Synthesis of Metal-Carbonyl–Dendrimer–Antibody Immunoconjugates: Towards a New Format for Carbonyl Metallo Immunoassay

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We report the preparation of metal-carbonyl–dendrimer–antibody conjugates. These metal-carbonyl-multilabeled antibodies are designed to be used in a new solid-phase-format carbonyl metallo immunoassay (CMIA). A fourth-generation polyamidoamine dendrimer was labeled with 10–25 (η^5-cyclopentadienyl)iron dicarbonyl (η^1-N-succinimidyl) entities. An antibody was chemically modified at its carbohydrate chains by a site-directed process used to preserve the antigen–antibody binding site. The antibody was then coupled with the dendrimer labeled with 10 metal carbonyl groups. An average of 1.4 labeled dendrimers were grafted per antibody molecule. These metal-carbonyl–dendrimer–antibody conjugates were used as new universal detection reagents that recognize their specific antigens. The antigens were spotted onto nitrocellulose membranes and detected by using the conjugates in combination with Fourier transform infrared spectroscopy. A detection level in the range 5–200 pmol per membrane was achieved. This approach opens the way to a new CMIA format.

Introduction

Bioorganometallic chemistry is emerging as an independent research field.[1–7] This type of chemistry concerns biologically significant species containing at least one direct metal–carbon (M–C) bond. Development in this field has led to the discovery of new potential therapeutics.[8–10]A recent review described the important biological role played by CO in mammals.[11] CO gas, for instance, has physiological properties such as antiinflammatory effects, and metal carbonyl complexes such as $[Ru(CO)_3Cl(glycinate)]$ show promise as CO-releasing molecules for use in medical applications, particularly those dealing with the cardiovascular system.

In the analytical research field, metal carbonyl complexes have been linked to bioactive flavonoids to allow the study of flavonoid–protein interactions in molecular signaling events by Fourier transform infrared (FTIR) spectroscopy.[12] Herein, we describe the application of metal carbonyl complexes in the field of immunoanalysis, where they are used as infrared probes for quantification of molecules present at trace level.

A decade ago, we designed a nonisotopic immunoanalytical method named carbonyl metallo immunoassay (CMIA). The technique involves quantification of compounds containing transition metal carbonyl labels, which can be detected by FTIR spectroscopy. The CMIA method was initially developed for the quantification of antiepileptic drugs.[13] A metal carbonyl probe (alkyne-$(Co)_2(CO)_6$, $CpMn(CO)_3$, $PhCr(CO)_3$, or CpFe(-CO)(L); L = ligand)[14] was chemically attached to the hapten to form a tracer. This tracer competed with the analyte for specific antibody binding in a liquid-phase immunoassay. The remaining free tracer was extracted with an organic solvent and quantified by FTIR spectroscopy. This approach takes advantage of the specific and intense absorption bands of the metal carbonyl label in the mid-infrared spectral range (1800–2200 cm^{-1}), where few others vibrators absorb. The assay format is well suited to therapeutic drug monitoring but is not sensitive enough to detect pesticides such as chlortoluron[15] and atrazine[16] in environmental matrices, compounds for which the maximum admissible concentration is set at 0.1 $\mu g L^{-1}$ by European guidelines. The low sensitivity of the technique is caused by the lack of signal amplification during the course of the experiment. In contrast, conventional techniques such as HPLC and GC, which use sample pretreatment,[17–19] or immunoassays like ELISA, which use an enzyme as a label, involve signal amplification.[20] Consequently, it seemed necessary to modify the CMIA format to improve the sensitivity of the method by increasing the intensity of the IR signal.

The intensity of the FTIR signal is proportional to the quantity of metal carbonyl units present in the analyzed sample.[21] Therefore, the only way to increase the signal is through metal carbonyl multilabeling of one of the protagonists of the assay, that is, the hapten or the antibody. Haptens such as pesticides or drugs are small molecules so multilabeling of these compounds would be detrimental to antibody–hapten recognition efficacy. Therefore, the antibody macromolecule, which be-

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longs to the immunoglobulin G (IgG) family and has a molecular weight of around 150 KDa, seems the protagonist of choice for metal carbonyl multilabeling. We have shown previously that random multilabeling of monoclonal IgG by treatment with *N*-succinimidyl esters of organometallic complexes[22,23] is not a reliable way of multilabeling as it sometimes leads to complete loss of hapten recognition. This problem is probably the result of coupling of some metal carbonyl units at the hapten–antibody binding site.[24] In the late 1980s, Diamandis achieved fluorescence signal amplification by preparing carrier proteins labeled with a large number of europium chelators. These multilabeled proteins were used as detection reagents and were linked to antibodies either directly[25] or through the avidin–biotin system[26] in an effort to design fluorometric immunoassays for biological compounds. More recently, this group has designed a new detection reagent in which the carrier protein is replaced by polyvinylamine.[27]

Starburst polyamidoamine (PAMAM) dendrimers are well-defined polymers[28] with a high number of terminal primary amine groups that have found promising biomedical applications in the last decade. For example, these polymers have been used as carrier molecules to increase the specific activity of radiolabeled antibodies for targeted drug therapy and diagnosis.[29–34] It has also been shown that immunoclusters composed of antibody–dendrimer–fluorescein conjugates complexed with antigens provide an amplified fluorescent signal.[35]

These results prompted us to use the fourth-generation (G4) PAMAM dendrimer as a carrier of a large number of metal carbonyl complexes for site-directed conjugation to an antibody. We took advantage of the carbohydrate moieties located in the crystallizable fragment (Fc) of every IgG molecule, which can be oxidized to aldehyde groups and then used to perform reductive amination of the antibody with several dendrimers. This process led to a multilabeled antibody. Specific chemical modifications at the carbohydrate sites are well known to have no effect on the remote antibody–antigen binding site integrity.[36]

We labeled the dendrimer PAMAM G4 with the iron carbonyl complex (η^5-cyclopentadienyl)Fe(CO)$_2$(η^1-*N*-maleimide), commonly named Fp-maleimide, and successfully conjugated the labeled molecule to goat antirabbit IgG. Preliminary immunological assays on nitrocellulose membranes were run by using this new universal metal-carbonyl–dendrimer–antibody detection reagent and FTIR detection.

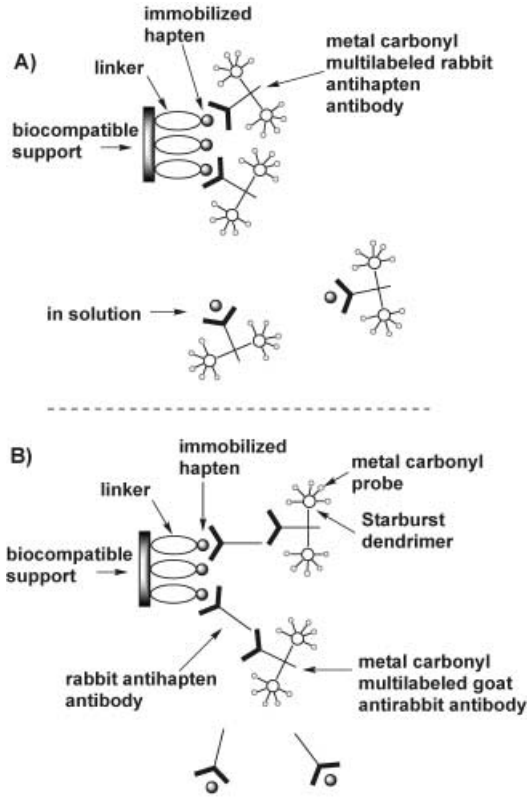


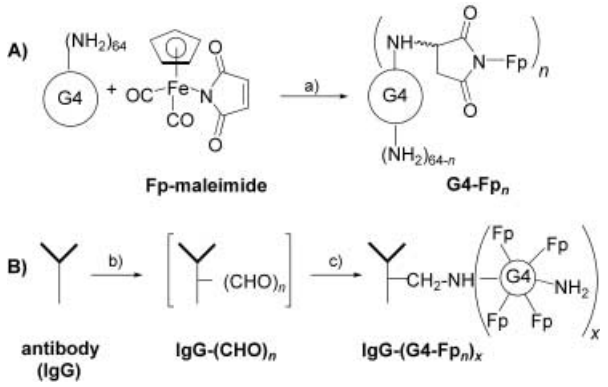
Figure 1. *The new solid-phase CMIA: Metal carbonyl multilabeling of the primary antipesticide antibody (A) or the secondary antibody (B). The metal carbonyl probes bound to the surface of the antibody are quantified by FTIR spectroscopy. Hapten: the molecule to be tested*

Results and Discussion

The new format we propose for CMIA is a solid-phase competitive immunoassay (Figure 1). The assay involves a biocompatible solid support that is able to bind the antigen or the hapten and is compatible with IR detection by transmission or reflection. Two labeling strategies can be considered: metal carbonyl probes can be attached either to the primary rabbit antihapten antibody for direct detection of the immobilized hapten (Figure 1 A), or to the secondary goat antirabbit antibody used to detect the first hapten–antibody interaction (Figure 1 B). The second strategy is preferable because multilabeling of the goat antirabbit antibody opens the way towards a new class of universal detection reagents for use in immunoassays involving primary antibodies produced in rabbits. We decided to use the PAMAM G4 dendrimer as a carrier of multiple metal carbonyl labels because this commercially available compound is water soluble and has 64 surface primary amino groups available for nucleophilic reaction with transition-metal carbonyl reagents and oriented attachment to the glycosylated moieties of an antibody. This polymer, with a calculated molecular weight of 14215, is also well suited to the purification techniques generally used for biomolecules, that is, dialysis, ultrafiltration, and size-exclusion chromatography.

Preparation of organometallic dendrimers

Controlled labeling of the amine groups on the surface of the dendrimer (Scheme 1 A) was achieved by treating PAMAM G4 in methanol with Fp-maleimide prepared as described previously.[37] Initial Fp-maleimide/G4 ratios varying from 24 to 47 were tried and reactions were run at room temperature (Table 1). When 32 molar equivalent or less Fp-malemide was used (i.e. no more than half as many Fp-malemide units as surface amine groups on the dendrimer), only about 10 amino groups were labeled per dendrimer molecule, even with a pro-



Scheme 1. *Synthetic route to labeled PAMAM G4 dendrimer and the IgG conjugate. a) MeOH; b) $NaIO_4$ (see Table 2); c) G4-Fp_n (7 equiv), $NaBH_3CN$.*

Table 1. *Synthesis of metal-carbonyl-labeled dendrimers containing Fp-maleinimide.*

	Fp-maleimide/G4 initial ratio	Reaction time [h]	Number of Fp units per G4 molecule
1	32	18	10
2	24	72	11
3	47	72	25

longed reaction time (entries 1 and 2). The number of Fp labels attached was increased to 25 solely by increasing the initial Fp-maleimide/G4 ratio to 47 (entry 3). Experiments performed at 37 °C did not have improved labeling efficiency (data not shown). The labeled dendrimers bearing *n* Fp groups (G4-Fp_n) were coarsely purified by lipophilic gel-permeation chromatography on Sephadex LH-20 in methanol to separate G4-Fp_n from unreacted Fp-maleimide. This purification step was followed by aqueous gel-filtration chromatography on Superdex 200 in phosphate buffer at pH 7.2. PAMAM dendrimers behave as if they were heavier than globular proteins of the same molecular weight, as has been described for PAMAM G5 dendrimers.[38] For example, myoglobin has a molecular weight of 17000 and eluted at 17.5 mL, whereas PAMAM G4, which has a lower molecular weight (14215), eluted earlier (16 mL). The typical chromatographic profile of G4-Fp_n shown in Figure 2 indicates that a heterogeneous mixture of organometallic dendrimers was produced under our reaction conditions: the elution profile contains one broad peak (13.9 mL) corresponding to heavy compounds, and one sharp peak (16.1 mL) corresponding to lighter labeled dendrimers. The broad peak was not suited to our purpose because its elution volume is too close to that of IgG (11.5 mL) and we planned to use the same gel-filtration column to separate IgG-(G4-Fp_n)$_x$ from excess G4-Fp_n in the next purification step. So we chose to work with the lighter fraction for conjugation of the dendrimers with goat antirabbit IgG. This fraction of organometallic dendrimer (G4-Fp_n) was characterized by FTIR and UV/Vis spectroscopy. The IR spectrum of a dried spot of G4-Fp_n on a nitrocellulose membrane showed two intense bands at $\tilde{\nu}_{CO} = 2000$ and 2049 cm^{-1}, which are characteristic of the Fp group. The number of Fp groups bound per dendrimer molecule was determined by spectrophotometric measurements. The Fp concentration in the sample was calculated by taking a direct reading of the absorbance at 370 nm ($\varepsilon_{Fp} = 600\ M^{-1}cm^{-1}$), and the concentration of residual primary amino groups was determined by using two colorimetric assays: the trinitrobenzene sulfonic acid (TNBS) assay[39] and a modified version of the ninhydrine assay.[40] The dendrimer labeled with 10 Fp units per G4 (G4-Fp_{10}) was chosen for studying the conditions required for coupling with secondary antibodies.

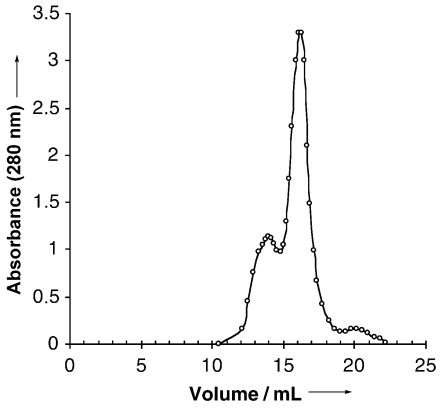


Figure 2. *Elution profile of G4-Fp_n passed through a 25 mL Superdex 200 column at 0.4 mL min^{-1}.*

Coupling of goat antirabbit IgG with labeled dendrimers: Preparation of IgG-(G4-Fp_n)$_x$

Labeled dendrimer G4-Fp_{10} was coupled to antirabbit antibody (IgG) as shown in Scheme 1B: Carbohydrate moieties located in the Fc region of the IgG molecule were oxidized by treatment with $NaIO_4$ to give IgG(CHO)$_n$. Wolfe et al.[41] have shown that the number of oxidized sites generated by $NaIO_4$ is pH dependent and varies between 1 at pH 7 and 8 at pH 3, with a strong effect between pH 4 (4 sites) and pH 3 (8 sites). Another important factor is the concentration of the oxidizing reagent. The same authors recommend the use of 5–10 mM periodate to avoid overoxidation and precipitation. Whatever conditions we used, aldehyde groups were produced during the first 30–60 minutes. Reductive amination of the antibody was then performed by treatment with the labeled dendrimer in the presence of $NaBH_3CN$ as a reducing agent. In the literature, this step is commonly run at a pH value above 9 but degradation of PAMAM dendrimers has been observed at pH 9,[42] which precluded use of these conditions. In light of these facts, we tried three combinations of pH value: pH 4.3 for oxidation and 8 for reductive amination, or pH 3.6 for oxidation and 8 or 7.2 for reductive amination. These trials were compared to a fourth experiment in which both oxidation and reductive amination were performed at physiological pH.

An antirabbit IgG solution in buffer at pH 3.6 or 4.3 was oxidized by treatment with $NaIO_4$ (10 mM) for 1 h at room temperature in the dark. The resulting IgG(CHO)$_n$ was quickly sepa-

rated from the oxidizing reagent by gel filtration with concomitant buffer exchange and was allowed to react with G4-Fp_{10} in phosphate buffer at pH 7.2 or 8 at room temperature for 1 h. $NaBH_3CN$ was then added to reduce intermediate imines to stable secondary amines. At the end of the reaction time, ethanolamine was added to block reaction of the remaining free aldehyde groups. The crude immunoconjugate was purified by size-exclusion chromatography on a Superdex 200 column. A

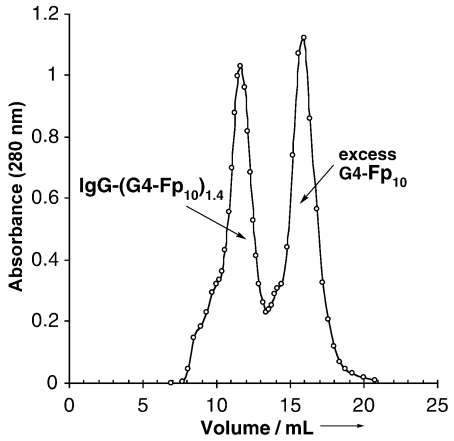


Figure 3. *Chromatographic profile of IgG-(G4-Fp_n)$_x$ obtained through purification on a 25 mL Superdex 200 column eluted at 0.4 mL min^{-1}.*

typical chromatographic profile is shown in Figure 3. Heavy compounds corresponding to the expected conjugates eluted between 8 and 13 mL. Fractions that eluted between 8.4 and 12.1 mL were pooled and concentrated. The G4-Fp_{10}/IgG ratio was determined by taking absorbance readings at 365 and 280 nm. Standard curves obtained by plotting A_{365} and A_{280} versus concentration of G4-Fp_{10} were used to determine both the concentration of G4-Fp_{10} bound to IgG and its contribution to the absorbance at 280 nm. This G4-Fp_{10} contribution was subtracted from the total absorbance at 280 nm to obtain the

Table 2. *Number of G4-Fp_n units linked to the IgG molecule when the two-step reaction was carried out at various pH values.*

Compound	pH_{ox} 4.3 pH_{amin} 8	**pH_{ox} 3.6 pH_{amin} 8**	pH_{ox} 3.6 pH_{amin} 7.2	pH_{ox} 7.2 pH_{amin} 7.2
G4-Fp_{10}	0.9	**1.4**	1.1	0.6
G4-Fp_{25}	–	**0.5**	–	–

pH_{ox} = pH value in the oxidation step; pH_{amin} = pH value in the amination step.

IgG concentration (A_{280} = 1.4 for 1 mg mL^{-1} IgG solution). The results are summarized in Table 2. Oxidation at pH 4.3 (followed by reductive amination at pH 8) led to attachment of 0.9 G4-Fp_{10} units per IgG molecule. Oxidation at pH 3.6 (and reductive amination at pH 8) increased this value to 1.4 G4-Fp_{10} per IgG molecule. When the experiment was run at pH 7.2 for both oxidation and coupling, 0.6 G4-Fp_{10} units were attached per IgG. The best pH combination (oxidation at pH 3.6 and reductive amination at pH 8) was applied to conjugate the highly labeled dendrimer G4-Fp_{25} with IgG. The dendrimer/IgG ratio dropped abruptly to 0.5 with this change in labeling, which suggests that the steric hindrance generated by the presence of a high number of Fp residues prevents efficient coupling of IgG to the remaining free amine groups of the labeled dendrimer. Such poor conjugation between highly functionalized dendrimers and an enzyme has already been observed.[43] This result can be rationalized by looking carefully at the topology of the reaction protagonists. PAMAM G4 is spherical and has a theoretical diameter of 4.5 nm.[28] This value becomes even greater when Fp groups are grafted onto the surface of the molecule. The crystal structure of an intact IgG shows that the two heavy chains of the Fc region are folded and form a hollow ring with carbohydrate moieties inside.[44] The external diameter of this region is about 6.5 nm so access to generated aldehydes is restricted. Consequently, one can anticipate that no more than two PAMAM G4 dendrimers, one on each side of the hollow ring, can be linked to one IgG molecule through the glycosylated residues.

IR analysis of IgG-(G4-Fp_n)$_x$ on a nitrocellulose membrane gave a spectrum containing the two $\tilde{\nu}_{CO}$ bands at 2052 and 2000 cm^{-1} characteristic of the Fp group, which confirms the presence of the metal carbonyl units on the antibody molecule.

Immunoreactivity of the labeled antibodies IgG-(G4-Fp_n)$_x$

The next step was to check the effect of the presence of such bulky substituents on the immunoreactivity of these labeled

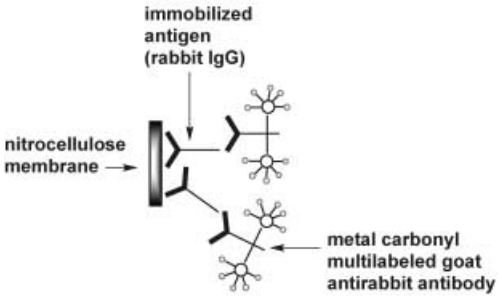


Figure 4. *The interaction between rabbit IgG and goat antirabbit IgG-(G4-Fp_{10})$_{1.4}$ at a nitrocellulose surface can be detected by FTIR spectroscopy.*

antibodies. An immunological assay was performed on nitrocellulose membranes with commercial rabbit IgG as the antigen (Figure 4). This test is the key step for the development of the new CMIA format and corresponds to the revelation step shown in Figure 1 B since we assume that the first hapten–antihapten antibody interaction at the membrane surface is classical.

Nitrocellulose was chosen because it is a well-known support used for protein blotting or transfer and for immunoblotting. Nitrocellulose is transparent in the mid-infrared spectral range, as shown in Figure 5 (spectrum A), and is both easy to handle and cheaper than a gold support.

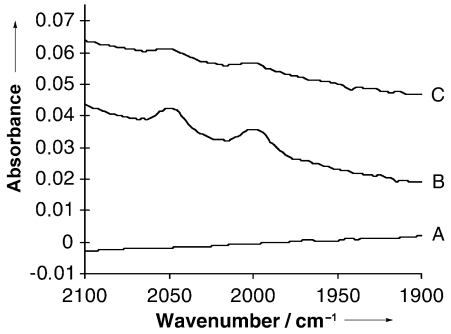


Figure 5. *FTIR spectra of nitrocellulose membranes; detection by transmission. A) Untreated nitrocellulose membrane. B) Rabbit-IgG-coated membrane incubated with Fp-labeled antirabbit IgG solution. C) β-LG-coated membrane incubated with Fp-labeled antirabbit IgG solution.*

We used a membrane coated with rabbit IgG to demonstrate the feasibility of our approach. A second membrane was coated with the same quantity of β-lactoglobulin (β-LG), which is not recognized by goat antirabbit IgG. This second membrane was used to assess nonspecific interactions with the support. After drying, the two membranes were incubated with 4% goat serum to block unoccupied sites and minimize nonspecific interactions. The membranes were incubated with Fp-labeled antirabbit IgG solution, washed several times with phosphate-buffered-saline-Tween, dried, and analyzed by IR spectroscopy (Figure 5, spectra B and C). The spectrum of the membrane coated with rabbit IgG (spectrum B) contains the two $\tilde{\nu}_{CO}$ bands characteristic of the Fp label, which indicates that the Fp-labeled antirabbit IgG bound to the membrane. The spectrum of the membrane coated with β-LG (spectrum C) shows very weak bands. The difference between the intensities of the absorbance peaks at 2052 cm^{-1} in spectra B and C is 4.46×10^{-3} a.u. This result clearly demonstrates that specific interactions occurred between the antigen and the labeled antibody and that few nonspecific interactions with the membrane took place.

In a second experiment, decreasing quantities of rabbit IgG (40–0.1 μg) were spotted onto membranes with concomitant increasing quantities of β-LG so that the total amount of protein adsorbed per membrane remained the same (40 μg). Unoccupied sites on the membranes were blocked with goat serum. The membranes were then incubated with Fp-labeled antirabbit IgG, washed, and dried. The absorbance at 2052 cm^{-1} decreased with decreasing quantity of coated antigen (Figure 6), which demonstrates that this absorbance can be related to the amount of specific antigen bound to the membrane. The new reagent enabled the amount of solid-phase-immobilized rabbit IgG to be measured with a detection level in the range 5–200 pmol per membrane. To the best of our knowledge, this is the first example of the use of nitrocellulose membranes for quantitative immunodetection of a tracer by FTIR spectroscopy. On the basis of this preliminary result, we can envisage the development of an immunoassay on a solid support with quantification through FTIR detection.

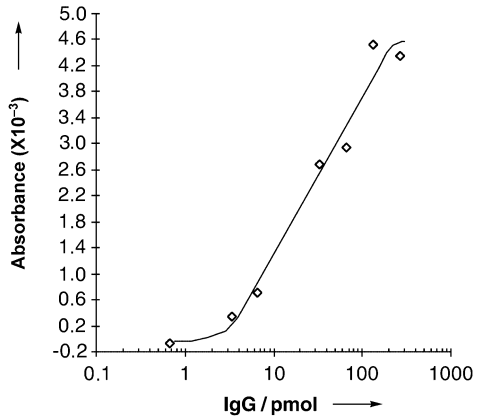


Figure 6. *Absorbance at 2052 cm^{-1} ($\tilde{\nu}_{CO}$) of the Fp label (corrected by measurements taken from the blank) versus amount of rabbit IgG on the membrane.*

Conclusion

We have prepared and characterized PAMAM G4-$(Fp)_n$ dendrimers with 10–25 organometallic Fp groups per dendrimer. The number of Fp groups per molecule is dependent upon the initial reaction conditions. These labeled dendrimers were successfully coupled to oxidized immunoglobulin G. Coupling of the dendrimer with the fewest labels led to an immunoconjugate with 1.4 G4-Fp_{10} per IgG, whereas use of G4-Fp_{25} led to attachment of 0.5 G4-Fp_{25} per IgG. This result suggests that the size of the G4 dendrimer and the steric hindrance around the remaining free NH_2 groups of the polymer govern the efficacy of conjugation of this molecule with IgG. Nevertheless, we have achieved signal amplification since about 14 Fp units were grafted onto one antibody molecule, compared to the 1:1 ratio achieved with the CMIA format used previously.

Preliminary immunological assays were carried out on nitrocellulose membranes, which are cheap, easy to handle, and transparent in the mid-infrared spectral range. The following results were obtained:

1) Chemical modification of a goat antirabbit IgG with a bulky Fp-labeled PAMAM G4 dendrimer did not affect antigen recognition of the Fp-labeled antibody.
2) Only weak nonspecific interactions between the membrane and the Fp-labeled antibody molecules were observed.
3) Quantitative detection of the metal carbonyl tracer on nitrocellulose was achieved.

The development of a new format for the carbonyl metallo immunoassay on biocompatible solid supports can now be envisioned. This new solid-phase format is solvent free, whereas the old format uses isopropyl ether and carbon tetrachloride as solvents.The metal carbonyl dendrimer linked to a secondary antibody is a new universal detection reagent that could be of general interest in the field of immunoanalysis.

Experimental Section

Materials: PAMAM G4 dendrimer was purchased from Aldrich. Fp-maleinimide was synthesized as described previously.[37] Goat anti-

rabbit IgG (product no. R3128), rabbit IgG (product no. I5006), goat serum, and β-lactoglobulin were purchased from Sigma. Sephadex LH20 and Superdex 200 gel-filtration media and a Hi Trap fast-desalting ready-to-use column were purchased from Amersham Biosciences. Aqueous solutions of proteins and modified dendrimers were concentrated by ultrafiltration with a Centricon-10 or Centricon-30 device (Millipore–Amicon Bioseparation). Dialysis of crude IgG–dendrimer–Fp conjugates was performed with a Slide-A-Lyzer Dialysis Cassette 10000 MWCO (Pierce). Nitrocellulose membranes (pore size, 0.45 μm) were purchased from Bio-Rad (product no. 162-0117). A peristaltic pump (Minipuls 3, Gilson) was used to maintain a 0.4 mL min^{-1} flow rate during gel-filtration chromatography on a Superdex 200 column. UV/Vis spectra were recorded on a UV/mc^2 spectrometer (Safas).

Buffers: NaPB buffer (10 mM, pH 7.2) contained NaH_2PO_4 (0.2 M, 11 mL), Na_2HPO_4 (0.2 M, 39 mL), and NaCl (0.15 M) diluted with deionized water (final volume, 1 L). NaPB buffer (10 mM, pH 8.0) contained NaH_2PO_4 (0.2 M, 2.6 mL), Na_2HPO_4 (0.2 M, 47.4 mL), and NaCl (0.15 M) in deionized water (final volume, 1 L). KPB buffer (pH 7.2) contained KH_2PO_4 (0.95 g), K_2HPO_4 (3.2 g), and NaCl (3.8 g) in deionized water (500 mL). Ethanolamine·HCl (1 M, pH 9.6) solution was produced by adding ethanolamine (300 μL) to deionized water (5 mL) and adjusting the pH value by addition of concentrated HCl.

FTIR spectroscopy: FTIR spectra were recorded on a bench-top spectrometer (Bomem Michelson MB 100 FT) equipped with a liquid-nitrogen-cooled mercury-cadmium-telluride detector and a membrane holder (6 mm diameter) positioned perpendicular to the beam. FTIR data were manipulated on a PC with the WinBomem-Easy program. Typically, 44 scans were coadded in about 1 min and the resulting interferogram was apodized with a cosine function and then Fourier transformed to yield a 4 cm^{-1} resolution spectrum. The baseline-corrected absorbances were calculated by the Quant method included in the program.

Methods: All experiments were conducted in the dark.

Preparation of G4-Fp_n conjugates: PAMAM G4 (1.43 μmol, 250 μL) in methanol (2 mL) was incubated with various amounts of Fp-maleinimide under the conditions listed in Table 1. The solution was then passed through a 16 mL Sephadex LH 20 column. The organometallic dendrimer was eluted with methanol and fractions were concentrated under vacuum (not to dryness as this process appeared to be damaging).[42] NaPB (600 μL, pH 7.2) was immediately added and the crude product was purified on a 25 mL Superdex 200 column in sample volumes of no more than 200 μL. Labeled dendrimer was eluted with NaPB (pH 7.2) at 0.4 mL min^{-1}. Fractions were pooled and concentrated by ultrafiltration. The concentrated solution of organometallic dendrimer was stored at −20 °C.

Analysis of G4-Fp_n conjugates: The concentration of Fp groups bound to G4 was measured spectroscopically by recording the absorbance at 370 nm ($\varepsilon_{370} = 600\ M^{-1}\ cm^{-1}$). The concentration of dendrimer residual amino groups was assayed by two methods: the TNBS assay[39] and a modified version of a ninhydrine test described by Luo et al.[40] This second assay was carried out as follows: A calibration curve was constructed for G4 (17 nmol mL^{-1}) in ethanol. This solution (80–250 μL) was combined with ninhydrine (750 μL, 0.4 wt% in ethanol) and diluted with ethanol to give a final volume of 1 mL. These standard solutions where heated at 75 °C in a water bath for 5 min without evaporation and cooled before dilution with ethanol (1 mL). The UV absorbance at 590 nm was measured. The G4-Fp_n conjugates were assayed at two dilutions in duplicate.

Preparation of IgG-(G4-Fp_n)$_x$ conjugates: $NaIO_4$ (0.1 M) in citric acid (10 mM, 36 μL) was added to a solution of goat antirabbit IgG (2.46 mg) in NaPB (200 μL, pH 7.2) and the pH value was adjusted to 3.6 with citric acid (10 mM, 124 μL). This solution was incubated for 1 h at RT then quickly purified on a 5 mL Hi Trap fast-desalting column (NaPB, pH 7.2). Oxidized IgG solution was concentrated on a Centricon-30 device and transferred into NaPB (240 μL, pH 8.0). This solution was treated with G4-Fp_n (7 mol equiv) and the pH value was adjusted to 8.0 with Na_2HPO_4 (0.2 M). The resulting solution was incubated for 1 h at RT then $NaBH_3CN$ (2.5 M) in NaPB (20 μL, pH 8.0) was added. The incubation was continued for 1 h at RT, then overnight at 4 °C. Ethanolamine·HCl (50 μL, pH 9.6) was added and the solution was incubated for 2 h at RT then dialyzed against NaPB (pH 7.2) for 24 h at 4 °C. After concentration to 150–200 μL, the crude solution was passed through a 25 mL Superdex 200 column and compounds were eluted at 0.4 mL min^{-1} with NaPB (pH 7.2).

Determination of the ratio G4-Fp_n/IgG: The concentrations of IgG and G4-Fp_n were measured spectroscopically by recording the absorbance of the sample at 280 and 365 nm, respectively. Standard curves of absorbance at 365 and 280 nm were plotted as a function of the quantity of G4-Fp_n and used to determine both the concentration of G4-Fp_n bound to IgG and the contribution made by G4-Fp_n to the absorbance at 280 nm. This contribution was subtracted from the total absorbance to obtain the IgG concentration ($A_{280} = 1.4$ for 1 mg mL^{-1} solution).

Immunological tests on nitrocellulose membranes: Rabbit IgG solutions (0.1–40 μg IgG) were spotted onto punched nitrocellulose disks (6 mm diameter) with the amount of β-LG solution necessary to coat each membrane with the same total amount of protein (40 μg). Two other membranes were coated with β-LG (40 μg), one to provide a reference IR spectrum (reference) and the other for measuring nonspecific interactions (blank). Membranes were air-dried for 45 min then incubated with blocking buffer (KPB + 4% goat serum, 500 μL per membrane) for 90 min at RT. Each membrane was then incubated for 2 h at RT with Fp-labeled goat antirabbit IgG (0.8 nmol) in diluted blocking buffer (KPB + 3% goat serum, 150 μL), except for the reference membrane, which was incubated without antibody. Each membrane was washed three times with KPB + 0.1% Tween 20 (1 mL per membrane, first a fast soaking, then two 5–10 min soakings). The washing step ended with a fast soaking in KPB (1 mL per membrane). The membranes were air-dried for 2 h before their IR spectra were recorded.

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