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**Research Papers**

# Sorptive properties of antibodies onto cyanoacrylic nanoparticles

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## Summary

Further studies were carried out on the interaction of monoclonal antibodies (MAbs) with cyanoacrylic nanoparticles (NP). Even after incubation in culture media with seric proteins, these immunoparticles conserved a good immunoreactivity and a sufficient stability for potential usefulness in targeting. On the other hand, optimal conditions were determined for using these NP as a solid phase for immunoassays. More surprisingly, it was observed that radiolabelling of monoclonal antibodies with iodine induced important changes in the physicochemical interaction between the protein and the polymeric support.

## Introduction

Cyanoacrylic nanoparticles (NP) were firstly designed as a lysosomotropic and biodegradable drug carrier (Couvreur et al., 1979a and b; Lenaerts et al., 1984). However, the major uptake of these particles by the reticuloendothelial system (mainly liver and spleen) could be a drawback for cancer targeting. An increased uptake by the tumoural cells might be obtained after coating the surface of NP with monoclonal antibodies against tumour-associated antigens. In a preliminary paper (Manil et al., 1986a), we demonstrated the feasibility of the preparation of such immunoparticles which, in addition, were efficient for the immunoradiometric assay (IRMA) of tumoural markers such as α-fetoprotein (AFP). Parameters useful for improving both the immunoreactivity and the stability of these conjugates, however, had not been tested. Therefore, the present study describes methodological improvements able to optimize the efficiency of NP-IRMA and the stability of MAb-NP binding.

Furthermore, owing to the disappointing results obtained in the in vivo targeting with such carriers (Illum et al., 1984), we investigated the interaction of NP with MAb in seric conditions in particular, with special attention to the bioerosion of the polymer, potentially responsible for a quick release of the antibody.

## Materials and Methods

### *Chemicals and monoclonal antibodies (MAbs)*

Isobutylcyanoacrylate (IBC) and isohexylcyanoacrylate (IHC) monomers were obtained from Ethnor (Paris, France) and Weil Chemische Fabrik

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(Mannheim, F.R.G.), respectively. Dextran 70, D Glucose and polyethyleneglycol (PEG, mol. wt. 6000 Da) were purchased from Prolabo (Paris, France). $^{125}I$ Na was supplied by the Commissariat à l'Energie Atomique (Saclay, France). Iodogen was a Pierce chemical product (Rockford, IL, U.S.A.). Fetal calf serum (FCS) was obtained from Seromed (Biockrom, Berlin, F.R.G). AFP standard solutions were used as presented in RIA-Gnost kits (Behring, Marburg, F.R.G.) or serially diluted with fetal calf serum. Bovine serum albumin (BSA) was supplied by Merck (Darmstadt, F.R.G.)

Two previously described anti-AFP MAbs were selected for this study: AF01 (Bellet et al., 1984) and AF04 (Manil et al., 1986b). Their isotypes are $IgG_2$ and $IgG_1$, and their affinity constants for AFP are $1.6 \times 10^{10}$ $M^{-1}$ and $0.8 \times 10^{10}$ $M^{-1}$, respectively.

*Radiolabelling of MAbs*

The antibodies were labelled with $^{125}I$ by the iodogen method (Fraker and Speck, 1978): 25 $\mu$g of MAb in phosphate-buffered saline (PBS) were incubated at 20 °C with $0.5 \times 10^{-3}$ Ci $^{125}I$ Na in polypropylene tubes coated with 2.5 $\mu$g iodogen. After 5 min, the reaction was stopped by adding 100 $\mu$l of a solution containing 830 mg ascorbic acid, 29 g KI and 9 g NaCl per litre. Free iodide was separated with a Sephadex G25 chromatography column (Pharmacia, Uppsala, Sweden) previously saturated with PBS containing 1% BSA.

*Nanoparticles preparation*

Nanoparticles were prepared separately with each monomer: 100 $\mu$l of monomer were added to a $10^{-3}$ M $H_3PO_4$ solution (10 ml) containing 1% Dextran 70 and 5% glucose, under continuous mechanical stirring. After 2 h (IBC) or 6 h (IHC) polymerization, an homogeneous milky suspension was obtained. In some experiments, either $^{125}I$-labelled ($250 \times 10^3$ cpm/ml) or unlabelled AF01 MAb (20 $\mu$g/ml) was dissolved in the polymerization medium 10, 30 or 60 min after addition of the cyanoacrylic monomer. In all cases, the NP suspension was lyophilized for 24 h in a freeze-dryer (Piccolo-CRIP, Argenteuil, France). Resuspension of preparations of dry nanoparticles (100 mg cyanoacrylate per vial) was achieved by the addition of 10 ml of 0.1 M pH 7.4 PBS. The size of the nanoparticles, controlled before and after lyophilization by a laser nanosizer (Coulter, Margency, France), was found to be 120 nm (IBC) and 170 nm (IHC).

*Adsorption of MAbs to NP*

Radiolabelled MAbs were added to the NP suspension (10 mg cyanoacrylate per ml pH 7.4 PBS) and incubated for 2 h at 20 °C. Separation of coated NP from free MAbs was performed by PEG-induced differential centrifugation as described before (Manil et al., 1986a). This method was, however, slightly modified as follows: PEG 6000 was diluted in pH 7.4 PBS in place of distilled water and added to the NP suspension in suitable quantity to obtain a 7% PEG final concentration. A centrifugation (3000 *g*) was used for precipitating MAb-coated nanoparticles without sedimentation of the free antibody fraction.

*Immunoreactivity measuring of NP-adsorbed MAbs by IRMA*

In this sandwich assay, a first solid phase-adsorbed MAb (AF01, unlabelled) was used for selective capture of AFP. The second MAb (AF04, labelled), directed against another epitope of this antigen, was used for detection of the bound antigen. For this, NP (10 mg/ml) were incubated with AF01 (50 $\mu$g/ml) for 2 h at 20 °C unless the antibody was already added during the polymerization. Unoccupied sites on the nanoparticles' surface were then saturated by incubating (2 h at 20 °C) with FCS (50% final concentration, corresponding to about 25 mg protein/ml). Then 50 $\mu$l of AF01-coated NP were mixed with the AFP-containing sample (50 $\mu$l) and $^{125}I$-AF04 (200 $\mu$l in PBS, 50,000 cpm). Each assay was performed in triplicate. After incubation overnight at 20 °C, separation of the fraction of radiolabelled antibodies bound to NP was achieved with PEG 7%, as explained above. These conditions with IBC NP, defined as standard protocol, will be used throughout unless otherwise specified for testing each of the parameters separately.

*Stability of MAb-NP binding*

For these experiments, unlabelled antibody was

mixed together with radiolabelled antibody. This solution was then added to the nanoparticles suspension (10 mg/ml) in order to obtain a final antibody concentration ranging from 0 to 50 $\mu$g/ml and displaying $250 \times 10^3$ cpm/ml. Then the NP, diluted or not, were incubated at 37 °C in the presence of various concentrations of FCS or purified BSA. In some experiments, *AF01-coated and saturated (50% FCS) NP were further incubated with AFP and unlabelled AF04 (8.3 ng/ml) in the same conditions described for IRMA.

## Results and Discussion

*Immunoreactivity investigations (IRMA)*

In order to optimize the technical conditions of immunoradiometric assays, several parameters were successively investigated. By slight modifications of such parameters as NP saturation (with 50% FCS instead of 10%), composition of PEG solution (7.0% in pH 7.4 in PBS instead of distilled water) and centrifugation speed (3000 *g*, 10 min), we succeeded in reducing the background activity (due to non-specific coating of radiolabelled-AF04 to nanoparticles) below 3% (previously 6%) of the total amount of radioactivity present in the incubation medium (Fig. 1). In all NP-IRMA, standard deviation between triplicates was always lower than 3%, while day-to-day reproducibility was generally kept under 7% (Fig. 1).

Furthermore, in view of improving the sensitivity of NP-IRMA, 3 concentrations of antibody were tested for coating. Although satisfactory results were obtained with 2 $\mu$g MAb per mg NP, a slightly higher sensitivity was reached with 5 $\mu$g antibody (Fig. 2). In contrast, most of the immunoreactivity disappeared when using only 1 $\mu$g MAb/mg NP. In other experiments, the capture antibody was added during the polymerization process, instead of after it. In these conditions, neither size nor homogeneity of nanoparticles was modified. In contrast, sensitivity of the IRMA was dramatically reduced when the MAb was added during the first 10 min of the reaction (Fig. 3). However, when the MAb was added more than 60 min after starting of polymerization, results were

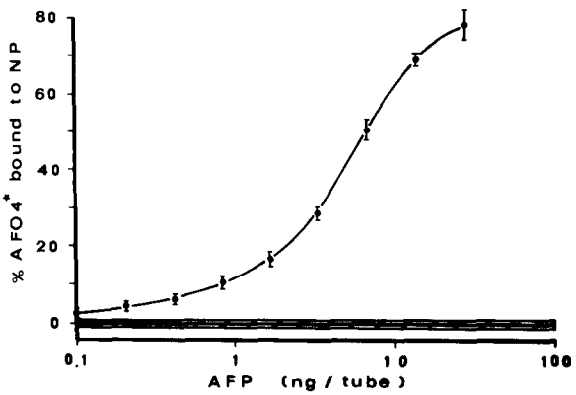


Fig. 1. NP-IRMA. Day-to-day reproducibility ±S.D. of 5 assays. Capture antibody coated onto NP: 5 $\mu$g AF01 per mg of polymer. Tracer antibody: $^{125}$ I-AF04, 50,000 cpm.

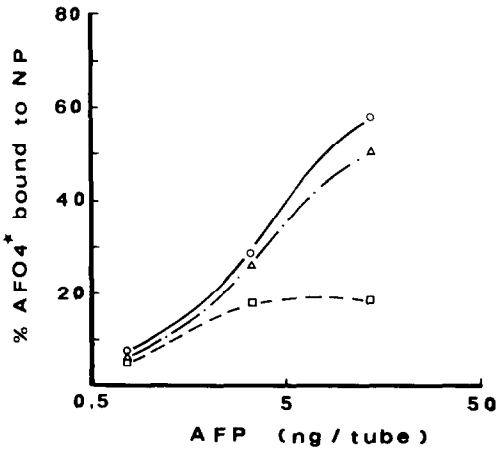


Fig. 2. Influence of the concentration of capture antibody for the NP-IRMA of AFP. (○) 5; (△), 2; (□), 1 $\mu$g AF01 per mg of NP.

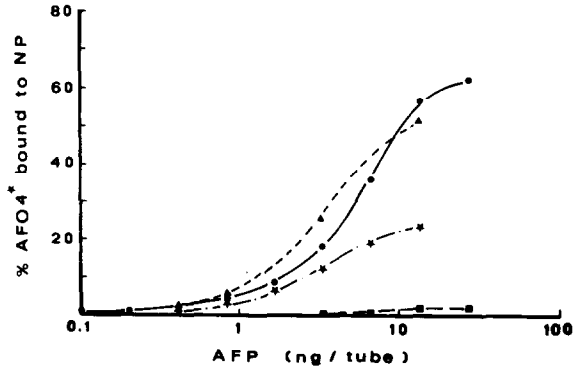


Fig. 3. Comparison of NP-IRMA using the capture antibody (AF01: 2 $\mu$g per mg NP) either coated in standard conditions (▲) or added 10 min (■), 30 min (★) or 60 min (●) after the beginning of the polymerization.

obtained that were almost the same as with the standard coating process. At 30 min, the loss of immunoreactivity was intermediate and especially marked for higher AFP levels.

The influence of NP concentration on IRMA efficiency is illustrated in Fig. 4. Higher concentrations than 0.83 mg/ml did not allow an improved efficiency of the IRMA. But with lower concentrations (0.41 mg/ml), the detection of AFP was lowered, especially for antigen concentrations higher than 100 ng/ml.

Temperature and time conditions were also investigated at two stages of the assay. For saturation times ranging between 2 h and 40 h at both 20 and 37 °C (with NP concentration of 5 mg/ml and 50% FCS), full immunoreactivity was conserved for subsequent IRMA (Fig. 5). In contrast, when varying the incubation time at 37 °C of more diluted NP suspensions (0.83 mg NP/ml), only 70% and 40% of the specific binding of radiolabelled AF04 obtained in standard conditions could be achieved after 24 h and 48 h, respectively (for 530 ng AFP/ml) (Fig. 6).

Finally, the last parameter we have tested was the NP chemical composition: as shown in Fig. 7, a lower antigen binding capacity was obtained with IHC-NP in place of IBC-NP coated with the same quantity of AF01.

Taken as a whole, immunoreactivity investigations have shown that maximum efficiency was reached for NP concentrations higher than or equal to 0.83 mg/ml, each mg NP being coated with 5 μg AF01. Early addition of MAb in the polymerization medium hid the whole immunoreactivity which, however, was conserved when the MAb was added to almost formed nanoparticles. More interesting was the fact that the lower the NP concentration for long incubation times at 37 °C (20 h and more), the weaker was the remaining immunoreactivity on the antibody-coated NP. These results led us to study the stability of the MAb-NP binding further.

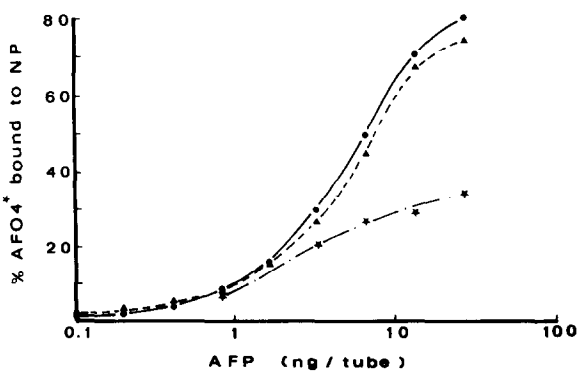


Fig. 4. Influence of the NP concentration on the immunoradiometric detection of AFP. (●) 3.3; (▲) 0.83; (★), 0.41 mg per ml.

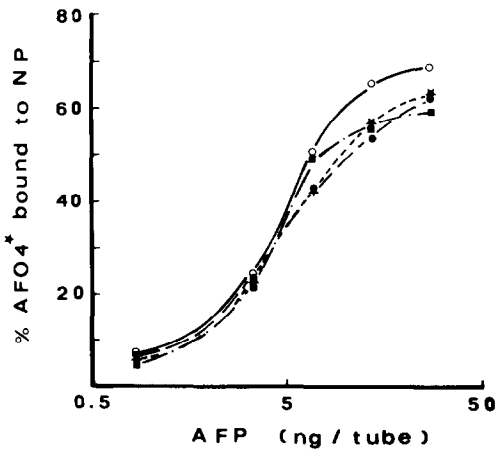


Fig. 5. Influence of the saturation protocol (with NP: 5 mg/ml and 50% FCS) on anti-AFP NP-IRMA (incubation 4 h at 37 °C). Saturation conditions: (○), 2 h at 20 °C; (★), 20 h at 20 °C; (●), 20 h at 37 °C; (■), 40 h at 37 °C.

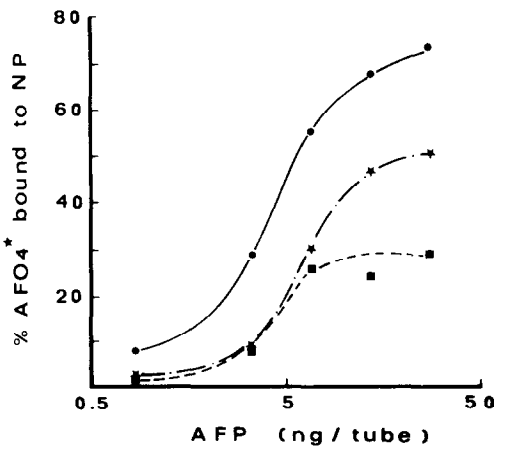


Fig. 6. Comparison of 3 incubation protocols of IRMA. Saturation was performed in standard conditions. Incubation conditions: (●), 20 h at 20 °C; (★), 20 h at 37 °C; (■), 40 h at 37 °C.

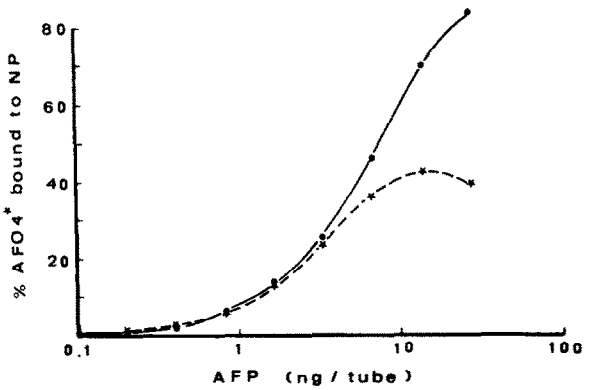


Fig. 7. IRMA performed in standard conditions with isobutylcyanoacrylate (●) or isohexylcyanoacrylate (★) nanoparticles.

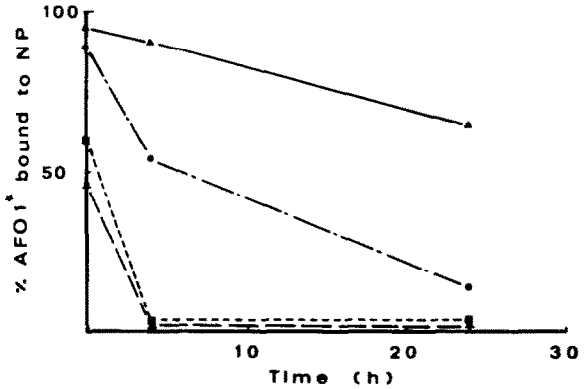


Fig. 8. Fraction of radiolabelled AF01 associated with NP after incubation at 37°C with FCS (22.5 mg/ml). NP concentrations : (▲), 5 mg/ml; (●), 2.5 mg/ml; (■) 1.25 mg/ml; (★) 0.83 mg/ml.

*Stability of MAb-NP binding*

This study was performed with $^{125}$I-labelled monoclonal antibody. It was firstly found that for NP concentrations ranging from 0.83 mg/ml to 5 mg/ml, the linkage of MAb remained unchanged after incubation for 20 h at 37°h whatever the amount of cold antibody present with the radiolabelled antibody. In view of this result, further experiments were carried out only with radiolabelled antibodies. When NP concentration was lower than 2.5 mg/ml in the incubation medium, desorption of radiolabelled MAb occurred after 4 h incubation at 37°C in the presence of FCS as well as in the presence of BSA (Figs. 8 and 9). In contrast, for NP concentrations beyond 2.5 mg/ml, the stability was satisfactory in the presence of BSA (Fig. 9) whereas in the presence of FCS, an almost complete desorption was noted after 4 h (Fig. 8). When the MAb was dissolved in the polymerization medium 30 min or 60 min after addition of the monomer in place of simply coated at the surface of well-formed nanoparticles, the stability of MAb-NP binding was not significantly improved. In the same way, no marked improvement of stability of MAb-NP binding was obtained when MAb was coated on IHC-NP. Furthermore, in the IRMA standard conditions, we found that leakage of radiolabelled capture antibody was so important (95%) that IRMA turned out to be inefficient in contrast to what it seemed. This discrepancy was at first difficult to explain. Indeed the fact that MAb was more importantly desorbed from its polymeric support in the presence of FCS (esterase-rich medium) than in the presence of purified BSA (esterase-free medium) let suppose that MAb linkage was a consequence of esterase-induced bioerosion of the polymer. This was, however, not consistent with the high efficiency observed in the IRMA performed in FCS-rich medium. For the same reason, chemical degradation of the MAb was not conceivable. Likewise, deiodination of radiolabelled monoclonal antibody could not explain this difference since no free iodide was detected (after G25 gel

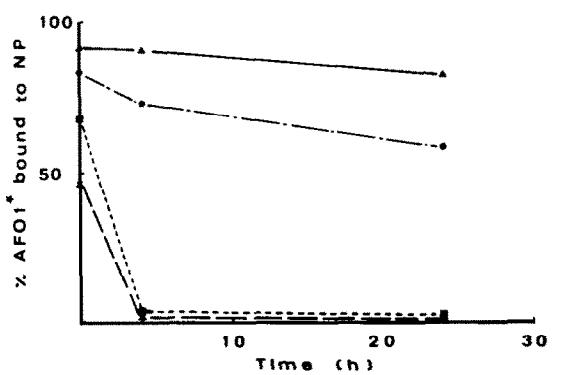


Fig. 9. Fraction of radiolabelled-AF01 associated with NP after incubation at 37°C with BSA (22.5 mg/ml). NP concentrations: (▲), 5 mg/ml; (●) 2.5 mg/ml; (■) 1.25 mg/ml; (★), 0.83 mg/ml.

filtration) even after 20 h incubation at 37 °C with 50% FCS.

In consequence, it was supposed that the methodology used to study the stability of MAb-NP binding should induce artefacts. Indeed, the separation of NP from the incubation medium was carried out by precipitating the polymer with a PEG solution (7%). A first hypothesis was that this precipitation could be dependent on the constitution of immune complex networks (NP-MAb1-Ag-MAb2) whereas only incomplete precipitation could be achieved when the single capture antibody was coated to NP (NP-MAb1*). But this hypothesis was discarded after testing in the IRMA conditions that neither the PEG-induced precipitation nor the stability of the (NP-MAb1*-Ag-MAb2) complex was improved comparatively to the single NP-MAb1*.

Finally, the discrepancy between the efficiency of IRMA and the weak stability of radiolabelled MAb-NP linkage was consistent with a quite different behaviour of the same radiolabelled and cold monoclonal antibodies for their interaction with NP surface. Practically, the following experiments were carried out: nanoparticles coated with cold AF01 were compared, in a IRMA experiment, to the same particles but covered with both cold and $^{125}$I-labelled capture antibody. After 4 h at 37 °C with AFP and $^{125}$I-labelled tracer antibody (AF04), the radioactivity associated to the solid phase was found in both cases proportional to the AFP concentration, with, however, a constant increment in the presence of radiolabelled capture antibody (Fig. 10). In contrast, when the incubation was carried out for 24 h, parallel curves were also obtained in the presence as well as in the absence of radiolabelled capture antibody but with a reduced difference between the two curves. In other words, when NP were coated with both cold and labelled antibody, a preferential release of the radioactive antibody was noted. This led us to the conclusion that the labelling of antibodies by the iodogen method dramatically modified their physicochemical interaction with the nanoparticulate surface.

## Conclusions

In view of the conserved immunoreactivity and of the stability of immunonanoparticles in the presence of seric proteins, we showed their potential value for eventual in vivo use. Furthermore, by modifying various experimental parameters, we determined the optimal conditions for using nanoparticles as a solid support for IRMA. But the most surprising observation was the fact that iodine labelling by using the iodogen method importantly modified the physicochemical interactions between the protein and the polymeric phase, leading to an early release of the labelled antibody. Therefore, it is noteworthy that such a

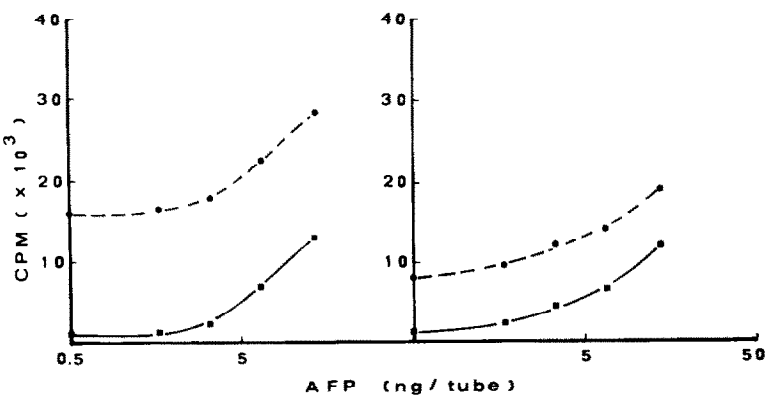


Fig. 10. NP-IRMA (AF01-AF04*) with (●------●) or without (■———■) addition of radiolabelled capture antibody (AF01*). Left: incubation time: 4 h at 37 °C; right: incubation time 20 h at 37 °C.

labelling could lead to erroneous conclusions in terms of immunonanoparticles distribution in vivo. Therefore the conclusions reached by Illum et al. (1984) concerning the targeting with MAb-coated nanoparticles in vivo should be further discussed in the light of the results of the present study.

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