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Specific binding of a biotinylated, metallocarbonyl-labelled dendrimer to immobilized avidin detected by diffuse-reflectance infrared Fourier transform spectroscopy

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Molecular recognition between avidin covalently immobilized at the surface of acrylic resin beads and a transition metallocarbonyl tracer of the biotin ligand was detected using diffuse reflectance infrared Fourier transform spectroscopy. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: biotin; avidin; acrylic beads; metallocarbonyl complex; DRIFT spectroscopy; Starburst dendrimer

INTRODUCTION

A large variety of bioanalytical applications make use of the detection of high-affinity receptor–ligand (enzyme–substrate, antibody–antigen) recognition processes. The biotin–avidin system is particularly exploited, mostly because of the extremely high affinity between the two molecules, giving rise to one of the strongest non-covalent bonds known.[1] This is of great interest in immunoassays, in general to mediate the binding of the reporter group to the antibody.[2] Most studies devoted to this bioaffinity system have shown that the association reaction is fast and irreversible and that the biotin–avidin couple resists breakdown even in very aggressive conditions.[3]

On the other hand, transition metallocarbonyl complexes are useful IR-detectable markers, endowing labelled molecules with strong absorption in the 1800–2150 cm^{-1} spectral range, which is virtually transparent for biomolecules or biological matrices. This property provided the basis for a new liquid-phase, competitive immunochemical method (carbonyl metallo immunoassay) that has been applied to the quantification of analytes such as antiepileptic drugs in serum samples.[4,5] However, most immunoassays involve the immobilization of one of the protagonists of the immunoreaction onto a solid support compatible with the detection method. This is the reason why studies devoted to the IR reflection–absorption detection of biotin–avidin complex formation, where biotin is immobilized on planar gold substrates and avidin is labelled with a metallocarbonyl probe, have recently been published.[6–8] However, this type of solid substrate is seldom used in immunoassays, probably because of its high cost and also because it requires rather sophisticated biomolecule immobilization procedures. Conversely, beads made of natural or synthetic polymers are widely used as solid phases for immunoassays.[9] Beads covered with a large variety of proteins, including avidin, are commercially available. Alternatively, protein immobilization techniques on chemically derivatized beads are well described.[10]

Herein, we report the detection of the interaction between avidin immobilized onto acrylic resin beads and biotin labelled with a metallocarbonyl tag via an amine-terminated polyethylenimine dendrimer. Direct detection of the biomolecular association was achieved by diffuse-reflectance infrared Fourier transform (DRIFT) spectroscopy.

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This technique enables one to study many substances, such as powders or rough solid surfaces, in their natural state, and is based on the following principle: The sample is submitted to an incident beam and reflects part of it in a specular (i.e. mirror-like) mode and part of it in all directions. The resulting spectrum exhibits both absorbance and reflectance features due to contributions from transmission, internal and specular reflectance components. DRIFT spectroscopy enables quantitative analysis provided that specular reflection is small and particle size and packing methods are strictly controlled.[11] For example, this technique was recently applied to study the formation of (η^6-arene) chromium tricarbonyl complexes within the confines of NaX zeolites.[12]

EXPERIMENTAL

Materials

Generation-5 poly(ethylenimine) dendrimer (DAB5) and diisopropylethylamine (DIPEA) were purchased from Aldrich. Biotin aminocaproic acid and *N,N,N′N′*-tetramethyl-(*O*)-(*N*-succinimidyl) uronium tetrafluoroborate (TSTU) were purchased from Fluka. (η^5-Cyclopentadienyl)iron dicarbonyl (η^1-*N*-maleimidato), Fp-maleimide, was synthesized according to a previously published procedure.[13] Avidin-coated acrylic beads (ref. A4808), horseradish peroxidase–biotinamidocaproyl conjugate (biotin–HRP) and *ortho*-phenylenediamine (OPD) were purchased from Sigma.

Methods

Synthesis of biotin–DAB5-Fp

A solution of biotin aminocaproic acid in dry DMF (0.01 M) was treated with one molar equivalents of TSTU and DIPEA to form the *N*-succinimidyl ester *in situ*. After 15 min, DAB5 in MeOH (0.007 M, 0.1 molar equivalents) was added to the solution. After 2 h, Fp-maleimide (40 molar equivalents) was added to the solution and the mixture was incubated for 48 h at room temperature in the dark. The solution was submitted to gel filtration chromatography using a 16 ml volume column filled with Sephadex LH20 resin (Pharmacia) conditioned with methanol. Species were eluted with methanol. 1 ml fractions were collected manually and analysed for the presence of the Fp tag at 366 nm. Fractions containing the biotinylated, labelled dendrimer were pooled. The resulting sample was assayed for biotin concentration by the HABA assay[14] based on the decrease of absorption at 500 nm (ε_{500} = 35 500 M^{-1} cm^{-1}), characteristic for 4′-hydroxyazobenzene-2-carboxylic acid (HABA) bound to avidin,[15] and for Fp tags concentration spectrophotometrically at 366 nm ($\varepsilon^{Fp}_{366\ nm}$ = 600 M^{-1} cm^{-1}). The organic solvent was evaporated under reduced pressure. The residue was immediately dissolved in the same volume of water and the solution kept at 4 °C until use.

Synthesis of DAB5-Fp

A mixture of DAB5 in solution in methanol (0.007 M) and Fp-maleimide (40 molar equivalents) was incubated for 48 h at room temperature in the dark. The labelled dendrimer was purified and analysed as above. The organic solvent was evaporated under reduced pressure. The residue was immediately dissolved in the same volume of water and the solution kept at 4 °C until use.

Biotin–DAB5-Fp and DAB5-Fp binding assays

Avidin-coated resin beads (5 mg) were suspended in a 30 μM aqueous solution of biotin–DAB5-Fp or DAB5-Fp (0.5 ml) placed in the upper compartment of a 0.2 μm Micro-spin filter tube (Alltech) and shaken for 30 min. The beads were filtered by centrifugation at 4000 rpm for 5 min and washed twice with water (0.5 ml). The filtrates were pooled and analysed spectrophotometrically at 366 nm. The beads were further treated with a 500 μM aqueous solution of biotin or by water for 30 min. The suspension was filtered by centrifugation, the beads were washed twice with water, and the filtrates were pooled and analysed spectrophotometrically at 366 nm.

Biotin binding assay

Avidin-coated resin beads (5 mg) were suspended in a 500 μM aqueous solution of biotin (0.5 ml) placed in the upper compartment of a 0.2 μm Micro-spin filter tube and shaken for 30 min. The beads were filtered by centrifugation at 4000 rpm for 5 min and washed twice with water (0.5 ml). The beads were further treated with a 60 μM aqueous solution of biotin–DAB5-Fp for 30 min. The suspension was filtered by centrifugation, the beads were washed twice with water, and the filtrates were pooled and analysed spectrophotometrically at 366 nm.

Biotin–HRP enzymatic assay

Two sets of avidin-coated resin beads (5 mg) were suspended in a 10 mg l^{-1} aqueous solution of biotin–HRP (0.5 ml) placed in the upper compartment of a 0.2 μm Micro-spin filter tube and shaken for 30 min. The beads were filtered by centrifugation at 4000 rpm for 5 min and washed twice with water (0.5 ml). One set of beads was further treated with a 500 μM solution of biotin for 30 min, and the other set was treated with the same volume of water. The suspensions were filtered and the beads washed twice with water. The filtrates were pooled and the presence of enzyme was assayed in each sample at 492 nm after addition of a 0.7 g l^{-1} solution of OPD containing 0.04% H_2O_2 (v : v) in citrate–phosphate buffer pH 5.

DRIFT spectroscopic measurements

IR spectra were recorded on a Biorad FT F175 spectrometer equipped with a Pyke Easy Deff device. Vacuum-dried bead samples (2 mg) were deposited as a uniform layer on a small pad (~1 cm^2) of abrasive paper. 125 spectra were accumulated at 4 cm^{-1} resolution and ratioed by a reference spectrum recorded for uncovered abrasive paper. The DRIFT spectrum of untreated avidin-coated acrylic beads was recorded separately and used to subtract the contribution of the beads from the IR absorption.

RESULTS AND DISCUSSION

A multi-labelled biotin metallocarbonyl tracer was synthesized according to a strategy previously described.[14] This strategy takes advantage of the 64 primary amines functions carried by the Starburst™ generation-5 poly(ethylenimine) dendrimer to enable conjugation of both biotin ligands and (η^5-Cp)iron dicarbonyl (Fp) groups (Fig. 1). Measurement of absorbance at 366 nm indicated that the resulting biotin–DAB5-Fp tracer contained an average 24 Fp groups and the HABA test revealed two biotin entities per dendrimer molecule. Accordingly, conjugation of Fp-maleimide to the same dendrimer yielded the new compound DAB5-Fp which contained 33 Fp groups per dendrimer molecule.

The avidin-coated acrylic resin beads used in this work (Sigma, ref. A4808) have a medium diameter of 150 μm and a binding capacity of 200–400 ng (0.8–1.6 nmol) biotin per milligram of beads. A known amount of beads was suspended in a solution of biotin–DAB5-Fp of known concentration (Fig. 2) and the filtrate solution was recovered after 30 min. Its absorbance at 366 nm, i.e. where the Fp group displays a maximum of absorption, happened to be lower than that of the starting biotin–DAB5-Fp solution. This decrease of absorption at 366 nm was quantitatively related to the amount of biotin–DAB5-Fp bound to the beads (Table 1, experiment A, first step). Surprisingly, the corresponding amount of bound biotin was two to four times higher than the announced binding capacity, even when inferring that only one biotin entity per dendrimer molecule is able to interact with immobilized avidin.

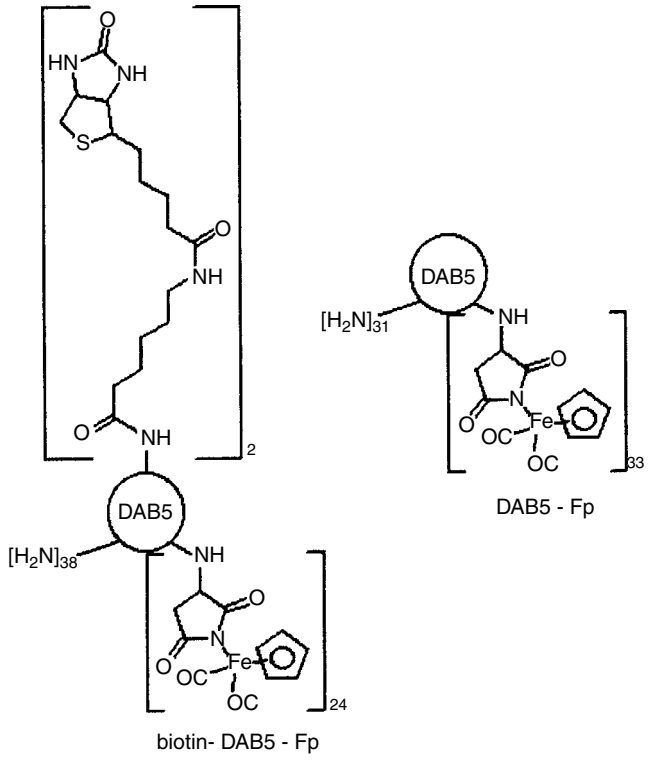

Figure 1. Structure of biotin–DAB5-Fp and DAB5-Fp.

To find out whether the binding of biotin–DAB5-Fp to the avidin-coated beads occurred via a specific biotin–avidin interaction, the same amount of beads was first treated with a solution of biotin to saturate the avidin binding sites and the beads were then treated with an aqueous solution of biotin–DAB5-Fp at the same concentration as in experiment A. UV–visible difference data on the filtrates (Table 1, experiment B) seemed to indicate that the biotin–DAB-Fp trace was still able to bind to the biotin-saturated avidin beads. However, the amount of bound biotin–DAB5-Fp was lower than after experiment A. Consequently, the unexpectedly high amount of biotin–DAB5-Fp observed after experiment A may be explained by two concomitant mechanisms of interaction of the tracer with avidin-coated beads, i.e. specific interaction by formation of biotin–avidin complexes and non-specific interaction with the solid support itself.

To ascertain that non-specific interaction between the tracer and avidin-coated beads occurred, we performed another experiment where the initial biotin–DAB-Fp solution was replaced by a DAB5-Fp solution at the same concentration. UV–visible analysis of the filtrate showed that the non-biotinylated dendrimer did bind to avidin-coated beads, but to a minor degree (Table 1, experiment C, first step).

The DRIFT spectrum of avidin-coated beads is shown in Fig. 3. It displays only weak and broad absorption in the 1800–2150 cm^{-1} spectral range where the metallocarbonyl reporter groups absorb. This should, in principle, allow one to detect any metallocarbonyl tag bound to the beads. Indeed, the DRIFT spectrum of biotin–DAB5-Fp-treated acrylic beads

Table 1. Quantity of Fp label (Q_{Fp}) and of dendrimer tracer (Q_T) bound to avidin-coated acrylic resin beads as measured by UV–visible spectrometry on the filtrates

	First step		Second step	
	Q_{Fp} (nmol/mg beads)	Q_T, (nmol/mg beads)	Q_{Fp} (nmol/mg beads)	Q_T (nmol/mg beads)
Experiment A (biotin–DAB5-Fp then water)	74.4	3.1	69.6	2.9
Experiment B (biotin then biotin–DAB5-Fp)	—	—	40.8	1.7
Experiment C (DAB5-Fp then biotin)	40	1.2	37.4	1.1
Experiment D (biotin–DAB5-Fp then biotin)	72	3.0	36.0	1.5

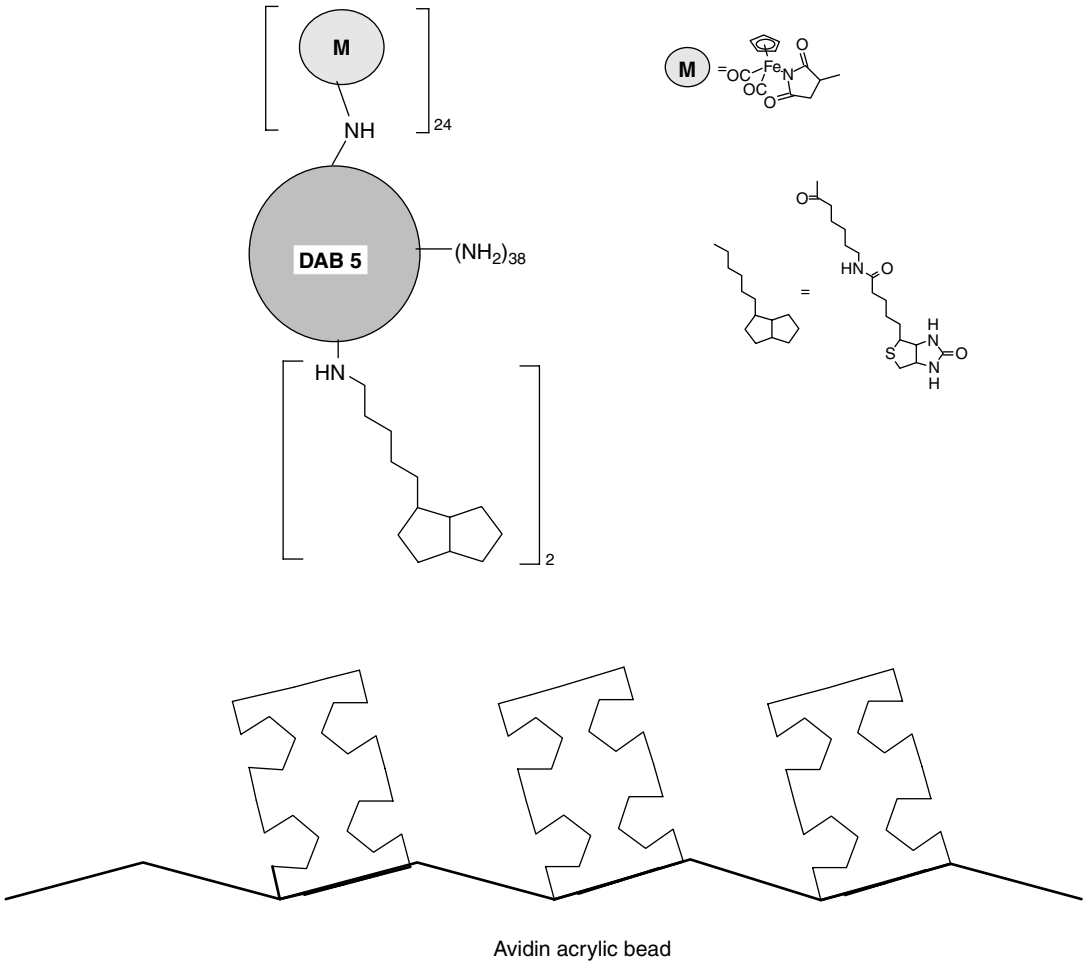

Figure 2. Schematic representation of the binding of biotin–DAB5-Fp to avidin covalently bound to acrylic resin beads.

(experiment A), shown in Fig. 4 (trace A), displayed two ν_{CO} bands, at 2047 and 1999 cm^{-1}, that are characteristic of the $CpFe(CO)_2$ unit. This finding confirmed that biotin–DAB5-Fp tracer was bound. DRIFT spectral analysis of the beads treated according to experiment B also confirmed that binding of biotin–DAB5-Fp to beads resulted both from specific and non-specific interactions with the solid substrate. This non-specific interaction was also observed on the DRIFT spectrum measured after experiment C, which showed that avidin-coated acrylic beads treated with non-biotinylated DAB5-Fp gave the two characteristic ν_{CO} bands.

We also studied the behaviour of biotin–DAB5-Fp-treated beads in the presence of a large excess of biotin: avidin-coated beads were first shaken in an aqueous solution of biotin–DAB5-Fp as in experiment A, then treated with an aqueous solution of biotin. The DRIFT spectrum of the beads (Figure 4, trace B) still displayed the two ν_{CO} bands, but a marked decrease in their intensity was observed as compared with trace A. Accordingly, Fp groups were detected in the final filtrate by UV–visible spectroscopy (Table 1, experiment D, second step), corresponding to desorption of *ca* 50% of initially bound biotin–DAB5-Fp tracer. A similar experiment, where the biotin solution was replaced by water, yielded only 6% of desorption of initially bound biotin–DAB5-Fp tracer (Table 1, experiment A, second step). Very weak desorption was also observed when the initial biotin–DAB5-Fp tracer solution was replaced by a solution of DAB5-Fp and treated by biotin solution (Table 1, experiment C, second step). Thus, both DRIFT spectroscopy of the beads and UV–visible spectroscopy of the filtrate indicated that biotin–DAB5-Fp tracer bound to immobilized avidin could be partly exchanged by biotin.

To find out whether the affinity between biotin covalently linked to a bulky substituent and immobilized avidin was less high than with biotin itself, we performed an enzyme colorimetric assay on the beads using a biotin–horseradish peroxidase conjugate. Two bead samples were first treated with the biotinylated enzyme; then, one of them was shaken in a solution of biotin and the other one was shaken in water. Both final filtrates were assayed for the presence of enzyme by addition of a specific substrate. Only the filtrate of the beads treated with biotin contained some enzyme. In this case also,

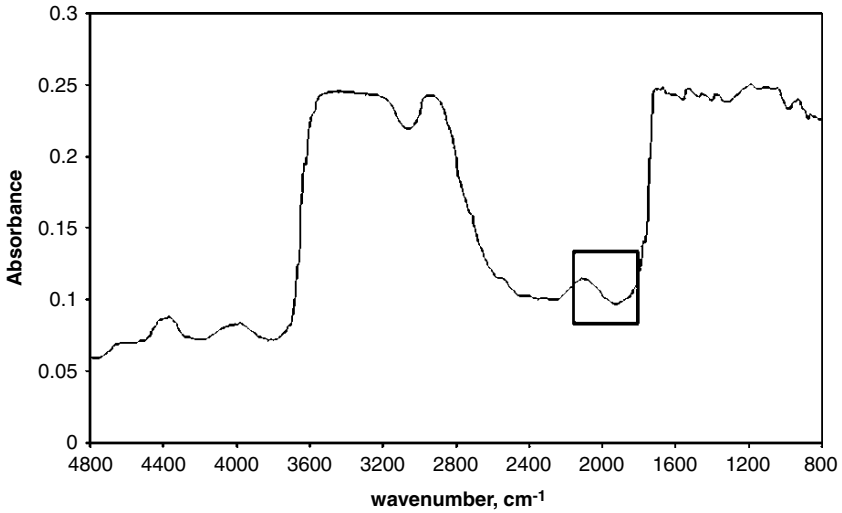

Figure 3. DRIFT spectrum of avidin-coated acrylic resin beads.

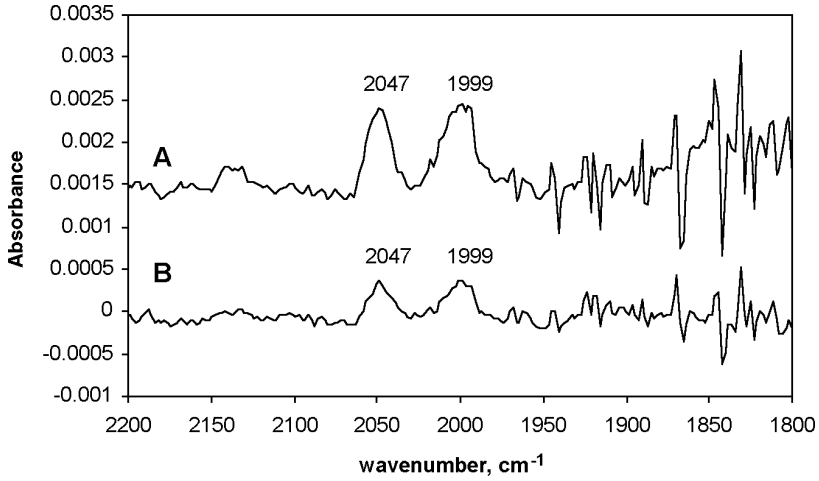

Figure 4. Carbonyl ligand stretching vibration region of the DRIFT spectrum of avidin-coated acrylic resin beads treated with biotin–DAB5-Fp tracer: (A) at the end of experiment A; (B) at the end of experiment D. These spectra were corrected from the intrinsic absorption of the beads by spectral subtraction.

biotin was able to desorb biotin–HRP partially from immobilized avidin. Partial exchange of biotin–HRP conjugate and biotin–DAB5-Fp tracer by biotin itself is probably due to the particular arrangement of the avidin subunits. Avidin is a tetramer with four binding sites that are arranged in two pairs on opposed faces of the molecule. Avidin that is fully saturated by biotin derivatives carrying a bulky substituent has been shown to be less stable than the protein saturated by biotin itself, and one half of the biotinylated ligands can be exchanged relatively rapidly by biotin.[1]

CONCLUSIONS

In summary, this study showed for the first time that DRIFT spectroscopy was a sensitive spectral method to detect the interaction between avidin covalently bound to acrylic resin beads and a biotin dendrimer metallocarbonyl complex conjugate. This was made possible because the protein immobilized onto this kind of solid support displayed a window of absorption in the 1800–2150 cm^{-1} spectral range where the characteristic bands of all metallocarbonyl complexes are observed. We were also able to show that binding of the biotin tracer to the biofunctionalized solid support involved both specific and non-specific interaction modes. In addition, and contrary to a generally admitted rule, biotin binding to avidin could be made partially reversible when biotin is covalently bound to a large and bulky substituent (here, the generation-5 dendrimer or the HRP enzyme). Associated with a metallocarbonyl marker, DRIFT spectroscopy, with its very simple sampling method (no

particular sample preparation) and high sensitivity, appears to be a very attractive alternative to IR reflection–absorption spectroscopy on planar gold surfaces for the detection of interfacial biomolecular interactions.

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