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Isotopically enriched nanoparticles in combination with mass spectrometry for the assessment of nanoparticle-biomolecule stoichiometries in engineered nanoassemblies

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ABSTRACT

Determination of stoichiometries between engineered nanoparticles and species attached to their surface after chemical functionalization or interaction with biological media has been a hotspot during the last years, still unsatisfactory solved. Herein we propose a new approach only requiring for a simple mass spectrometric sulfur isotope ratio measurement in the final solution. Of course, enriched ^{34}S isotope has to be previously introduced in the engineered nanoparticle structure. Conversely to existing methodologies, mostly based on relative analyte measurements, proposed method does not require any previous knowledge of the molecule concentration in the starting solution. Such rather generic approach can be applied to study physical and/or chemical interactions between any sulfur-containing molecule and a wide variety of engineered nanoparticles containing sulfur as well, either in their own core-shell structure or in the ligand used for their surface functionalization (*e.g.* any thiol-derivative). In this paper, an inexpensive standard synthetic procedure was carried out to incorporate the required stable isotopic ^{34}S into the ZnS shell of CdSe quantum dots (QDs). Such isotopically enriched nanoparticles were then successfully applied to the quantitative assessment of stoichiometries between QDs and different target biomolecules, such as surface-functionalizing molecules (*e.g.* biotin) and proteins (*e.g.* bovine serum albumin). Of course, any biological conclusion and/or optimized bioconjugation conditions obtained using such isotopically enriched QDs analogues could be easily translated to nanoassemblies obtained using regular non-enriched QDs since they are chemically and physically identical.

1. Introduction

Quantum dots (QDs) are semiconductor nanoparticles (NPs) that have been extensively applied as targeted luminescent probes for *in vivo* and *in vitro* bioanalytical and bioimaging studies [1]. They are also currently used in more dynamic and sophisticated applications such as scaffolds in nanosensors and drug delivery or theranostics assemblies [2–4]. In such bioanalytical applications, QDs need to be previously functionalized with appropriate biomolecules (*e.g.* ligands, proteins) to guarantee their specific interaction with target analytes. As a matter of fact, such controlled functionalization of the QDs surface with appropriate functional ligands or proteins is a key issue that is unfortunately still far from being routinely achieved. In fact, it is widely recognized that new and reliable analytical methods are urgently demanded for this purpose [5].

Additionally, application of QDs in biological media is strongly affected by their interaction with proteins from the medium, bringing about the so called 'protein corona' associations. Once the NPs enter in the biological medium, protein adsorption onto NPs surface occurs [6]. This naturally formed biological layer plays a crucial role in the NPs physicochemical properties. The study of this 'protein corona' formation and its characterization has been a hotspot in bionanoresearch during the last years, and a lot of work has been devoted to characterize, quantify and understand this process [7–9]. In this context, bovine serum albumin (BSA) has been typically selected as model protein to study the protein corona formation [10, 11], and strong evidences of its importance to protect and make NPs more stable in biological media have been reported [12]. A critical parameter that characterizes such protein corona is the average number of proteins that are linked to the surface of the NP under given experimental

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conditions. However, adequate analytical approaches to carry out such protein:NP ratio determinations are still under demand [5]. Among the different strategies already described in the literature to estimate biomolecule:NP ratios, sulfur determination appears to be a promising choice as it is present in most proteins [9]. However, very often, S is also present in either the core-shell NPs (ZnS, PbS, CdSe/ZnS) and/or in the functional ligands attached to the surface in order to render them water stable (*e.g.* dihydrolipoic acid or cysteine). This makes hard to distinguish the S originally present in the engineered NP from the sulfur later incorporated as functionalizing agents (*i.e.* biotin) or as present in the proteins constituting the 'protein corona' in biological media (*i.e.* BSA).

Herein we have synthesized conventional fluorescent CdSe QDs with a shell consisting of Cd/Zn sulfide isotopically enriched in ^{34}S (~95%), clearly different from natural sulfur where ^{32}S accounts for almost 95% abundance. In that way, the incorporation of S-containing biomolecules to the QD environment can be mathematically computed from the S isotope ratios: ^{34}S (mostly coming from QDs) to ^{32}S (mostly coming from the biomolecule). Such sulfur isotope ratios can be accurately and precisely measured by inductively coupled plasma tandem mass spectrometry (ICP-MS/MS). The synthesis strategy of ^{34}S-enriched QDs (QD_{enr}) together with the new analytical methodology proposed is illustrated in Fig. 1. To produce such isotope-enriched nanotool, the corresponding S-containing reagent, used as precursor during the NP shell formation, had to be first synthesized using isotopically enriched elemental ^{34}S as starting reagent. Then, QD_{enr} could be synthesized and thoroughly characterized. Finally, the great potential of the QD_{enr} has been evaluated by tackling two of the most challenging tasks nowadays in bionanotechnology. First, the quantitative determination of the protein corona population in different mixtures of QD_{enr} and BSA, selected as a model protein. Second, the accurate determination of the number of functionalizing molecules (biotin selected as a model in this case) intentionally incorporated to the QD_{enr} surface, *via* chemical bioconjugation, under different experimental conditions.

It should be noted that although isotopically enriched NPs have been scarcely synthesized so far, they have proved to show the same physico-chemical properties as their natural analogues [13–16]. In those recent works, enriched NPs have been used as isotopic tracers in standard isotope dilution studies aimed at studying NPs behavior in complex matrices (*e.g.* the study of fate and transformations of NPs in sediment-slurry matrices, standard estuarine sediments, biological tissues, *etc.*). To our knowledge this manuscript reports for the first time a simple approach based on isotopically enriched NPs (QD_{enr}), to quantify precisely the ratio between those NPs and chemically bound (bioconjugated) biomolecules or alternatively, biomolecules adsorbed (protein corona) to the NPs surface.

2. Methods and theory

2.1. Synthesis and characterization of the quantum dots isotopically enriched in ^{34}Sulfur

Standard CdSe QDs were synthesized following the organometallic route described elsewhere using CdO as precursor [17]. Prior to the growth of the isotopically enriched Zn/Cd^{34}S shell over the CdSe core, it was necessary to synthesize and characterize the ^{34}S-enriched precursor (^{34}S-hexamethyldisilathiane). Such compound was obtained under N_2 atmosphere from the reaction of chlorotrimethylsilane with the resulting mixture of $^{34}S_8$ with the strong reducing $LiHBEt_3$ in organic media (see Appendix for the details).

The product of such synthesis, was evaluated by ^{1}H and $^{13}C\{^{1}H\}$-NMR and the observed spectra are given in Fig. S1 (Appendix). Further evidences for identification and purity determination of the ^{34}S-hexamethyldisilathiane were obtained by GC–MS. Fig. 2a shows clearly that one single peak was observed at 6.3 min in the GC–MS chromatogram.

Retention time matched very well with that of a commercial hexamethyldisilathiane with natural S isotopic abundance. The purity of the synthesized ^{34}S-reagent was established to be > 99%. Comparison of the Electron Ionization fragmentation pattern obtained for the ^{34}S-enriched (Fig. 2b) and the natural abundance standard (Fig. 2c) showed the expected mass shift of 2 mass units for the molecular ion peak (m/z = 180) and the sulfur containing fragment corresponding to the loss of one $-CH_3$ group (m/z = 165) due to the vast presence of ^{34}S instead of ^{32}S in the enriched and natural products, respectively. Conversely, the fragment at m/z = 73, corresponding to $(CH_3)_3Si^+$ and that does not contain sulfur, was observed in both mass spectra.

The ^{34}S-hexamethyldisilathiane was then used for the shelling of the CdSe core previously synthetized. The so-obtained core-shell QD_{enr} were further surface-coated with an amphiphilic polymer for aqueous stabilization (see Appendix for the details) following the procedure previously described elsewhere [18]. The size of the final QD_{enr} was determined using transmission electron microscopy (see Fig. S2, Appendix). An average QD_{enr} diameter value of 4 nm, with low size dispersion (± 0.4 nm) was obtained. The UV–vis absorbance and fluorescence spectra of the synthesized QD_{enr} are shown in Fig. S3 (Appendix). As can be seen, the use of the isotopically enriched ^{34}S-precursor did not affect the photoluminescent properties of the resulting NPs. This is in agreement with other reports showing that isotopically enriched NPs show the same physical and chemical properties as their natural analogues [13, 16].

2.2. Elemental characterization of water-soluble QD_{enr}

After polymer coating of the QD_{enr}, several purifications were carried out. First, samples were passed through syringe filters (0.22 μm) (Teknokroma, Spain) to remove eventual nanoparticle aggregates generated during the QD_{enr} solubilization process. Second, buffer was exchanged by ultrapure water using two rounds of dilution and preconcentration through a centrifuge filter (molecular weight cut-off = 100 kDa, Millipore, Spain, 3000 rpm centrifugation speed).

Elemental analysis of the QD_{enr} was performed using an ICP-MS equipped with a triple quadrupole (Agilent 8800, Agilent Technologies, Japan) by flow injection analysis (FIA). A two-position and six-port injection valve (Rheodyne, IDEX Health & Science, Germany) was used to allow on-line flow injection the QD_{enr} using the peristaltic pump of the instrument and ultrapure water as carrier. Operation conditions were daily optimized using a tuning solution and typical values are given in Table S1 (Appendix). S and Cd isotopes were detected using "mass shift mode" ($^{48}SO^+$, $^{49}SO^+$ and $^{50}SO^+$) after their reaction with oxygen in the cell and "on mass mode" ($^{106}Cd^+$ and $^{111}Cd^+$), respectively. Dwell time for each of the targeted isotopes was 100 ms. Integration of FIA peaks was performed using the software provided with the instrument (MassHunter, Agilent).

2.2.1. Determination of sulfur isotope ratios after correction for the mass bias

For determination of sulfur isotope ratios, mass bias was evaluated by measuring the corresponding $^{34}S/^{32}S$ ratio in a prepared 600 μg L^{-1} natural S ICP standard solution from a certified 1000 mg L^{-1} standard (Merck, Germany).

A power law algorithm (Eq. (1)) was used for the correction of the mass bias (K) [19], typically observed in ICP-MS, due to the more efficient transmission of the heavier ions:

$$Ln\left(\frac{R_{std}}{R_{theo}}\right) = K \cdot \Delta M \tag{1}$$

where R_{theo} and R_{std} are the theoretical (2009 IUPAC Technical Report [20]) and experimentally determined, in the natural sulfur abundance standard, $^{34}S/^{32}S$ isotope ratio, respectively. ΔM is the mass difference between sulfur isotopes. Mass bias was determined daily obtaining a

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