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Preparation and characterization of poly(amidoamine) dendrimers functionalized with a rhenium carbonyl complex and PEG as new IR probes for carbonyl metallo immunoassay

Jan-Martin Heldt, Nathalie Fischer-Durand, Michèle Salmain, Anne Vessières *, Gérard Jaouen

Ecole Nationale Supérieure de Chimie de Paris, Laboratoire de Chimie Organométallique, UMR CNRS 7576, 11 rue P. et M. Curie, 75231 Paris cedex 05, France

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Abstract

The first poly(amidoamine) (PAMAM) dendrimers tethered with both (η^5-cyclopentadienyl) rhenium tricarbonyl ($CpRe(CO)_3$) units and polyethylene glycol (PEG) chains were prepared and characterized by combining NMR spectroscopy and Fourier-transform IR spectroscopy. Grafting of $CpRe(CO)_3$ units was achieved by reductive amination of formyl-$CpRe(CO)_3$ with the peripheral amines of generation 3 and 4 PAMAMs to yield dendrimers labeled with a variable number of $CpRe(CO)_3$ units, ranging from 8 to 14 for PAMAM-G3 and 17–30 for PAMAM-G4. PEG chains of different lengths were then attached to some of the remaining peripheral amines, and their respective ability to improve the solubility of the metallodendrimers in aqueous buffered media was evaluated. These metallodendrimers represent new infrared probes designed to be coupled to immunological reagents for the amplification of the IR signal in carbonyl metallo immunoassay (CMIA).

Keywords: Dendrimer; Polyethylene glycol; Metal carbonyl; Rhenium; FT-IR spectroscopy

1. Introduction

Highly branched dendritic macromolecules (dendrimers) [1–4] have been extensively used in recent years as new chemical architectures to build well-organized nanostructures. Dendrimers incorporating organometallic moieties at the periphery or within the framework exhibit attractive electrochemical, optical and catalytic properties [5–8]. In the biomedical area, dendrimers have found promising applications for magnetic resonance imaging or boron neutron-capture therapy [9]. In the field of sensors, ferrocenyl-tethered dendrimers have been investigated as immobilized redox species mediating the biocatalytic oxidation of glucose, by glucose oxidase. This formed the basis of the design of glucose [10,11] and biotin biosensors [12,13]. Ferrocenyl-tethered dendrimers have also been designed for the molecular recognition of phosphate ion [14].

Our group has been interested for some time in the potentiality of using dendrimers as carriers of transition metal carbonyl complexes. Our objective aims at the development of new generations of universal detection reagents that allow the amplification, by multiple labeling, of the infrared detection signal in the carbonyl metallo immunoassay (CMIA) we have developed since the early nineties [15–17]. For this purpose, we have recently

* Corresponding author. Tel.: +33 1 44 27 67 29; fax: +33 1 43 26 00 61.
E-mail addresses: natduran@ext.jussieu.fr (N. Fischer-Durand), vessiere@ext.jussieu.fr (A. Vessières).

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described the preparation of the 4th generation poly(amidoamine) (PAMAM-G4) dendrimer tethered with 45 $CpFe(CO)_2$-succinimidato (Fp) units and four biotin entities [18]. More recently, we have reported the conjugation of such PAMAM-G4 bearing 10–25 Fp units with secondary antibodies, following a site-directed multi-labeling procedure involving their glycosylated residues [19]. It is noteworthy that the use of dendrimers to achieve an amplification of the signal level in non-isotopic immunoassays is a relatively new concept [20,21].

We report herein the first synthesis of PAMAM-G3 and G4 dendrimers labeled with (η^5-cyclopentadienyl)rhenium tricarbonyl ($CpRe(CO)_3$) units. These metallodendrimers were characterized following an original method that combines NMR spectroscopy and Fourier-transform IR (FT-IR) spectroscopy in the transmission mode on nylon membrane. In this study, we decided to work with the $CpRe(CO)_3$ entity which is known to be more photochemically stable than the previously used Fp entity. Moreover, we have experimentally shown that the rhenium complex exhibits a higher absorbance of its analytical IR band than the iron-carbonyl complex at the same concentrations. Thus, amplification of the IR signal should be higher with $CpRe(CO)_3$-labeled dendrimers than with Fp-labeled dendrimers. We also describe the attachment of polyethylene glycol (PEG) moieties of various chain lengths to some of the remaining amino end groups of the dendrimers surface. Their abilities to improve the solubility of the metallodendrimers in aqueous media are presented.

2. Experimental

2.1. General

PAMAM-G3 and G4 dendrimers, methoxy poly(ethylene glycol) (MW = 350, 750) (mPEG), 4-fluoro-3-nitrobenzoic acid, triethylamine (Et_3N), $NaBH_4$, 1,3-dicyclohexyl-carbodiimide (DCC) and 4-dimethylamino-pyridine (DMAP) were purchased from Sigma-Aldrich. Compounds **2a**–**b** were prepared from 4-fluoro-3-nitrobenzoic acid and mPEGs as described previously [22,23]. $CpRe(CO)_3$ was synthesized according to a previously published procedure [24]. Sephadex LH 20 was from Amersham Biosciences. Flash chromatography was performed on silica gel 60 (Merck, 40–63 μm). About 0.2 μm pore size Nalgene nylon membranes were purchased from VWR International. NMR spectra were recorded on BRUKER AC 200, BRUKER Avance 300 and BRUKER Avance 400 spectrometers. UV–Vis spectra were recorded on a UV/mc^2 spectrometer (Safas). 10 mM NaPB buffer pH 7.2 contained NaH_2PO_4 0.2 M (11 mL) and Na_2HPO_4 0.2 M (39 mL) diluted to 1 L of deionised water with 0.15 M NaCl.

2.2. FT-IR spectroscopy

FT-IR spectra were recorded on a bench-top MB 100 spectrometer (Bomem) equipped with a liquid nitrogen-cooled MCT detector, and a 6-mm diameter membrane holder perpendicularly positioned to the beam. FT-IR data were recorded and manipulated on a Windows-operating PC using the WinBomemEasy software. Routinely, 44 scans were coadded in about 1 min and the resulting interferogram was apodized using a cosine function and then Fourier-transformed to yield a 4-cm^{-1} resolution spectrum. The baseline corrected absorbances were measured using the Quant method included in the software.

The calibration curve used for the quantification of the metallodendrimers was established as follows:

Standard solutions of $CpRe(CO)_3$ in methanol in the range 6–1.5 nmol per membrane were spotted (5 μL) onto punched nylon membranes (diameter = 6 mm). Membranes were dried for 20 min at 37 °C before IR recording. The calibration curve was constructed by plotting absorbance at 2019 cm^{-1} versus quantity of complex spotted onto the membrane. This experiment was repeated several times and gave reproducible standard straight lines.

2.3. Synthesis of (η^5-cyclopentadienyl carboxaldehyde) rhenium tricarbonyl (*1*)

Compound **1** was prepared following a modification of the procedure described by Kolobova et al. [25]:

$CpRe(CO)_3$ (0.5 g, 1.49 mmol) was dissolved under argon in anhydrous THF (5 mL) in a Schlenk tube. The flask was cooled at −50 °C, and *t*-BuLi 1.7 M in pentane (936 μL, 1.07 equiv.) was slowly added. The flask was stirred 15 min at −50 °C, then cooled to −70 °C. Freshly distilled DMF (288 μL, 3.73 mmol) was then slowly added, and the reaction mixture was stirred for 4 h at −5 °C. The reaction was quenched with saturated aqueous ammonium chloride (3 mL). The aqueous phase was extracted with diethyl ether (4 × 5 mL) and dried ($MgSO_4$). Column chromatography on silica gel (eluent diethyl ether: petroleum ether, 80:20) afforded compound **1** as an off-white powder (427 mg, 79%); m.p. = 90 °C (lit. 91 °C); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.4 (t, 2H, J = 2.1 Hz, α-Cp); 5.94 (t, 2H, J = 2.3 Hz, β-Cp); 9.52 (s, 1H, CHO).

2.4. General procedure for the labeling of PAMAM G4 or PAMAM G3

2.4.1. Example of preparation of $G4Re_{30}$

PAMAM-G4 (200 μL, 1.144 μmol, 10% solution in methanol) was placed under argon in a round bottom flask. HCl 37% (3 μL) was added, followed by the slow addition of compound **1** (12.5 mg, 30 equiv.) in metha-

nol (1 mL). The solution was stirred for 2 h, then $NaBH_4$ (4.7 mg, 0.124 mmol) was added. The reaction mixture was stirred for 1 h, then concentrated to ~200 μL under vacuum. This crude solution was passed through a 16-mL LH 20 column and eluted with methanol. Fractions containing the metallodendrimer (detection by UV at 257 nm) were pooled and concentrated to ~700 μL. The colorless solution of $G4Re_{30}$ was stored at −20 °C.

^{1}H NMR (400 MHz, CD_3OD) δ 2.3–2.4 (broad m, 248H, CH_2CO); 2.6 (broad m, 126H, CH_2N); 2.8 (broad m, 374H, CH_2N); 3.3 (broad m, 314H, CH_2NHCO + MeOH + CH_2NHCH_2Cp); 3.53 (s, 66H, CH_2Cp); 5.48 (s, 60H, β-Cp); 5.61 (s, 60H, α-Cp)⇒30 $CpRe(CO)_3$/G4.

^{13}C NMR (100 MHz, CD_3OD) δ 34.82; 38.71; 39.92; 41.98; 47.22; 51.18; 53.53; 85.61; 85.91 (α,β-Cp); 109.01 (CH_2–Cp); 174.67 (CONH); 175.03 (CONH); 175.23 (CONH); 195.78 (C≡O).

$CpRe(CO)_3$ concentration was determined by FT-IR using the calibration curve established in Section 2.2, by deposition of 5 μL of the metallodendrimer solution in duplicate on nylon membranes. PAMAM-G4 concentration was then deduced from the found value and the coupling rate evaluated by ^{1}H NMR.

2.5. General procedure for the PEGylation of metallodendrimers

2.5.1. Example of PEGylation of $G4Re_{29}$ with Compound 2b

$G4Re_{29}$ (93 nmol) in a methanol solution was placed in a flask and the solvent was evaporated under vacuum. DMF (200 μL) was immediately added, and argon was bubbled into the flask. Compound **2b** (0.726 mg, 9 equiv.) and Et_3N (1.2 μL, 10 equiv.) were added and the resulting solution was stirred overnight at room temperature. The solution was diluted with methanol (400 μL) and passed through a 16-mL Sephadex LH-20 column with methanol as the eluent. Fractions containing the double-labeled dendrimer (detection by UV at 257 nm) were pooled and concentrated to ~200 μL. The bright yellow solution of $G4Re_{29}PEG_4$ was stored at −20 °C.

The concentration of mPEG was determined by colorimetric measurement at 428 nm (nitroaniline chromophore, $\varepsilon = 5450\ M^{-1}\ cm^{-1}$) [23]. The concentration of the double-labeled dendrimer was determined by IR-FT on nylon membrane, as described above (Section 2.4).

2.6. Solubility test

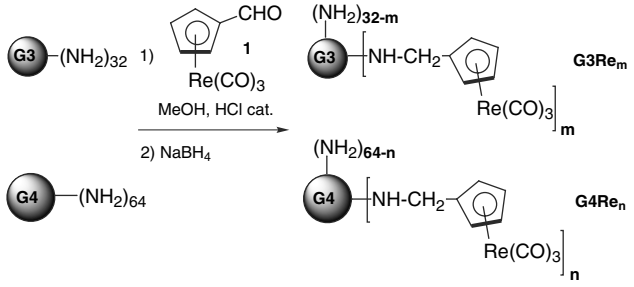
The solubility of the metallodendrimers in water or 10 mM NaPB pH 7.2 was estimated by dropping 5 μL of each sample in 1 mL of water or buffer, and by examining visually the clearness of the resulting solution. Results are given in Table 2.

3. Results and discussion

3.1. Synthesis and characterization of metallodendrimers

Partial functionalization of the surface primary amines of PAMAM dendrimers (32 for PAMAM-G3 and 64 for PAMAM-G4) was achieved following the reductive amination procedure with formyl-$CpRe(CO)_3$ **1** in the presence of the reductant $NaBH_4$ (Scheme 1). The crude product was purified by lipophilic gel-permeation chromatography on Sephadex LH-20 in methanol to separate the metallodendrimer from excess organometallic complex.

First, the average number of organometallic complexes attached per dendrimer was evaluated by ^{1}H NMR spectroscopy in CD_3OD. Fig. 1 shows the typical ^{1}H NMR spectra for commercial PAMAM-G4 (a) and PAMAM-G4 labeled with $CpRe(CO)_3$ (b). The four Cp protons of the organometallic complex were easily identified as two signals at 5.5 and 5.6 ppm, and the two methylene protons next to the Cp appeared as an intense sharp peak at about 3.5 ppm. Fortunately, these characteristic signals of the rhenium complex appeared at chemical shifts well separated from those of the dendrimer, which allowed to use the integrated areas of NMR peaks to quantify the number of rhenium complexes grafted per PAMAM dendrimer. With the assumption that the broad signal at 2.3–2.4 ppm corresponds to the 248 methylene protons next to the carbonyl groups (Fig. 2) as previously assigned in the literature [26], the integral ratio of this signal to the signal at 5.5 or 5.6 ppm which corresponds to two protons of the Cp led to an average number of 30 $CpRe(CO)_3$ units per PAMAM dendrimer for this example. This conjugate was named $G4Re_{30}$. Several $G3Re_x$ and $G4Re_x$ samples were synthesized by reacting PAMAM-G3 and PAMAM-G4 with various molar equivalents of **1** (Table 1). As shown, the coupling rate was almost quantitative in the range of molar equivalents of **1** per PAMAM-G3 or G4 studied (11–15 per PAMAM-G3 and 20–30 per PAMAM-G4), which means that we readily can control the number of metal carbonyl entities grafted per dendrimer.



Scheme 1. Labeling of PAMAM-G3 or G4 dendrimers with compound **1**.

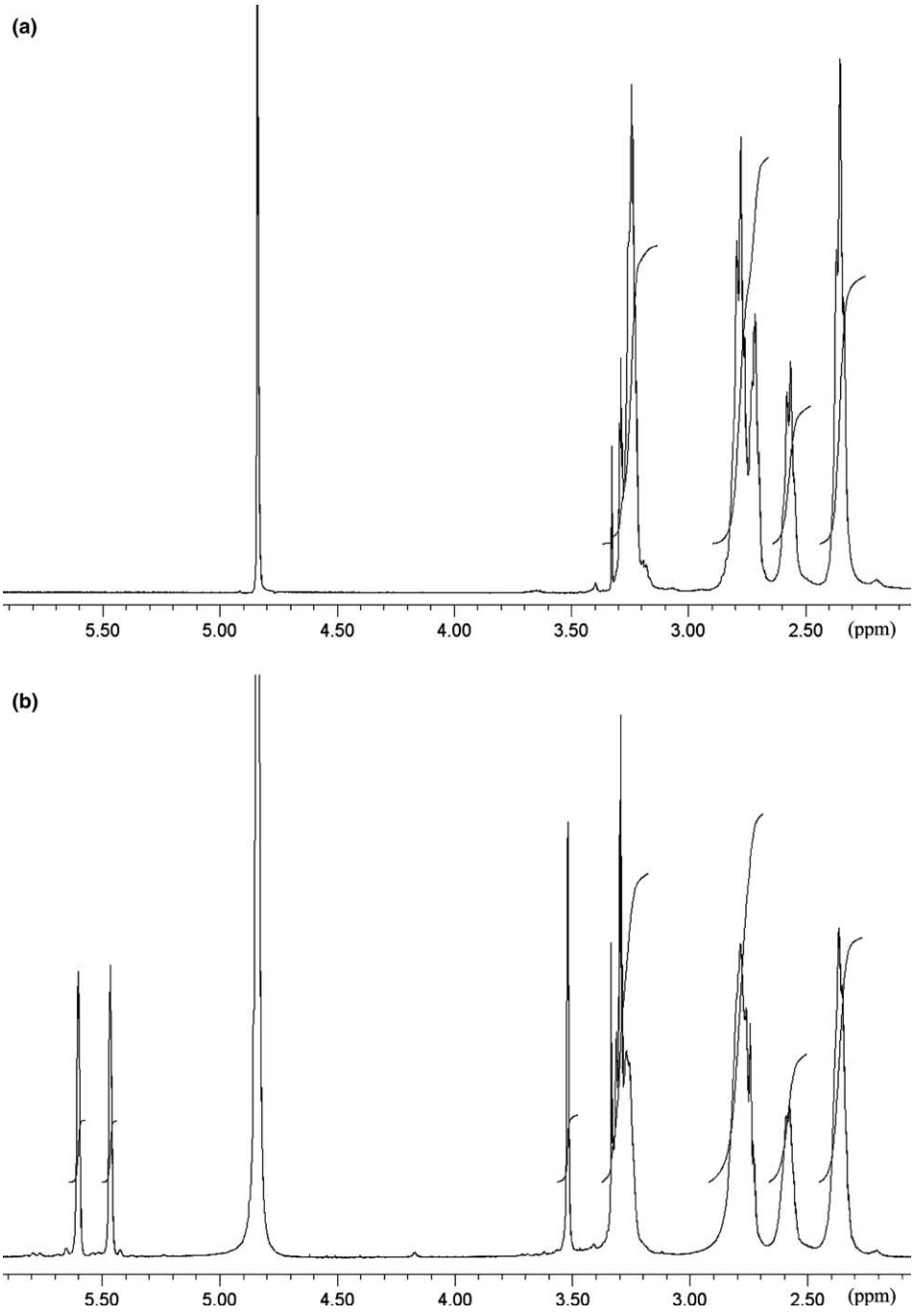


Fig. 1. ^{1}H NMR spectra for PAMAM-G4 dendrimer (a), and PAMAM-G4 labeled with $CpRe(CO)_3$ (b) in CD_3OD.

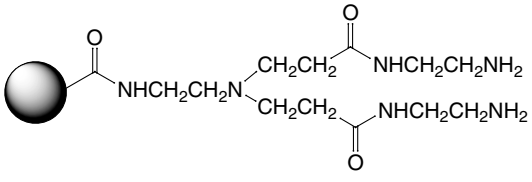


Fig. 2. Chemical structure of the outer part of a branch of PAMAM dendrimer.

These compounds must be kept in solution (here methanol) to maintain their stability, since we and others [27] have experienced the degradation of dendrimers when they were stored without solvent. Consequently, the concentration of the metallodendrimers in the samples has to be determined before using them in the next step of the synthesis. In principle, this could be achieved either by measuring the concentration of the remaining free amino groups on the dendrimer surface, or by measuring the concentration of the organometallic complex.

Table 1
Synthesis of $CpRe(CO)_3$-attached dendrimers

Generation of PAMAM dendrimer	**1**/dendrimer initial ratio (mol/mol)	Number of $CpRe(CO)_3$ units bound per dendrimer[a]	Coupling rate[b] (%)
G3	10	8	80
G3	11	10	91
G3	15	13	87
G3	15	14	93
G4	20	17	85
G4	25	21	84
G4	30	29	97
G4	30	30	100

[a] Determined by ^1H NMR.
[b] Coupling rate = column 3/column 2.

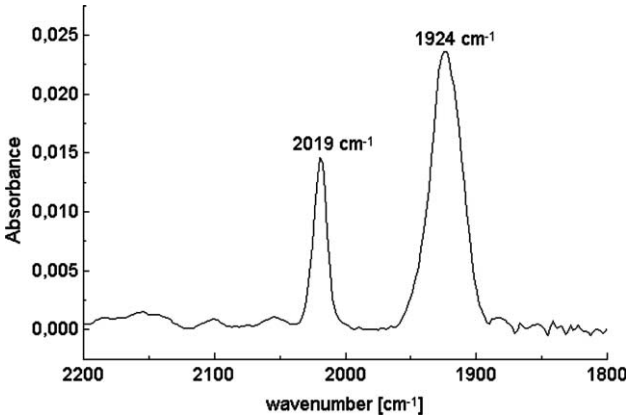


Fig. 3. FT-IR spectrum of $G3Re_8$ (0.26 nmol) on nylon membrane in the 1800–2200 cm^{-1} spectral range.

The dendrimer concentration could then be easily deduced by combining this result with the coupling rate evaluated by ^1H NMR spectroscopy (see above). Colorimetric assays such as the trinitrobenzene sulfonic acid (TNBS) assay [28] or the ninhydrin assay [29,30], which are commonly used to determine the concentration of free amino groups in proteins or dendrimers, gave results that were not satisfactory. Indeed, we and others [30] have also experienced that some free amines can be shielded and do not react with TNBS or ninhydrin depending on the steric hindrance generated by the molecules attached to the surface of the dendrimer, resulting in underestimation of the free amine concentration. Consequently, it seemed more judicious to determine the concentration of the organometallic complex. In UV–Vis, $CpRe(CO)_3$ shows a maximum of absorption at 257 nm where the dendrimer also absorbs, which precludes simple quantification of the rhenium complex, and thus the PAMAM dendrimer, by UV measurement. On the other hand, taking advantage of the specificity of the transition metal carbonyl complexes that display intense absorption bands in the mid-infrared spectral range (1800–2200 cm^{-1}) where few other vibrators absorb, we have previously demonstrated that peak heights were proportional to the quantity of complex in solution [16]. This was the basis of the development of the CMIA method [17]. More recently we have also shown that Fp-labeled immunoconjugates could easily be quantified by infra-red spectroscopy on nitrocellulose membranes, by deposition of aqueous solutions and drying in air [19]. In the present study, quantitative analysis of $CpRe(CO)_3$ was successfully achieved by FT-IR spectroscopy in the transmission mode, using for the first time nylon membranes. This type of membrane, compatible with organic solvents, was chosen since the metallodendrimers were stored in methanol. In the 1800–2200 cm^{-1}, the IR spectrum of $G3Re_8$ displays two intense bands at 2019 and 1924 cm^{-1} that are characteristic of the a_1 and e vibration modes of the $CpRe(CO)_3$ moiety (Fig. 3). A calibration curve was constructed by spotting known quantities of a cyclopentadienyl rhenium$(CO)_3$ derivative on nylon membranes and plotting the absorbance of the narrow a_1 vibration mode band as a function of quantity of complex. The choice of $CpRe(CO)_3$ as a standard compound was justified by the position of its analytical band at 2021 cm^{-1}, close to that of the metallodendrimer. Fig. 4 shows the typical calibration straight line of $CpRe(CO)_3$ in the range 6–1.5 nmol per membrane. The linear regression was then used to determine the concentration of $CpRe(CO)_3$ entities in the $CpRe(CO)_3$-labeled dendrimer solutions. By combining, for the first time in the PAMAM series, ^1H NMR spectroscopy and direct IR transmission measurement, it was thus possible to evaluate the number of $CpRe(CO)_3$ attached per dendrimer, as well as the concentration of the metallodendrimer in the final solution.

At this stage, the ability of these metallodendrimers to be used as IR probes for quantitative analysis was tested. Known quantities of $G4Re_{30}$ in the range

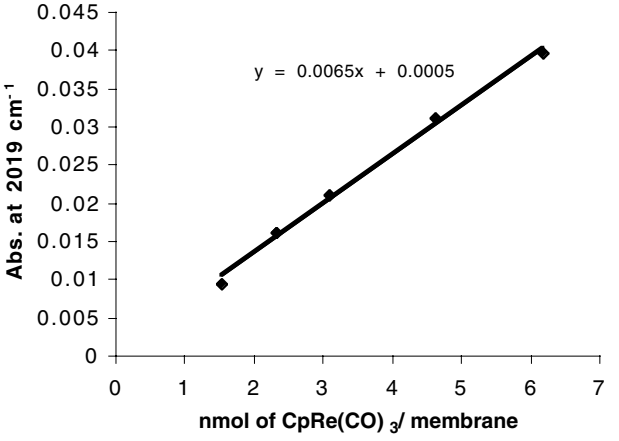


Fig. 4. FT-IR calibration curve of $CpRe(CO)_3$ in the range 6–1.5 nmol, on 6 mm-diameter nylon membranes (5 μL per membrane, solutions in methanol).

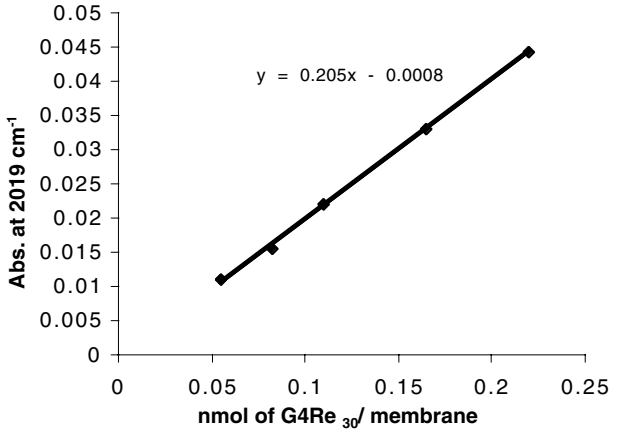


Fig. 5. FT-IR calibration curve of $G4Re_{30}$ in the range 0.055–0.22 nmol, on 6 mm-diameter nylon membranes (5 μL per membrane, solutions in methanol).

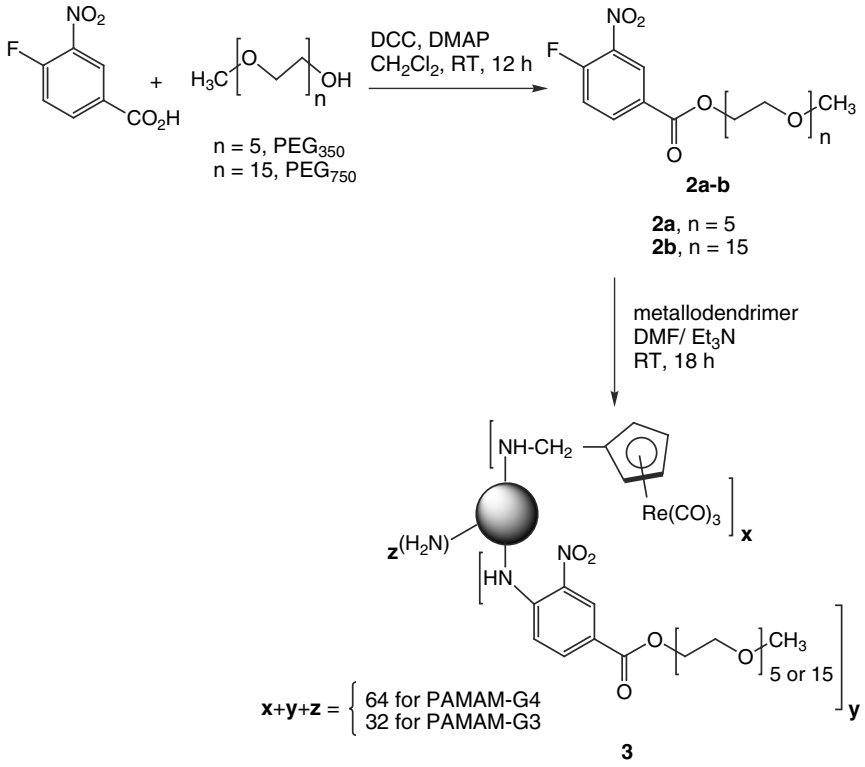
0.055–0.22 nmol (5 μL of methanol solution) were deposited on nylon membranes, and IR spectra were recorded. This experiment is summarized in Fig. 5. The linear relationship observed between the quantity of $G4Re_{30}$ spotted on membranes and the absorbance of the carbonyl band at 2019 cm^{-1} clearly demonstrates that these metallodendrimers could be used as new probes for quantitative analysis by FT-IR spectroscopy.

3.2. Grafting of PEG units to improve aqueous solubility

Our first objective was reached with the amplification of the IR signal by the multi-labeling of dendrimers with $CpRe(CO)_3$ units. However, we need not only a high number of rhenium carbonyl complexes attached to the dendrimers, but also a good solubility in buffer media for their further coupling to biomolecules such as avidin or antibodies. Despite the high hydrophilicity of PAMAMs, tethering of small hydrophobic $CpRe(CO)_3$ entities [31] led to metallodendrimers with poor solubilities in aqueous and buffered media. Here, we have observed that the limit of solubility in water was reached with about 10 $CpRe(CO)_3$ per PAMAM-G3, and 17 $CpRe(CO)_3$ per PAMAM-G4. By comparison, PAMAM-G4 labeled with 25–30 Fp entities were still soluble in aqueous and buffer media [19]. To solve this problem, PEGylation of the metallodendrimers was undertaken. Indeed, one of the reported benefits of PEG modification of molecules is the improved aqueous solubility of the resulting conjugates thanks to the strong association between water molecules and ethylene oxide units [32,33]. PEGylation was achieved by attachment of suitable PEG chains to a fraction of the remaining peripheral amines. To avoid crosslinking, monomethoxy-polyethylene glycols (mPEGs) were chosen. Two mPEGs with molecular weights of 350 and 750 were selected to evaluate the impact of the chain length on the solubility of the PEGylated dendrimer. PEGs with higher molecular weights were not tested to limit the steric hindrance, and as a result, the shielding of the remaining primary amines at the surface of the dendrimer that could prevent further coupling reaction [34].

First, 4-fluoro-3-nitrobenzoic acid was coupled to the hydroxyl end group of mPEGs in the presence of a catalytic amount of DMAP, to yield esters **2a**–**b** (Scheme 2) as previously reported [22,23]. Covalent attachment of activated mPEGs to the metallodendrimers proceeded by aromatic nucleophilic substitution of the fluorine of esters **2** by some of the surface amines of PAMAM yielding chromophoric *o*-nitroaniline derivatives **3**. The dendrimers were then purified by lipophilic gel permeation chromatography as mentioned above. The quantification of bound mPEG was performed by measuring the absorbance of the chromophore at 428 nm [22,23]. By combining this spectrophotometric quantification of mPEG and direct IR transmission measurement of the organometallic complex, these dendrimers bearing a double labeling were fully characterized. Results are listed in Table 2.

Solubility was then evaluated by dropping 5 μL of the labeled dendrimer in 1 mL of water or buffer, and by examining visually the clearness of the resulting solution. With $mPEG_{350}$, a number as high as 21 was necessary to obtain solubility in water with G4 bearing 25 $CpRe(CO)_3$ (entry 1) corresponding to the substitution of 46 out of 64 surface amines. On the other hand, eight $mPEG_{350}$ were not sufficient to allow aqueous solubility for G3 labeled with 14 $CpRe(CO)_3$ units (entry 2). None of them were soluble in buffered media. PEGylation with $mPEG_{750}$ proved to be much more efficient. A number as low as four was sufficient to obtain solubility in water with $G4Re_{29}$ (entry 3). Solubility in buffer (NaPB 10 mM pH 7.2) was reached with 9 $mPEG_{750}$ for $G4Re_{24}$ (entry 4), which means that 31 surface amines are still available for further reaction. In the G3 series, 3 $mPEG_{750}$ did not improve the solubility of $G3Re_{13}$ in aqueous media (entry 5). Five $mPEG_{750}$ allowed its solubility in water (entry 6), and solubility in buffer pH 7.2 was reached with 7 $mPEG_{750}$ (entry 7). Therefore, 12 amines remain still available on the G3 surface. This experiment clearly demonstrated the positive effect of the conjugation of a PEG with a medium chain length on the solubility of PAMAM dendrimers bearing hydrophobic organometallic compounds. More PEG chains were needed to reach solubility in buffered media. This different behavior between water and buffer is probably due to a salting out effect. A good compromise was found between the number of $CpRe(CO)_3$ labels to reach a high IR signal level, and the number of PEG chains added to maintain solubility in buffered media, allowing enough remaining surface amines for further reaction with immunological reagents. This balance was reached with 24 Re complexes and 9 PEG_{750}



Scheme 2. Synthetic route to PEGylated metallodendrimers.

Table 2
PEGylation of the metallodendrimers and solubility of the resulting conjugates

Entry	Metallodendrimer	Number of PEG bound per dendrimer		Number of remaining surface NH_2	Solubility	
		PEG_{350}	PEG_{750}		Water	Buffer pH 7.2
1	$G4Re_{25}$	21		18	Yes	No
2	$G3Re_{14}$	8		10	No	No
3	$G4Re_{29}$		4	31	Yes	No
4	$G4Re_{24}$		9	31	Yes	Yes
5	$G3Re_{13}$		3	16	No	No
6	$G3Re_{13}$		5	14	Yes	No
7	$G3Re_{13}$		7	12	Yes	Yes

for the PAMAM G4, and with 13 Re complexes and 7 PEG_{750} for the PAMAM G3.

4. Conclusion

Our two objectives were reached. First, we have increased the IR signal by the multi-labeling of dendrimers with $CpRe(CO)_3$ units. Eight to fourteen $CpRe(CO)_3$ were attached to G3, and G4 was labeled with seventeen to thirty $CpRe(CO)_3$. The coupling rate was evaluated by ^1H NMR spectroscopy, and the concentration of these metallodendrimers were evaluated following an original method conbining FT-IR spectroscopy in the transmission mode on nylon membrane and ^1H NMR spectroscopy. The calibration curve obtained with one of these metallodendrimers demonstrates their ability to be used as new probes for quantitative analysis by FT-IR spectroscopy. Second, these hydrophobic metallodendrimers were then transformed into hydrophilic conjugates by attaching suitable PEG_{750} chains, which allowed solubility in buffer pH 7.2 while allowing enough free amino end groups on the surface for further reaction. Coupling of these metallodendrimers with immunological reagents is now under investigation.

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