\right]} \qquad (1)$$

where C_S^0 and C_P^0 are the bulk concentrations of substrate and cosubstrate, C_E^0 is the enzyme film concentration, F is the faraday constant, S is the electrode surface area, D_P is the diffusion coefficient of the cosubstrate, k_1 and k_2 are the bimolecular rate constants of diaphorase toward the substrate and the oxidized form of cosubstrate, respectively, and k_{cat} is the enzyme turnover.

The cyclic voltammograms c and d reported in Figure 2 were also unchanged upon further increase of the NADH concentration, suggesting that $k_1 C_S^0 \gg k_{cat}$ and so that eq 1 can be simplified as follows:

$$i_{pl} = 2FS\sqrt{k_{cat} C_E^0 C_P^0 D_P \left[1 - \frac{\ln\left(1 + \frac{k_2 C_P^0}{k_{cat}}\right)}{\frac{k_2 C_P^0}{k_{cat}}}\right]} \qquad (2)$$

Both eqs 1 and 2 show that i_{pl} is proportional to the square root of the enzyme concentration inside the thick film deposited over the electrode surface. Using the kinetic rates constant values previously determined for b-DI toward the FcMeOH mediator,[20] i.e., $k_2 = 3.5 \times 10^8$ M^{-1} s^{-1}, $k_{cat} = 700$ s^{-1}, a mediator diffusion coefficient of $D_P = 6.7 \times 10^{-6}$ cm^2 s^{-1}, and considering that the b-DI activity is not significantly perturbed when incorporated within the aggregates, it was possible to estimate, using eq 2, the concentration of diaphorase within the magnetic film from the magnitude of i_{pl} (Figure 2). Values of $C_E^0 = 120$ and 30 μM b-DI were calculated from the curves c and d of Figure 2, respectively. The ratio between these two concentrations (i.e., 120/30 = 4) is in good agreement with the relative proportion of the number of active b-DI bound per nanoparticle contained within each enzyme aggregate (i.e., 1.25/0.24 = 5.2). It is interesting to note that the highest enzyme film concentration found here (130 μM) is competitive with the high enzyme loading previously obtained in multilayer films of glucose oxidase assembled through biomolecular recognition (enzyme film concentrations ranging from 145 to 250 μM).[7,10]

With the aim to gauge the capacity to deposit high enzyme coverage on an electrode surface using magnetically controllable nanostuctured enzyme aggregates, the b-DI/avidin/nanoparticle aggregates were replaced by commercially available magnetic streptavidin-coated beads of micrometer size. The streptavidin-coated microbeads were saturated on their surface with b-DI, and the binding capacity of active b-DI per milligram of micrometric particles was determined spectrophotometrically in the presence of NADH and DCIP. A value of 12 pmol mg^{-1} was obtained, which is in agreement with the 10 pmol mg^{-1} binding capacity announced by the manufacturer for a 1.5-kb biotin-labeled dsDNA fragment, but it is 10−100 times lower than the binding capacity of the magnetic aggregates of nanoparticles (Table 1). The micrometric beads loaded with b-DI were then magnetically attracted onto the surface of a SPE and electrochemically characterized. Increasing amounts of microbeads were first tested until a maximal catalytic current was reached, indicative of the formation of an enzymatic film of semi-infinite thickness. The resulting voltammetric curve is reported in Figure 3a, and it shows that a much lower catalytic current was obtained with the micrometric beads than with the enzyme aggregates of nanoparticles (Figure 3c). From the plateau current and eq 2, an enzyme loading film concentration of 10 μM b-DI was estimated, which is ~10 times lower than for the film of magnetic enzyme aggregates prepared from procedure B. Using the specific gravity of the micrometric beads (average value of 1.25 g cm^{-3}), their dimension (1-μm diameter), and their b-DI binding capacity (12 pmol mg^{-1}), it was interesting to estimate, on the basis of purely geometrical perspective, the enzyme concentration (assumed homogeneous) in the close-packed assemblage of the beads (i.e., a stack of parallel hexagonal layers of spheres having a compactness of 0.74). A value of ~11 μM b-DI was calculated, which is compatible with the b-DI concentration (10 μM) determined electrochemically within the magnetic film.

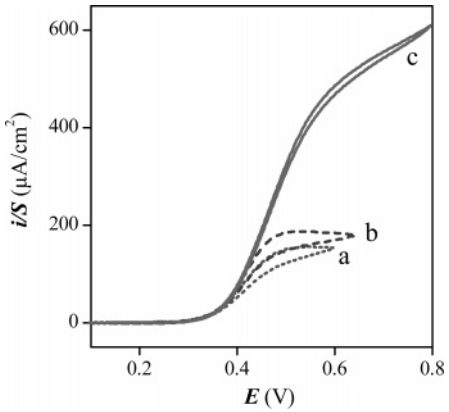


Figure 3. Cyclic voltammetric curves recorded at different b-DI-modified electrodes in a Tris buffer (pH 8.5) containing 20 μM FcMeOH and 2 mM NADH. (a) Dotted line: SPE electrode covered with magnetic streptavidin microbeads saturated on their surface with b-DI. (b) Dashed line: glassy carbon electrode coated with a saturated monolayer of neutravidin/b-DI. (c) Solid line: SPE covered with magnetic aggregates of b-DI-modified nanoparticles prepared from procedure B and the 1:2.4:4.8 P/Av/Enz ratio. Scan rate, 100 mV s^{-1}.

It was also interesting to compare the overall current responses obtained with films of magnetic particles to the catalytic current response recorded at a glassy carbon electrode modified by a saturated monolayer of b-DI. The latter electrode was prepared as previously described (b-DI surface coverage of 1.5 pmol cm^{-2}),[20] and its catalytic current response shown in Figure 3b was comparable to the response recorded at an electrode covered with b-DI-coated microbeads but definitely much lower than that obtained at an electrode covered with a film of magnetic enzyme aggregates of nanoparticles (Figure 3c). Such a gain of catalytic current density can be useful for increasing the analytical sensitivity of some biosensing applications. It is particularly important when the enzyme used to build the biosensor has a slow catalytic rate.[29]

Finally, the stability of the magnetic enzyme aggregates upon storage in their buffer solution was found particularly good since no significant decrease of the catalytic response was observed after several weeks in the refrigerator at 4 °C.

CONCLUSION

We have proposed here a very simple and fast approach to self-assemble three-dimensional magnetic enzyme aggregates with a high enzyme binding capacity. These enzyme aggregates were proved to be very useful in the construction of magnetically controllable enzyme electrodes with a high catalytic activity. The method was exemplified with the biotinylated diaphorase and its bioelectrocatalytic oxidation of NADH, but it should be easily extended to many other types of biotinylated enzymes and also to assembling of plurienzymatic systems. The versatile platform

(29) Yoon, H. C.; Kim, H.-S. *Anal. Chem.* **2000**, *72*, 922.

of avidin−biotin offers also the opportunity to build magnetic bioaggregates with many other kinds of biotinylated macromolecules such as antibody or DNA, opening thus new routes for the development of magnetic electrochemical immunosensors and DNA sensors, with potentially improved analytical performances compared with those based on micrometer-sized magnetic beads.[25,30−33]

(30) Gehring, A. G.; Crawford, C. G.; Mazenko, R. S.; Van, Houten, L. J.; Brewster, J. D. *J. Immunol. Methods* **1996**, *195*, 15.
(31) Perez, F. G.; Mascini, M.; Tothill, I. E.; Turner, A. P. F. *Anal. Chem.* **1998**, *70*, 2380.
(32) Wijayawardhana, C. A.; Purushotama, S.; Cousino, M. A.; Halsall, H. B.; Heineman, W. R. *J. Electroanal. Chem.* **1999**, 468, 2.
(33) Zacco, E.; Pividori, M. I.; Alegret, S. *Anal. Chem.* **2006**, *78*, 1780.

ACKNOWLEDGMENT

The authors acknowledge P. Beaunier (LRS, Paris), M.-J. Vaulay (ITODYS, Paris), and Y. Li (LCIM2, Paris) for access to the TEM, TGA, and SQUID equipment, respectively.

Received for review July 26, 2006. Accepted October 18, 2006.

AC061367A