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Immunochemical characterization of antibody-coated nanoparticles

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Abstract. A new method using surface plasmon resonance (SPR) through the BIAcore was used to demonstrate the specific interaction between an anti-CD4 monoclonal antibody (IOT4a), adsorbed on poly(methylidene malonate 2.1.2) (PMM 2.1.2) nanoparticles, and the CD4 molecule. The results obtained were compared with the interaction of the same immunonanoparticles with rabbit anti-mouse Fc antibodies. The molar ratio (Fc)/(Fab) was 1, suggesting that the same number of epitopes on the Fc and the Fab fragments were accessible after IOT4a adsorption onto nanoparticles. Comparing the observed association rates of free antibody and antibody adsorbed on nanoparticles, the number of molecules of IOT4a antibody on PMM 2.1.2 nanoparticles was estimated as between 2.6 and 3 per nanoparticle. The properties of the antibody-coated nanoparticles are compatible with their use as antibody-targeted pharmacophores.
Key words. Immunonanoparticle; methylidene malonate; surface plasmon resonance.

Abbreviations. rsCD4 = recombinant soluble cluster differentiation 4; HIV-1 = human immunodeficiency virus type 1; RU = resonance unit; Np = nanoparticles; Np-Ab = anti-CD4 immunonanoparticles.

The controlled delivery of drugs and their specific release into the target tissues represent one of the most important pharmacological challenges. Several solutions to this problem have been proposed. For instance, liposomes and other soluble macromolecular drug carriers have been shown to be effective for peptide delivery[1,2]. Moreover, drugs can be released to specific cells or tissues using monoclonal antibody-mediated targeting devices[3].

An alternative to liposomes is the use of biodegradable nanoparticles[4]. Poly(alkyl-2-cyanoacrylate) (PACA) nanoparticles are the most extensively studied drug delivery systems[5]. However, because of the extreme pH conditions required for polymerization and the cytotoxic effects of PACA nanoparticles, other colloidal suspensions have been prepared from monomers such as asymmetric methylidene malonate 2.1.2 (MM 2.1.2)[6,7]. As PACA nanoparticles[8], these PMM 2.1.2 nanoparticles were able to adsorb proteins on their surface[9].

Poly(methylidene malonate 2.1.2) nanoparticles carrying an anti-CD4 monoclonal antibody have been shown in vitro to inhibit the entry of human immunodeficiency virus type 1 (HIV-1) into MT4 cells[10]. Thus it was of particular interest to analyse the binding parameters of IOT4a-adsorbed nanoparticles on rsCD4, in order to define the nature of this interaction and thus improve our targeting.

Previous work has defined the physico-chemical parameters of the anti-CD4 IOT4a monoclonal antibody on rsCD4 using surface plasmon resonance (SPR) (BIAcore™, Pharmacia Biosensor AB, Uppsala, Sweden)[11]. This technology is a new method that allows real-time analysis of molecular association. To summarize the principle, SPR is an optical phenomenon arising in thin metal films (e.g. gold) under conditions of total reflection. The phenomenon produces a sharp dip in the intensity of reflected light at a specific angle. The position of this resonance angle is correlated to the refractive index that is dependent on the concentration of dissolved material in solution close to the surface layer of the carboxylated dextran-coated gold film. To allow interaction measurements in real time with a relevant time resolution, BIAcore uses continuous flow technology. One interactant is immobilized on the sensor surface of carboxylated activated dextran, which forms one wall of a micro-flow cell. As molecules from the solution bind to the immobilized interactant, the resonance angle changes and the response is registered in resonance units (RU). The BIAcore has mainly been used in antigen-antibody interaction studies. The aim of the present paper is to demonstrate the specific interaction of antibody-adsorbed nanoparticles with immobilized rsCD4 molecules using the BIAcore system, and to define several parameters of this binding.

Materials and methods

Reagents. Recombinant soluble CD4 (rsCD4), at a concentration of 0.2 mg/ml in phosphate buffered saline, pH

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7.2, was purchased from Intracel (Cambridge, MA, USA). Anti-CD4 antibodies (IOT4a, clone 13B8-2) were provided by Immunotech (Marseille, France). Rabbit anti-mouse Fc (RAM-Fc), *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N'*-(dimethylaminopropyl)-carbodiimide (EDC), ethanolamine and surfactant P20 were purchased from Pharmacia (Uppsala, Sweden). *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid) (Hepes), EDTA and 2-(*N*-morpholino) ethanesulphonic acid (Mes) were obtained from Sigma (St Louis, MO, USA).

Immobilization of ligands on the sensorchip surfaces. Immobilization of RAM-Fc ligand was performed according to the general procedure for immobilization recommended by the BIAcore constructor (Pharmacia Biosensor AB, Uppsala, Sweden)[12,13]. For rsCD4 immobilization we proceeded as previously described[11]. Briefly, after equilibration of the instrument with HBS buffer (10 mM Hepes, 150 mM NaCl, 0.05% surfactant P20, pH 7.4), the following samples were automatically injected in succession into the BIAcore: NHS/EDC in a mixed solution (1/1, v/v), then 30 µl of rsCD4 (25 µg/ml) in 100 mM Mes buffer, pH 6.0, 1 M ethanolamine, pH 8.5, to inactivate the remaining NHS ester residues, and 100 mM HCl to wash the unbound material. The immobilization procedure was performed at flow rates of 2 µl/min for rsCD4 and 5 µl/min for RAM-Fc.

Purification of immunonanoparticles. Lyophilized nanoparticles were resuspended in ultra-pure water at a concentration of 10 mg/ml. Five milligrams was diluted in 2 ml of phosphate buffer (KH_2PO_4/Na_2HPO_4, 66 mM, pH 7.4) containing 20 µg of native IOT4a monoclonal antibody[9]. The mixture was maintained under gentle stirring for 2 h at room temperature. To separate the immunonanoparticles from unbound antibodies and BSA, two different methods were used. First, a gel filtration on Sepharose CL-4B (Pharmacia Biotech, St. Quentin en Yvelines, France) was performed; immunonanoparticles were eluted with phosphate buffer, pH 7.4[9,14]. Second, the immunonanoparticles were washed three times by centrifugation at 17,600*g* for 10 min at room temperature in phosphate buffer, pH 7.4. Stability of immunonanoparticles has been demonstrated elsewhere[9].

Binding of immunonanoparticles to the sensorchip. The binding of immunonanoparticles was performed at a flow rate of 2 µl/min at 25 °C. To maintain a homogenous suspension, the Np-Ab were kept in suspension by automatic aspiration/refluxing movements. The Np-Ab were allowed to interact for 4 min with rsCD4 or RAM-Fc.

Scanning electron microscopy. After passing the immunonanoparticles through the non-activated CD4 or RAMFc-bound channels, the sensorchip was washed in 0.1 M ammonium acetate, pH 7.5, to eliminate residual

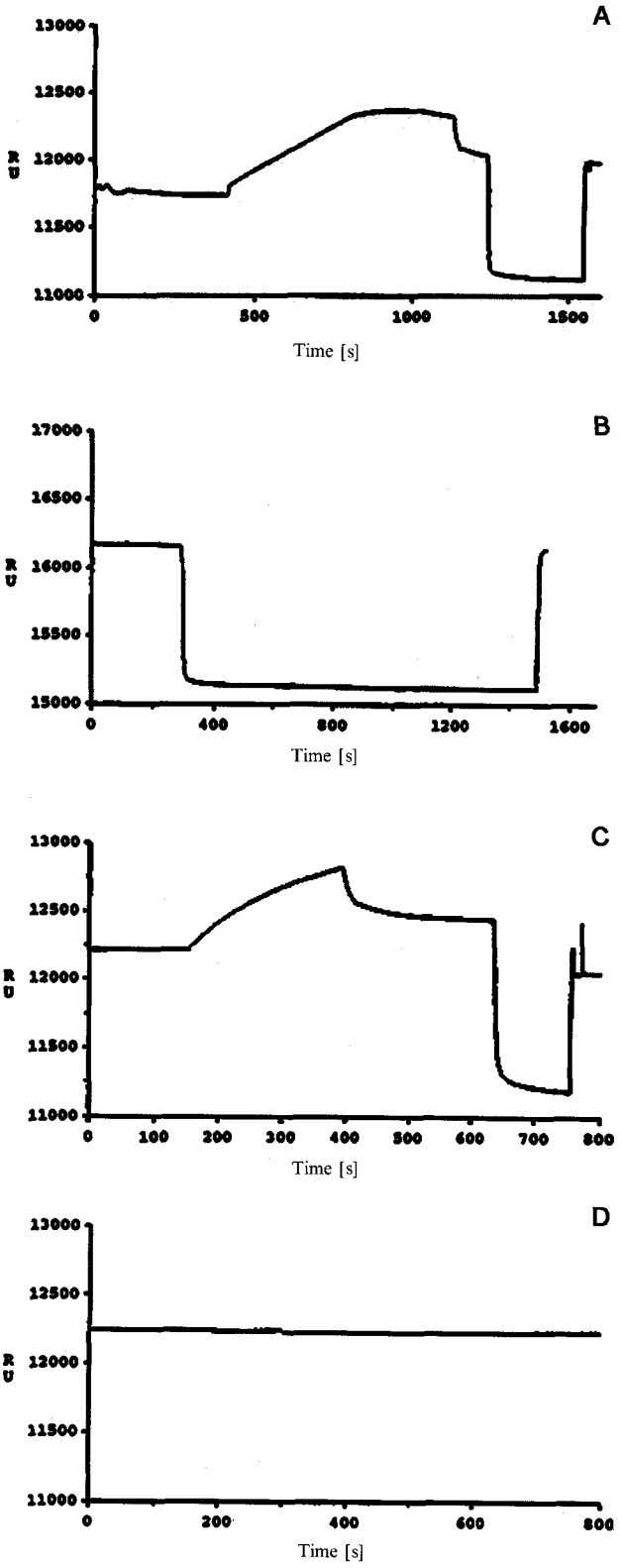

Figure 1. Sensorgrams of adsorption of anti-CD4 nanoparticles on protein-immmobilized sensorchips. Kinetics of adsorption of anti-CD4-adsorbed immunonanoparticles on rsCD4 (*A*) or RAM-Fc (*C*) immobilized on sensorchips. Ten microlitre immunonanoparticles at a concentration of 10 mg/ml were injected onto the protein immobilized sensorchips. The sensorgrams were recorded at a flow rate of 2 µl/min. Controls were performed with BSA nanoparticles injected under the same conditions (*B*, *D*).

salts from the surfaces to be observed. The sensorchips were gold-covered with a 100 Å layer using a JFC-1100 cathodic pulverizer (JEOL, Croissy sur Seine, France) and observed with a JSM 6400F high-field scanning electron microscope (JEOL). Primary electron acceleration voltage, working distance and primary beam intensity were respectively set at 5 keV, 15 mm and 6×10^{-12} A.

Immunoadsorption on cells. CD4 + CEM cells at a concentration of 2×10^6 per millimeter were incubated with freshly preparated immunonanoparticles (50 µg polymer/ml)[9] for 5 or 20 min at 37 °C and set on a Transwell filter (Costar, Cambridge, MA, USA). Cells were then fixed for 30 min in a 2% glutaraldehyde solution and washed three times in 0.2 M sodium cacodylate buffer, pH 7.4, supplemented with 0.012 M $CaCl_2$. Dehydration was conducted through four successive washes (5 min each) with aqueous solutions containing 30, 50, 70 and 90% ethanol and three washes (5 min each) in pure ethanol. For microscopic observation, the preparations were treated as for the sensorchips.

Results and discussion

Immobilization of RAM-Fc increased the RU signal to 3091, corresponding to 20 fmol/mm^2, while the immobilization of rsCD4 resulted in an increase of 2232 RU, corresponding to 40 fmol/mm^2.

After preparation of immunonanoparticles (Np-Ab), the suspension was allowed to flux through the channels of the sensorchip. As shown on the two sensorgrams (fig. 1A, C), specific binding of the Np-Ab to rsCD4 or RAM-Fc was observed. These interactions did not occur with BSA-adsorbed nanoparticles used as control (fig. 1B, D). Scanning electron microscopic analysis of the sensorchip surfaces confirmed the presence of immunonanoparticles on the rsCD4 bearing flowcell only (fig. 2).

Table 1. Kinetic parameters of binding of anti-CD4 nanoparticles.

Immobilized proteins on sensorchip	RU_{max} (RU)	k_{obs} (s^{-1})
RAM-Fc	746 ± 40	ND
rsCD4	1346 ± 229	0.004

From the binding curves as a function of time, the RU_{max} values at equilibrium could be calculated using a double exponential[11]. The RU_{max} values obtained were 746 ± 40 RU for Np-Ab on RAM-Fc and 1343 ± 229 RU for Np-Ab on rsCD4 (table 1). Since the molar surface density of rsCD4 was twice that of RAM-Fc, these results allowed us to conclude that the molar ratio of adsorbed immunonanoparticles as reflected in the RU_{max} is similar for the Fc fragment recognized by RAM-Fc and the Fab fragment recognized by rsCD4. This suggests that IOT4a adsorption onto nanoparticles does not significantly decrease the accessibility of the Fc fragment.

Figure 3 shows the linear dependence of the kinetic association rate measured (k_{obs}) at different concentrations of free IOT4a mAb. If we assume that the rate of IOT4a-rsCD4 binding was the same for free antibody as for antibody adsorbed on nanoparticles, k_{obs} calculated

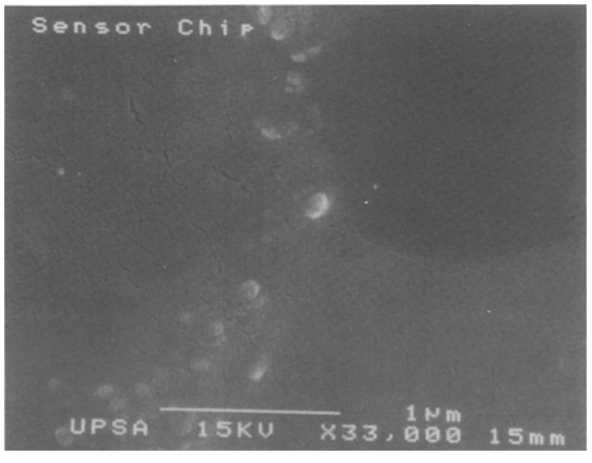

Figure 2. Scanning electron microscopy of immunonanoparticles bound on sensorchips. Scanning electron microscopy analysis of anti-CD4 (IOT4a mAb) immunonanoparticles bound onto rsCD4-immobilized sensorchips was performed. After the passage of immunonanoparticles, the sensorchips were washed three times with 0.1 M ammonium acetate, then covered with gold as described in 'Materials and methods'. Primary electron acceleration voltage, working distance and primary beam intensity were respectively set at 5 keV, 15 mm and 6×10^{-12} A. The scale is indicated on the pictures.

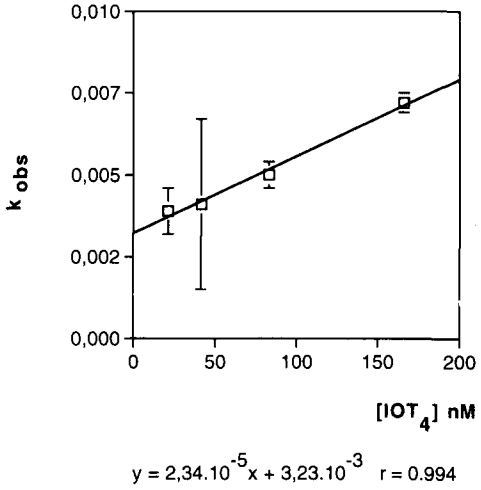

Figure 3. Plot of k_{obs} versus IOT4a MAb concentration. Kinetics of soluble IOT4a MAb on immobilized rsCD4 were performed using the BIAcore system. For each concentration, k_{obs} was determined by analysis using the double exponential method for the association phase of IOT4a on rsCD4[11].

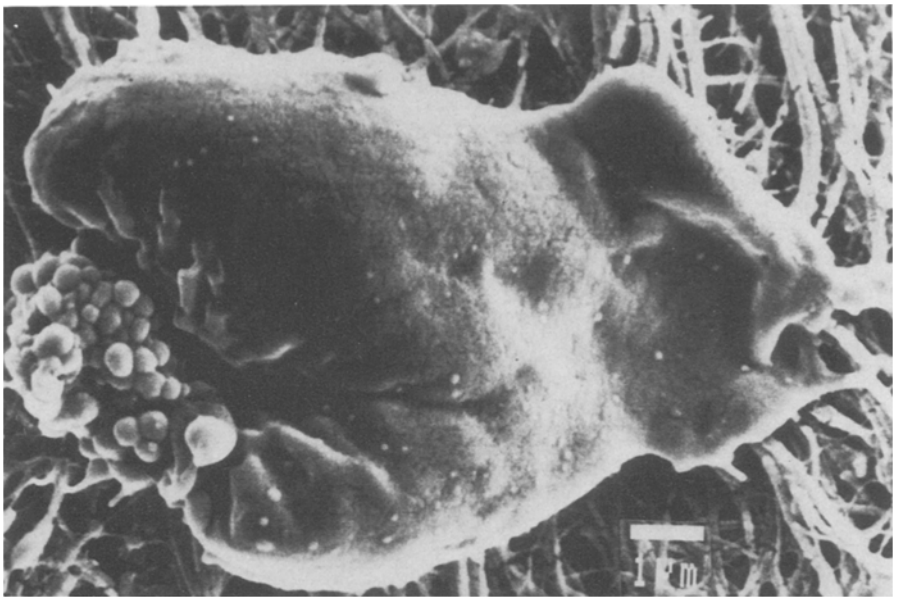

Figure 4. Scanning electron microscopic observation of immunonanoparticles bound to human $CD4^+$ cells. After incubation with the anti-CD4 immunonanoparticles, the cells were fixed and treated for electron microscopic observation as described in 'Materials and methods'. The scale is indicated on the pictures.

from the sensorgram in figure 1 can be used to determine the molar concentration of antibody in the nanoparticle suspension, using the linear equation derived from figure 3. We have found 33 nM of IOT4a for a suspension of 7.7×10^{14} nanoparticles/l. Using the following equation:

$$\text{Number of antibodies per Np} = \frac{[\text{IOT4a}] \times 6 \times 10^{23}}{\text{Number of nanoparticles/l}} \quad (1)$$

we calculated 2.6 to 3 IOT4a molecules/nanoparticle. This low amount is probably due to the simultaneous presence of bovine serum albumin at a molar ratio of 20 to 1 in the coating antibody solution. Immunonanoparticles were, however, able to induce a capping on CD4-expressing cells, in contrast to the free antibodies, suggesting that the former enhanced aggregation of membrane proteins, acting as IgG polymers (fig. 4).

The BIAcore has been used mainly for the studies of kinetics and equilibrium of protein-protein or DNA-protein interactions, in which one of the partners is immobilized. This work demonstrates the analytic possibilities of the BIAcore system on particulate material. It also confirms the specific cell-biological properties of antibodies adsorbed on nanoparticles, confirming their ability to target specific cells or tissues.

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