# Controlled Functionalisation of Gold Nanoparticles with Mixtures of Calix[4]arenes revealed by Infra-red Spectroscopy

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#### Abstract

Labile ligands such as thiols and carboxylates are commonly used to functionalise AuNPs, though little control over the composition is possible when mixtures of ligands are used. It was shown recently that robustly functionalised AuNPs can be obtained through the reductive grafting of calix[4]arenes bearing diazonium groups on the large rim. Here, we report a calix[4]arene-tetra-diazonium decorated by four oligo(ethylene glycol) chains on the small rim, which upon grafting gave AuNPs with excellent stability thanks to the C-Au bonds. Mixtures of this calixarene and one with four carboxylate groups were grafted on AuNPs. The resulting particles were analysed by infra-red spectroscopy, which revealed that the composition of the ligand shell clearly reflected the ratio of calixarenes that was present in solution. This strategy opens the way to robustly protected AuNPs with well-defined numbers of functional or postfunctionalisable groups.

# Introduction

Gold nanoparticles (AuNPs) have found applications in many different fields, ranging from electronics<sup>1,2</sup> and catalysis<sup>3</sup> to sensing<sup>4</sup> and nanomedicine,<sup>5,6</sup> thanks to their remarkable electronic and optical properties.<sup>7,8,9,10</sup> For these applications, nanoparticles are usually conjugated with organic ligands. The first purpose of these ligands is to ensure the stability of the AuNPs in the medium (typically organic solvent or aqueous solution) used for their processing or for their application in suspension, preventing the aggregation of the particles by electrostatic or steric repulsion (Figure 1a). Secondly, organic ligands can be used to introduce additional functionalities on the AuNPs. This requires ligands that have both a group to bind to the gold surface (*e.g.,* carboxylate, amine, phosphine, or thiol groups)<sup>11,12</sup> and a functional or post-functionalisable group.<sup>13</sup> Examples are AuNPs functionalised with peptides or antibodies for the detection of proteins or viruses<sup>14,15</sup> and elaborate supramolecular architectures built from AuNPs with DNA.<sup>16,17</sup>

For many of such applications control over the density of the functional groups on the particle surface is essential.<sup>18</sup> Molecular sensors based on the aggregation of AuNPs for the detection of biomolecules will only work in the adequate dynamic range if the number of functional groups present at their surface matches the analyte concentration.<sup>15,19</sup> Too low densities of functional groups will lead to saturation of the AuNPs with the analyte, whereas too high levels of functionalisation could prevent binding of the analyte or give uncontrolled aggregation (Figure 1b). In the case of Super-Paramagnetic Iron Oxide Nanoparticles (SPIONs) too, it has been shown that the surface density of ligands is an essential parameter that can affect the fate and excretion of these MRI contrast agents in the body.<sup>20</sup>

Since full coverage of the surface of AuNPs is required to ensure their stability (Figure 1c),<sup>21</sup> the main way to control the number of functional groups is by using mixed monolayers of ligands (Figure 1d). When citrate-protected AuNPs are used, a functionalised thiol can be added in small amounts to obtain a mixed layer of citrate and thiols (often with a limited stability of the AuNPs) or a mixture of two thiol-terminated compounds can be added.<sup>13</sup> Alternatively, simple thiols grafted on gold can be partially replaced by functionalised thiols, taking advantage of the dynamic nature of the S-Au bond.<sup>22,23</sup> This reversible exchange process will cause the systems to reach a thermodynamic equilibrium.<sup>8</sup> However, working with mixtures of ligands could result in the absorption of the strongest binding ligand only<sup>23</sup> or in the phase separation of the two ligands at the surface of the nanoparticles,<sup>24,25</sup> instead of the formation of a mixed monolayer. Hence, the resulting ratio of ligands is often unknown<sup>26,27</sup> and cannot be readily quantified without releasing the ligands from the surface,<sup>28,29</sup> especially when AuNPs larger than a few nm are used.<sup>30,31</sup> The aim of the current work is to develop a new class of organic ligands that allows controlling the composition of mixed monolayers on AuNPs.



**Figure 1.** Schematic representations of AuNPs (a) functionalised with stabilising ligands, (b) fully covered with functional ligands, (c) partially covered with functional ligands, limiting their colloidal stability, (d) covered with a mixed monolayer of two different ligands.

An alternative to the labile binding of carboxylates, amines, and thiols to gold surfaces is the grafting via covalent C-Au bonds.<sup>32,33,34,35</sup> These can be obtained using organic ligands bearing an aryl-diazonium group.<sup>36</sup> This group can be reduced *in situ*, generating an highly active aryl radical that readily reacts with the gold surface.<sup>37,38</sup> A drawback of this method is that it often leads to the formation of multilayers, because the generated aryl radicals easily react with the already grafted aryl groups.<sup>37,38</sup> However, this can be prevented by using calix[4]arene-tetra-diazonium compounds whose structure and geometry do not allow any additional grafting.<sup>39,40</sup> These cone-shaped macrocycles can be grafted via multiple C-Au bonds, leading to a remarkably robust ligand shell on surfaces and on AuNPs.<sup>39,41</sup> Furthermore, because the grafting of these calix[4]arenes via C-Au bonds is irreversible, homogeneous mixtures of calixarenes can be grafted.<sup>42</sup> On gold surfaces, the ratio between two different calixarenes grafted on the surface reflects the one in the solution.<sup>43</sup> Additionally, the use of calix[4]arenes with carboxylates allows the post-functionalisation of the surfaces to which it is grafted.<sup>39,41,43</sup>

Here we report the grafting of mixtures of two different calix[4]arenes on AuNPs via their diazonium salts (Scheme 1). The newly synthesised calixarene (2) bears four

oligo(ethylene glycol) groups on their small rim while the second one (1) bears four carboxylates. AuNPs functionalised with these calixarenes and mixtures thereof are extremely robust and form very stable dispersions in aqueous media. The ratios of the two calixarenes at the surface of the AuNPs were determined by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. Even though infra-red spectroscopy has been used to characterise ligands on AuNPs for several decades,<sup>44,45,46</sup> the method is not often used to quantitatively study mixtures of ligands grafted on nanoparticles.<sup>47</sup> The obtained ratios show a clear trend with the fractions of the calixarenes present in the grafting solution, validating this strategy for the formation of a ligand shell with controlled composition.



Scheme 1. Schematic representation of the grafting of tetra-diazonium compounds  $calix(CO_2^{-})_4(N_2^{+})_4$  1 and  $calix(oEG)_4(N_2^{+})_4$  2 on citrate-protected AuNPs in aqueous solutions. Six different ratios were used, varying from pure 2 (0/100) to pure 1 (100/0).

# **Experimental Section**

#### Grafting of calixarenes on AuNPs

The synthesis of calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub>(N<sub>2</sub><sup>+</sup>)<sub>4</sub> **1** was achieved according to previously reported procedures.<sup>39,41</sup> Calixarenes calix(oEG)<sub>4</sub>(N<sub>2</sub><sup>+</sup>)<sub>4</sub> **2a,b** were synthesised as outlined in Scheme 2 and detailed in the Supplementary Information. AuNPs were synthesized by the reduction of KAuCl<sub>4</sub> with citrate using a modified Turkevich method and dialyzed against a 1 mM solution of sodium citrate.<sup>48,49,15</sup> TEM characterisation showed mainly spherical AuNPs with an average core diameter of 17 nm. Aqueous solutions were prepared either in Millipore or in Lichrosolv water and all procedures were carried out at room temperature.

The grafting procedures used here are similar to the one we have reported previously to graft  $calix(CO_2^{-})_4(N_2^{+})_4$  **1** on AuNPs.<sup>41</sup> Compounds **1** and **2a** were dissolved in water to obtain solutions with a concentration of 10 mM. Mixtures of these solutions were prepared as specified in Table 1.

Sample	$calix(CO_2^-)_4(N_2^+)_4 1 (10 \text{ mM})$	calix(oEG) <sub>4</sub> (N <sub>2</sub> <sup>+</sup> ) <sub>4</sub> <b>2</b> (10 mM)	Total
0/100	0 μL	150 μL	150 μL
5/95	7.5 μL	142.5 μL	150 μL
10/90	15 μL	135 μL	150 μL
25/75	37.5 μL	112.5 μL	150 μL
50/50	75 μL	75 μL	150 μL
100/0	150 μL	0 µL	150 μL

Table 1. Mixtures of calixarene solutions used in grafting.

Gold nanoparticles (17 nm, 600  $\mu$ L, 24 nM, 14 pmol) were placed in 1.5 mL LoBind Eppendorf tubes with a 4 mm stir bar and an aqueous solution of NaBH<sub>4</sub> (7.5  $\mu$ L, 0.1 M, 0.75  $\mu$ mol) was added. Six of these tubes were prepared and, to each, a calixarene solution (150  $\mu$ L, 10 mM, 1.5  $\mu$ mol) was added over the course of 1 minute upon vigorous stirring, resulting in effervescence of the red colloidal suspension. The tubes were closed after 30 minutes and stirred gently for 16 hours, after which the stir bars were removed and water (750  $\mu$ L) was added. The samples were centrifuged for 30 min at 18000 g, after which the supernatant was removed and the AuNPs were resuspended in NaOH solution (1.5 mL, 10 mM). This process was repeated 3x with NaOH solution (1.5 mL, 1 mM) and 2x with water (1.5 mL). At the end of the final cycle, the AuNPs were suspended in water (600  $\mu$ L) and stored at room temperature for further characterisation.

#### **IR Spectroscopy**

ATR-FTIR spectra were recorded at 20°C, on a Bruker Equinox 55 spectrophotometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride (MCT) detector. The gold nanoparticles were deposited on a germanium internal reflection element (triangular prism of 6.8 x 45 mm, with an internal incidence angle of 45°, ACM France) as follows: 1  $\mu$ L AuNPs dispersion (~ 25 nM, optical density 10, in 0.1 mM NaOH) was placed on the Ge crystal and the water was removed with a flow of nitrogen gas. This procedure was repeated twice so that three 1  $\mu$ L droplets were superimposed on the Ge crystal. Opus software (4.2.37) was used to record 128 scans with a resolution of 2 cm<sup>-1</sup>, under a continuous flow of nitrogen gas over the sample. Data were processed and analysed using the "Kinetics" software<sup>50,51</sup> in MatLab 7.1 (Mathworks Inc, Natick, MA) by subtraction of water vapour, baseline correction, apodisation at 4 cm<sup>-1</sup>, and flattening of the CO<sub>2</sub> signal. Finally, the spectra were normalised at 1459 cm<sup>-1</sup> (aromatic ring stretch band from the calixarenes) to compensate for variations in the number of AuNPs present on the spot at the Ge crystal where the measurement was performed.

### Further optical characterisation of the AuNPs

Electronic absorption spectra were recorded with a Shimadzu UV3600 spectrophotometer in disposable semi-micro cuvettes (PMMA). AuNPs were diluted to obtain 1-2 nM dispersion in 1 mL aqueous solution, depending on the experiment.

Samples were characterised by dynamic light scattering (DLS) using a Malvern Zetasizer NanoZS (Malvern, UK) with back scattering (NIBS 173°). Measurements were performed at 25 °C, using a refractive index of 1.54 for the gold nanoparticles. AuNPs (10  $\mu$ L, ~25 nM) were dispersed in 0.1 mM NaOH to obtain 1 mL of AuNPs (~0.25 nM). DLS was performed on 450  $\mu$ L of these diluted AuNPs in disposable semi-micro cuvettes (PMMA). The reported values are the average hydrodynamic diameters obtained from three independent measurements, using the Z-average as calculated by the Zetasizer software.

## **Results & Discussion**

#### Synthesis

To challenge the hypothesis that calix[4]arenes tetra-diazonium compounds are ideal candidates to control the grafting densities of mixed layers of ligands at the surface of nanoparticles, AuNPs were functionalized with two different calixarenes. As aqueous dispersions of AuNPs are desired for most biomedical applications, calix[4]arenes decorated with hydrophilic groups were chosen. Calix[4]arene( $CO_2^-$ )<sub>4</sub>(N<sub>2</sub><sup>+</sup>)<sub>4</sub> **1** was selected because its grafting on AuNPs affords extremely stable aqueous dispersions.<sup>41</sup> Regarding the second calixarene, we decided to use a compound bearing oligo(ethylene glycol) (oEG) chains on the small rim to ensure water compatibility and steric stabilisation of the AuNPs. Ethylene glycol chains are furthermore the gold standard stealth polymer used to ensure biocompatibility and increased circulation lifetime in the blood to nanocarriers.<sup>5,8,13</sup> These chains were introduced by an amide coupling on calixarene tetra-acid **3**, which is also an intermediate in the synthesis of **1** (Scheme 2).<sup>39</sup> This modification of the calixarene via an amide coupling could also be readily used to introduce a wide range of other amines onto the calix[4]arene scaffold.

After the introduction of the oEG chains at the small rim of the calix[4]arene, target tetra-diazonium compound **2** was obtained by two subsequent functional group modifications at the large rim. First, the nitro groups of the functionalised calixarene **4** were reduced by SnCl<sub>2</sub>, affording tetra-amine **5** in 95% yield. Secondly, the amine groups were converted into diazonium groups either by using NOBF<sub>4</sub> in acetonitrile (**2a**), in analogy to the synthesis of **1** that we described previously,<sup>41</sup> or using NaNO<sub>2</sub> in aqueous solution at acidic pH (**2b**).



Scheme 2. Synthesis of calix(oEG)<sub>4</sub>( $N_2^+$ )<sub>4</sub> 2a,b.

#### Grafting calix(oEG)<sub>4</sub> on AuNPs

Similarly to the grafting of **1** on AuNPs, calix(oEG)<sub>4</sub>(N<sub>2</sub><sup>+</sup>)<sub>4</sub> **2a** (BF<sub>4</sub><sup>-</sup> as counter-ion) and **2b** (Cl<sup>-</sup> as counter-ion) were grafted on citrate-protected AuNPs by adding an aqueous solution of these calixarenes to a dispersion of AuNPs with NaBH<sub>4</sub> (10<sup>5</sup> calixarenes per AuNP, 0.5 eq. NaBH<sub>4</sub> per calixarene).<sup>41</sup> Subsequent washing cycles employing centrifugation afforded AuNPs coated with calix(oEG)<sub>4</sub>. The AuNPs were analysed by transmission electron microscopy (TEM; see Figure S7) and thermogravimetric analysis (TGA; see Figure S8). The results showed that on average 600 calixarenes **2** are grafted per AuNP, which corresponds to a grafting density of 0.7 calixarenes per nm<sup>2</sup>. This value is close to that obtained for the grafting on surfaces (0.53 per nm<sup>2</sup>) but significantly lower than the value we had found for grafting of calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub>(N<sub>2</sub><sup>+</sup>)<sub>4</sub> **1** on AuNPs (1.5 per nm<sup>2</sup>). This difference can be explained when considering the steric bulk caused by the four oEG chains on **2**, which increases the footprint of this latter compared to the less bulky calixarene **1** (see Figure S9 for a cartoon).

Dynamic light scattering measurements confirmed the larger steric bulk of **2**, giving an average hydrodynamic diameter of the AuNPs-calix(oEG)<sub>4</sub> of 39.4 ± 3.2 nm [Polydispersity Index (PDI) = 0.31], compared to 24.0 ± 0.5 nm (PDI = 0.38) found for AuNPs-calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub>.

UV-Vis spectroscopic studies in various aqueous solutions were performed in order to see if the stability of the AuNPs was affected by the lower grafting density of AuNPs-calix(oEG)<sub>4</sub> (Figure 2). Dispersions of 2 nM AuNPs-calix(oEG)<sub>4</sub> are not only stable in pure water, but also in physiological salt concentrations (150 mM NaCl, 50 mM phosphate buffer) and show no influence of pH in the 2 to 13 range. We showed previously that AuNPs functionalised with calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub> have a robust enough coating to not be affected by fluoride, which easily replaces other ligands, such as thiols, leading to the aggregation of the AuNPs.<sup>41</sup> AuNPs-calix(oEG)<sub>4</sub> are also stable in the presence of fluoride (up to 0.85 M), whereas AuNPs that were protected with hepta(ethylene glycol)thiolates start to aggregate in the presence of 0.3 M KF and are fully aggregated with 0.45 M KF (see Figures S10, S11). AuNPs-calix(oEG)<sub>4</sub> can furthermore be dried completely (forming a gold coloured film) and resuspended in a solution of NaOH upon sonication (Figure S12). All these experiments show that AuNPs-calix(oEG)<sub>4</sub> display the same remarkable robustness and high stability as AuNPs-calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub>,<sup>41</sup> which indicates that these properties arise from the covalent nature of the coating rather than from the negative charges of the carboxylate groups.



**Figure 2.** UV-Visible absorbance spectra of AuNPs-calix(oEG)<sub>4</sub> suspended in (a) water (black), in phosphate buffer (0.05 M, pH 7.4, blue), and in NaCl (0.16 M, red), and (b) under acidic conditions obtained by the addition of HCl to obtain pH 4.5 (blue), pH 3.5 (green), and pH 2 (red), showing the stability of the dispersion of AuNPs at acidic pH. Aggregation is observed at  $pH \le 1$  (purple), though this can be reversed by increasing the pH (pink, dashed).

#### Grafting mixtures of calixarenes on AuNPs

Having established that the tetra-diazonium salts  $calix(CO_2^-)_4(N_2^+)_4 \mathbf{1}$  and  $calix(oEG)_4(N_2^+)_4 \mathbf{2}$  can both be grafted individually on AuNPs by using identical procedures and both afford very stable AuNPs, we followed the same procedure to graft mixtures of these two calixarenes. Compounds  $\mathbf{1}$  and  $\mathbf{2a}$  were each dissolved in water at 10 mM and mixtures of both solutions were made with ratios 0/100, 5/95, 10/90, 25/75, 50/50, and 100/0 as described in the Experimental Section.

The resulting functionalised AuNPs were firstly analysed by UV-Vis absorption spectroscopy (Figure 3). All spectra showed a sharp absorbance due to the surface plasmon resonance band of the AuNPs, indicating stable dispersions. The location of the maximum absorbance ( $\lambda_{max}$ ) shifted upon grafting from 519.5 nm (in 1 mM citrate) to 523 nm for pure calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub> (sample 100/0) and to 527 ± 0.5 nm for all the mixtures containing some calix(oEG)<sub>4</sub>. When HCl was added, aggregation of AuNPs containing carboxylates started at pH 6, with the largest effects observed for the AuNPs with  $\geq$  25% calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub> (samples 25/75, 50/50, and 100/0; see Figure S13). At pH 3 the AuNPs covered by only calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub> were extensively aggregated ( $\lambda_{max}$ = 592 nm), whereas the LSPR band of the AuNPs with calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub> : calix(oEG)<sub>4</sub> 5/95 had only shifted to  $\lambda_{max}$ = 535 nm, and no shift was observed for the AuNPs with calix(oEG)<sub>4</sub> only. Aggregation could be reversed upon addition of NaOH (Figure S14). In other words, the behaviour of the AuNPs with mixtures of calixarenes upon acidification lies in between that of AuNPs at both extremes (calix(oEG)<sub>4</sub> only: no aggregation; calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub> only: fast aggregation). This gives a first indication that mixtures of these two calixarenes were successfully grafted.



**Figure 3.** Normalised UV-Vis absorption spectra at pH 7 of the AuNPs (1.4 nM) in citrate (gray) and with mixtures of calix( $CO_2^-$ )<sub>4</sub> and calix(oEG)<sub>4</sub> (colours, as indicated in the legend). The inset shows a zoom of the spectra around  $\lambda_{max}$ .

#### IR studies on AuNPs with mixtures of calixarenes.

The UV-Vis absorbance studies show that, upon grafting of mixtures of calixarenes, AuNPs with mixed properties can be obtained. However, it does not give any quantitative information about the composition of the layer. For that reason, infra-red spectroscopy (ATR-FTIR) was employed to quantify the ratio of the ligands on the AuNPs. Dispersions of AuNPs were deposited on a Germanium internal reflection element and dried under a flow of nitrogen, after which the infra-red spectra were recorded (Figure 4).



**Figure 4.** (a) FTIR absorbance spectra from 3000-2800 and 1800-800 cm<sup>-1</sup> of AuNPs with mixtures of calix( $CO_2^-$ )<sub>4</sub> and calix(oEG)<sub>4</sub>, deposited from a dispersion in 0.1 mM NaOH. All spectra were normalised with respect to the intensity at 1459 cm<sup>-1</sup>; (b) Plot showing the relation between the average observed fraction of calix( $CO_2^-$ )<sub>4</sub> in the IR spectra and the fraction added (error bars show the standard deviation). The solid line indicates the perfect equivalence of the fraction added and that grafted; the dashed line represents the expected grafted fractions taking into account the double grafting density of calix( $CO_2^-$ )<sub>4</sub> compared to calix(oEG)<sub>4</sub>.

The IR spectra clearly show the IR absorbance signals of the different calixarenes. The most distinctive signal of the calix(oEG)<sub>4</sub> is the asymmetric COC stretching at 1105 cm<sup>-1</sup>,<sup>52</sup> but the amide-I (1669 cm<sup>-1</sup>) and amide-II (1540 cm<sup>-1</sup>)<sup>53</sup> are also clearly visible. The carboxylate signals from calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub> appear at 1604 and 1420 cm<sup>-1</sup> for the asymmetric and symmetric stretching respectively.<sup>53</sup> The presence of these signals was found to be pH dependent (Figure S15) and the AuNPs were therefore deposited from a basic solution (0.1 mM NaOH). Other signals are present in all spectra and are attributed to the aromatic part of the calixarene structure, like the asymmetric COC<sub>Ar</sub> stretching around 1190 cm<sup>-1</sup> and the aromatic ring stretching at 1459

 $cm^{-1}$ ,<sup>54</sup> which was used to normalise the spectra. A table with the assignments of bands is given in the SI.

In the IR spectra of the AuNPs with mixtures of calixarenes, the distinctive ethylene glycol and amide signals of calix(oEG)<sub>4</sub> decrease gradually with decreasing fractions of this calixarene in the grafting solution. This is also the case for the CH<sub>2</sub> signals at 2919 and 2852 cm<sup>-1</sup>, which are present in the spectrum from calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub>, but much stronger in the spectra of calix(oEG)<sub>4</sub> thanks to the presence of many additional CH<sub>2</sub> groups in the oEG chains. In contrast, the signals related to the carboxylates increase with increasing amount of calix(CO<sub>2</sub><sup>-</sup>)<sub>4</sub> added to the AuNPs.

We have used the IR absorbance values at 1105 and 1669 cm<sup>-1</sup> from calix(oEG)<sub>4</sub> and those at 1604 and 1420 cm<sup>-1</sup> for calix(CO<sub>2</sub>)<sub>4</sub> to determine the fractions of the two different calixarenes at the surface of the AuNPs (see SI). These four values were averaged and the observed fractions of grafted calix  $(CO_2)_4$  as function of that in the solution are plotted in Figure 4b. When both calixarenes were added to the AuNPs in equal quantities, roughly equal quantities were found on the AuNPs (46  $\pm$  5% calix(CO<sub>2</sub>)<sub>4</sub>). When smaller amounts of the calix( $CO_2^{-}$ )<sub>4</sub> were added (5 or 10%) the amount found was roughly double the amount added. This could be explained by the grafting density of  $calix(CO_2)_4$  which was found to be approximately twice that of calix(oEG)<sub>4</sub> and the former calixarene could thus have the ability to graft in smaller voids on the gold surface. The expected grafted ratio, calculated taking into account the grafting densities of both calix( $CO_2^{-}$ )<sub>4</sub> and calix(oEG)<sub>4</sub>, is plotted in Figure 4b with a blue dashed line, whereas the solid gray line represents the perfect equivalence of the ratio used in the grafting solution and the ratio found at the surface after functionalization. It is noteworthy that all the fractions of grafted calix  $(CO_2)_4$  that we have found fall within the interval defined by these two curves, clearly indicating the control over the grafted ratios by the use of defined ratios in the grafting solution. This result is in sharp contrast with those obtained with mixtures of thiols.<sup>23,27,47</sup>

# Conclusions

Control over the composition of mixed layers of organic ligands is a major problem in the functionalisation of nanoparticles, hindering the adjustment of the number of functional groups to the optimum for the envisaged application. Here we have shown that we can control the composition of the mixed layer of two calix[4]arenes grafted onto AuNPs by adjusting the ratio of the two calixarenes in solution. This strategy takes advantage of the extreme robustness of the calixarene-based coating on AuNPs. Furthermore, we have demonstrated that ATR-FTIR spectroscopy is an effective tool to quantify the composition of such a mixed layer.

This strategy of grafting mixtures of differently functionalised calix[4]arenes on AuNPs could be used to introduce a controlled number of functional groups, which can be post-functionalised

with for instance DNA or peptide aptamers for sensing purposes. This would then allow evaluating the effectiveness and dynamic range of the sensor as function of the number of functional groups, which is currently hardly possible, requiring optimisation of the experimental conditions for every mixture. Also for medical purposes involving for example the recognition of cells, control over the number of functional groups on AuNPs would be desirable. Finally, thanks to the extreme versatility of the calixarene platform, the use of mixtures of three or more different calixarenes can be envisioned, allowing the introduction of higher degrees of complexity on the surface of AuNPs.

#### ASSOCIATED CONTENT

#### **Supporting Information**

The detailed synthesis and characterisation of calix(oEG)<sub>4</sub>, characterisation of AuNPs by TEM, TGA and UV-Vis spectroscopy, IR spectra of AuNPs-calix( $CO_2^-$ )<sub>4</sub> at different pH, and details on the quantification and assignments of IR signals. This material is available free of charge via the Internet at http://pubs.acs.org.

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#### Notes

The authors declare no competing financial interest.

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# **TOC Graphic**

