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Potentiality of an organometallic labelled streptavidin–biotin system in metalloimmunoassay*

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Abstract: Biotin labelled with a cymantrene moiety (cyclopentadienyl manganese tricarbonyl complex) is described for the first time. Because this metallo-biotin retains full recognition for the specific glycoprotein avidin (or streptavidin), the labelled streptavidin–biotin system is proposed for use in a solid-phase competition-type metalloimmunoassay in which bovine serum albumin (BSA) is used as a model of applications. Atomic absorption spectrometry is utilized for the detection of the cymantrene-labelled biotin.

Keywords: *Avidin; biotin; metalloimmunoassay; organometallic; Zeeman atomic absorption spectrometry.*

Introduction

Because of the strong affinity of avidin for biotin ($K_a = 10^{15}$ L/M), the avidin–biotin system has become one of the most useful tools in the biomedical analysis. Thus, it was successfully utilized in a number of immunoassays of antigens or antibodies [1]. Moreover, organometallic derivatives are potential alternatives to radioisotopic and non-isotopic labels in immunoassays [2]. The two main advantages of these labels are, firstly the large choice of structures capable of being used as recently described for the labelling of drugs with metallocenic fragments [3, 4] and secondly the variety of analytical methods for the detection of the label.

A recent work has used one of these metallo-tracers in a competitive metalloimmunoassay [5], but in an approach where each hapten must be labelled with an organometallic label. In contrast, the avidin–biotin system acts as a universal reagent in immunoassays.

Here we describe the structure of biotin labelled with cymantrenic fragment (Scheme 1) after reaction in mild conditions between the hydrazide group of hydrazido-biotin and the aldehyde function of the carboxaldehyde cymantrene, and its recognition for the streptavidin is studied. The use of this tracer, designated as Cy-biotin, in an homogeneous competitive metalloimmunoassay is also described.

The amount of the tracer was determined by a Zeeman atomic absorption spectrometer (ZAAS); the liquid phase was directly introduced into the furnace of the ZAAS.

Experimental

Chemicals and reagents

Biotin-hydrazide was purchased from Pierce Europe (Interchim, France). (η^5-Cyclopentadienyl) manganese tricarbonyl (cymantrene) was purchased from Ventron Chemical (Mallet S.A., France). Avidin from *Streptomyces avidinii* ('streptavidin') and biotinylated antibodies were obtained from Amersham (Les Ulis, France). Bovine serum albumin and antiserum were obtained from Sigma (La Verpilleres, France). Other chemicals were of reagent grade or better.

Buffers

Phosphate-buffered saline (PBS) was a 0.01 M sodium phosphate solution of pH 7.4 containing 9 g NaCl per liter. PBS-tween (wash solution) was PBS which contained 1 ml polyoxyethylene sorbitan monolaurate (Tween 20) per liter.

Apparatus

Atomic absorption measurements were carried out on a Zeeman Hitachi model Z.7000 and operating conditions were previously described [6].

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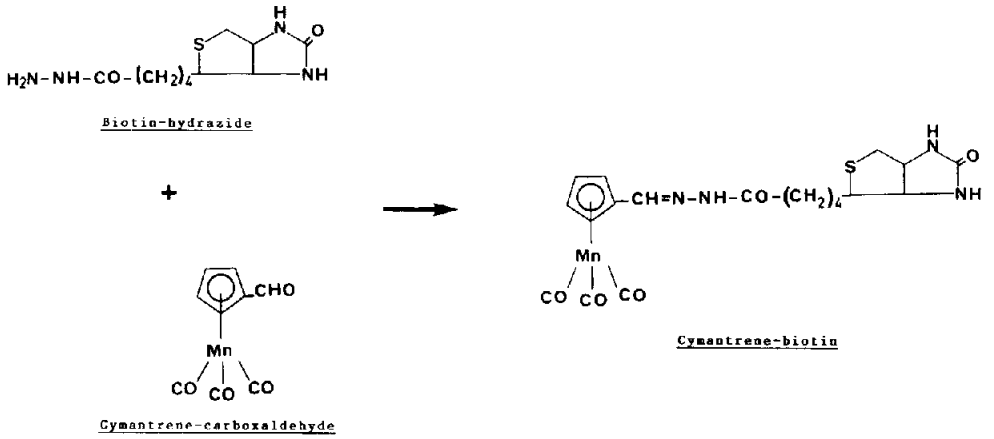
Synthesis of cymantrene-biotin (Cy-biotin)

A suspension of biotin-hydrazide (70.7 mg, 0.28 mmol) in 6 ml of a mixture of methanol–phosphate buffer was treated with cymantrene-carboxaldehyde (69.6 mg, 0.3 mmol) prepared according to the technique of Leblanc *et al.* [7]. The mixture was stirred for 15 days at ambient temperature and then the methanol evaporated under reduced pressure. The residue was extracted with methylene chloride and chromatographed on an alumina column (eluant: methylene chloride then methanol). The Cy-biotin was obtained as orange crystals (yield 42%) characterized by IR, ^{1}H NMR and mass spectrometry.

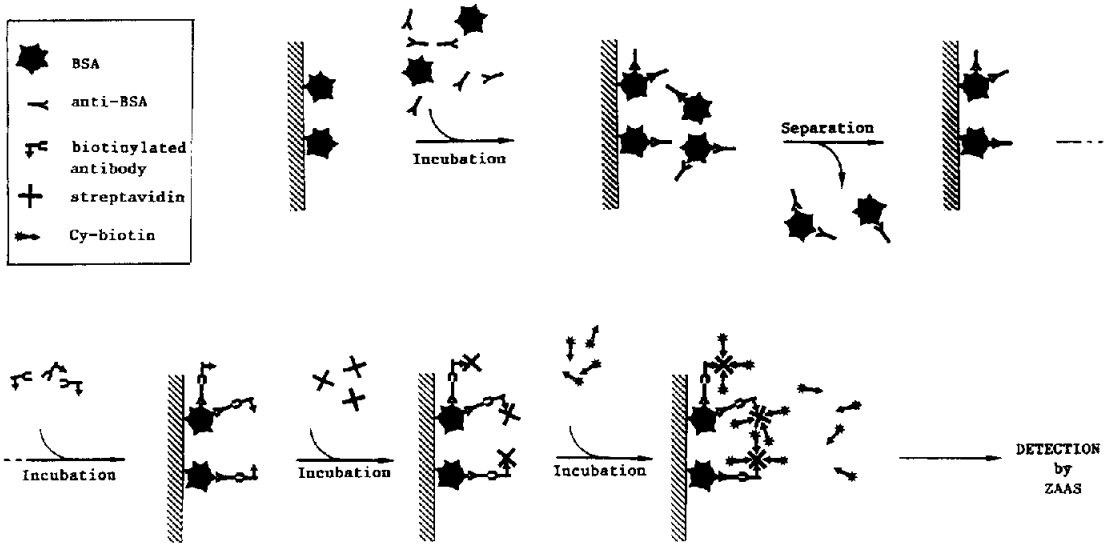
The structure of Cy-biotin is shown in Scheme 1.

Procedure of immunoassay

The general assay protocol is outlined in Scheme 2. Star-tubes (Nunc, Poly-labo, France) were coated with bovine serum albumin (BSA) by passive absorption according to the manufacturer's instructions. After the tubes were washed three times with PBS-tween, 450 μl of anti-BSA solution in PBS (1:1000) and 50 μl of standard solutions of BSA in PBS (0–2000 μg m^{-1}) were added and incubated for 1 h at 37°C. The tubes were washed as above and 500 μl of diluted (1:500) second biotinylated antibodies were added. After incubating for 1 h at 37°C, the tubes were washed again and 500 μl of streptavidin solution at 5 μg ml^{-1} were added and incubation was performed for 1 h at 37°C. The



Scheme 1
Schematic synthesis of cymantrene-labelled biotin (Cy-biotin).



Scheme 2
Schematic representation of a competitive metalloimmunoassay procedure using cymantrene-labelled biotin.

tubes were refrigerated at 4°C then 500 µl of Cy-biotin solution (5×10^{-8} M) were added. After incubating for 2 h at 4°C, 50 µl of the supernatant were introduced into the furnace of the ZAAS.

Results and Discussion

The product of condensation of a hydrazide and an aldehyde is a hydrazone, which forms slowly. Cymantrene-labelled biotin was obtained as hydrazone form in a water–methanol media after 15 days of reaction. After purification by chromatography, a concentrated solution of the metallo-biotin in methanol was prepared and found to be stable in the refrigerator for more than 1 month. Moreover, biotin is not inactivated upon labelling and retains good binding activity for the streptavidin as shown in the Scatchard plot (data not shown). Further dilutions of the Cy-biotin were performed in PBS and there was a good correlation between the metallo-biotin injected into the furnace of the ZAAS and the absorption signal. Furthermore, the detection of the label included in the Cy-biotin–streptavidin complex generated the linear dose–response curve shown in Fig. 1.

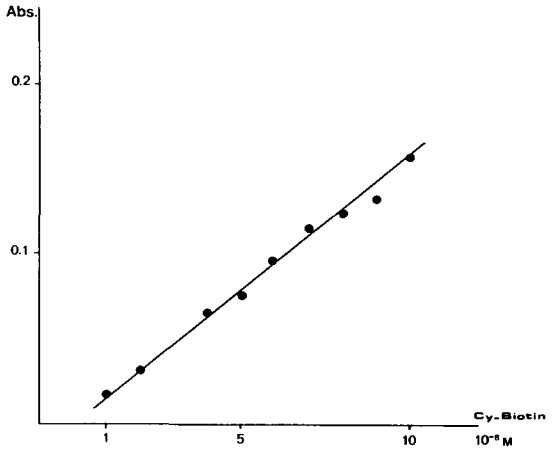


Figure 1
Dose–response curve of Cy-biotin by ZAAS.

This result is important for the metalloimmunoassay proposed here. In this assay the analyte competes with the immobilized antigen coated on the solid phase for binding to a limited amount of an analyte-specific antibody, then a second antibody labelled with biotin is introduced (as an alternative first biotinylated antibody may be used). After the reaction was completed and excess of reagent discarded, the assay was performed by adding excess of streptavidin followed by the metallo-biotin label. As described in Scheme 2, the excessive metallo-biotin–streptavidin complex is measured as above directly from the liquid phase by ZAAS.

In a pilot experiment, exemplified by the assay of bovine serum albumin, a typical standard curve was obtained in the presence of both 25 pmol of Cy-biotin and 0–1000 pmol of BSA per tube.

Conclusion

The metalloimmunoassay described in this paper for BSA, confirms that metallo-biotin is suitable for a wide range of antigens because the method requires only antibodies directed against the antigen (or biotin-labelled antibodies). Moreover, the combination of the advantages of organometallic complexes with those of the streptavidin–biotin system provides many possibilities for immunoassays of haptens or antibodies. Practical applications will be published in the near future.

Although this publication reports the detection of the label by atomic absorption spectrometry, other analytical methods can be considered for the detection of this type of tracer. Thus, infrared spectroscopy previously published for the titration of antisera [8] and electrochemical techniques are under investigation.

References

[1] M. Wilchek and E.A. Bayer, *Anal. Biochem.* **171**, 1–32 (1988).
[2] A.A. Ismail and P. Brossier, *Comments Inorg. Chem.* (submitted).
[3] I. Lavastre, J. Besançon, P. Brossier and C. Moise, *Appl. Organomet. Chem.* **4**, 9–17 (1990).
[4] I. Lavastre, J. Besançon, P. Brossier and C. Moise, *Appl. Organomet. Chem.* **5**, 143–149 (1991).
[5] F. Mariet, Ph.D. Thesis, Université de Bourgogne (1990).
[6] P. Cheret and P. Brossier, *Res. Commun. Chem. Pathol. Pharmacol.* **54**, 237–253 (1986).
[7] J.-C. Leblanc, C. Moise and J. Tirouflet, *J. Organomet. Chem.* **292**, 225–228 (1985).
[8] F. Mariet, P. Brossier, F. Dicaire and A.A. Ismail, *J. Pharm. Biomed. Anal.* **8**, 979–982 (1990).

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