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Asymmetric flow field-flow fractionation coupled to inductively coupled plasma mass spectrometry for the quantification of quantum dots bioconjugation efficiency

Mario Menéndez-Miranda, Jorge Ruiz Encinar*, José M. Costa-Fernández*, Alfredo Sanz-Medel

Department of Physical and Analytical Chemistry, University of Oviedo, Avda. Julian Claveria 8, E-33006, Oviedo, Spain

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ABSTRACT

Hyphenation of asymmetric flow field-flow fractionation (AF4) to an on-line elemental detection (inductively coupled plasma-mass spectrometry, ICP-MS) is proposed as a powerful diagnostic tool for quantum dots bioconjugation studies. In particular, conjugation effectiveness between a "model" monoclonal IgG antibody (Ab) and CdSe/ZnS core-shell Quantum Dots (QDs), surface-coated with an amphiphilic polymer, has been monitored here by such hybrid AF4-ICP-MS technique.

Experimental conditions have been optimized searching for a proper separation between the sought bioconjugates from the eventual free reagents excesses employed during the bioconjugation (QDs and antibodies). Composition and pH of the carrier have been found to be critical parameters to ensure an efficient separation while ensuring high species recovery from the AF4 channel. An ICP-MS equipped with a triple quadropole was selected as elemental detector to enable sensitive and reliable simultaneous quantification of the elemental constituents, including sulfur, of the nanoparticulated species and the antibody.

The hyphenated technique used provided nanoparticle size-based separation, elemental detection, and composition analysis capabilities that turned out to be instrumental in order to investigate in depth the Ab-QDs bioconjugation process. Moreover, the analytical strategy here proposed allowed us not only to clearly identify the bioconjugation reaction products but also to quantify nanoparticle:antibodies bioconjugation efficiency. This is a key issue in future development of analytical and bioanalytical photoluminescent QDs applications.

1. Introduction

Semiconductor nanoparticles (NPs) or Quantum dots (QDs) are nearly spherical, nanosized luminescent crystals (diameter ~2–10 nm), showing composition- and size-dependent unique optical properties [1]. In particular, cadmium selenide-based QDs, with a shell of an additional semiconductor material having a higher band gap, such as ZnS (e.g. CdSe/ZnS), are the most studied semiconductor nanocrystals so far specially for bioanalytical applications [2]. Potential and demonstrated analytical utilities of QDs range from more passive roles, such as targeted in vitro and in vivo fluorescent probes, to dynamic roles including active scaffolds in nanosensors and drug delivery or theranostic assemblies [3,4].

The final role of QDs in most biological applications will ultimately depend, to a large degree, on their ability to be bioconjugated to an appropriate recognition unit (i.e. antibodies, aptamers) able to bind specifically the analyte in the complex sample [5]. In this context, perhaps the largest obstacle to a wider implementation of QDs as fluorescent labels for immunoassays has been the absence so far of "a single bioconjugation reaction that fits all", whereby one can attach a variety of biomolecules to QDs by a simple and reproducible scheme. In fact, a rigorous control of the bioconjugation of the antibodies to the surface of the nanoparticles is mandatory for later biochemical applications success [6].

However the qualitative and, above all, quantitative assessment (efficiency) of the bioconjugation reactions is not a trivial task [7]. Few reports have been published so far for the qualitative evaluation of the bioconjugation of NPs to macromolecules. They are

* Corresponding authors.
E-mail addresses: ruizjorge@uniovi.es (J.R. Encinar), jcostafe@uniovi.es (J.M. Costa-Fernández).

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based on a wide variety of techniques such as X-ray photoelectron microscopy [8], Nuclear Magnetic Resonance (NMR) [9], fluorescence microscopy [10], UV/vis absorption (Bradford Assay) [11], fluorescence staining of the protein after dialysis and SDS-Page separation [12], or size exclusion chromatography with ICP-MS detection [13]. Unfortunately, all these techniques present some drawbacks, including the awkwardness to assess the bioconjugation efficiency. In most cases only a simple confirmation or rough estimations are given so far. In fact, to the best of our knowledge, very few reports have provided quantitative bioconjugation efficiency results. Ren and co-workers, first used capillary electrophoresis (CE) [14,15] and Size Exclusion Chromatography (SEC) [16] with laser-induced fluorescence (LIF) and fluorescence correlation spectroscopy (FCS) detection to provide a percentage of the protein-QD in the mixture. However, problems related to the adsorption of proteins to the inner wall of the capillary [14] and interactions of QDs with the SEC gel stationary phase [16] were reported. In other words, quantitative results were compromised by the species-specific column-capillary recoveries and undesirable unspecific interactions. Moreover, it is well known that optical properties (i.e. fluorescence and absorbance) of QDs after protein conjugation may be altered [7] and so direct comparison of spectroscopic peak areas of free and bioconjugated QDs could lead to biased results. Finally, the simultaneous detection of QDs species and proteins without resorting to extra laborious labelling [16] has proved to be difficult.

Asymmetrical flow field-flow fractionation (AF4) is one of the most promising techniques to achieve size-dependent separation of nanoscale particles ranging from <1 nm up to several μm. Analyte nanoparticles are injected here into a flat channel with a laminar tangential flow (having a parabolic velocity profile). A separation of the different size species is achieved by applying a transverse field that is a cross-flow perpendicular to the laminar flow. Nanoparticle separation is based on the relative diffusion coefficient of the particles which depends on their size, coating thickness, and coating material. Expected sample degradation or nanoparticles aggregation is low due to the lack of destructive forces during separation [17,18] and the minimal interaction with the separation channel. Also, AF4 can be coupled on-line to different types of detectors, such as UV/vis, dynamic light-scattering (DLS) or fluorescence spectroscopy providing additional information and specificity. However, those detectors neither provide nanoparticle elemental information nor respond directly to the elemental mass concentration of the nanoparticles. In this context, hybridation of the AF4 with elemental specific detectors (e.g. inductively coupled plasma mass spectrometry, ICP-MS), could provide nanoparticle sizing and compositional analysis, as well as limits of detection at least 10–100-fold lower than those provided by DLS or UV techniques [19]. What is more, ICP-MS signals can be made species-independent, that makes specific standards no longer needed [20].

In this work we present a strategy for both, qualitative and quantitative assessment of the bioconjugation of CdSe/ZnS QDs to proteins based on the use of an optimized AF4 separation. A novel ICP-MS (ICP-QQQ) detector is coupled on-line in order to be able to achieve the interference-free and sensitive simultaneous detection of Cd, Se and especially of S [21,22], a critical need for the assessment of the elution of the bioconjugated or free antibody. The ICP-MS elemental ratios measured in the different NP populations separated by the AF4 are used as the diagnostic tools. Conditions have been optimized first to attain complete separation of the QD bioconjugates from the excess of antibody and of QDs. Finally, the quantitative species recoveries achieved and the use of a detector whose response is directly proportional to the mass concentration of the NPs are the basis to the final bioconjugation efficiency computation obtained.

2. Materials and methods

2.1. Chemicals and materials

All chemicals were of analytical grade and used as received without further purification. The precursors used for the synthesis of the quantum dots were selenium powder (100 mesh, 99.99%), cadmium oxide (99.99%), hexamethyldisilathiane, 1.0 M diethyl zinc solution in hexane, trioctylphosphine (TOP, 90%), trioctylphosphine oxide (TOPO, 99%) and anhydrous chloroform (≥99%), all of them purchased from Sigma Aldrich (Schnelldorf, Germany) and hexylphosphonic acid (HPA) obtained from Alfa Aesar (Karlsruhe, Germany).

Water solubilization of our QDs was achieved by coating their surface with an amphiphilic polymer synthesized in our laboratory [23]. For such purpose, poly(isobutylene-alt-maleic anhydride) and bis(6-aminohexyl)amine were used (both of them purchased from Fluka, Basel, Switzerland). Dodecyl amine and tetrahydrofuran anhydrous were obtained from Sigma Aldrich. A saline borate buffer solution (SBB, 50 mM sodium borate, 100 mM NaCl) at pH = 12 was used to solubilize the QDs samples. Syringe filters (0.22 μm) obtained from Teknokroma (Madrid, Spain) were employed to remove nanoparticle aggregates generated during the QDs solubilization process. Amicon Ultra-4 100 kDa centrifugal filters from Millipore (Madrid, Spain) were used to carry out QDs buffer exchange by ultracentrifugation (5 min at 2000 × *g*, Biofuge Stratos Heraeus, Thermo Scientific, Germany).

The rat monoclonal IgG-2a antibody (0.5 mg/mL) was obtained from Acris-Antibodies (Herford, Germany). Ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), was used as pseudocatalyst reagent (Fluka) for quantum dot bioconjugation to the antibodies.

Ammonium acetate, Tween 20 and ammonia (Sigma Aldrich), were used to prepare the eluent solutions employed as carriers in the AF4 experiments. All carrier solutions, filtered on 0.1 μm cellulose membrane filters, were prepared using Milli-Q deionized water and analytical grade reagents.

2.2. Synthesis and surface modification of CdSe/ZnS quantum dots

CdSe/ZnS QDs were synthesized using CdO as precursor via the organometallic route described elsewhere [24]. Once the synthesis was finished, the mixture was centrifuged to remove excess of reagents and then the precipitated NPs were redispersed in anhydrous chloroform and stored at room temperature in the dark. The so synthesized hydrophobic QDs were coated with an amphiphilic polymer following a procedure previously described elsewhere [25]. Such polymer-coated QDs were dispersed in 50 mM SBB at pH = 12 at a concentration of ~3.0 μM (stock solution).

2.3. Bioconjugation of the QDs to the antibody

The assayed strategy for creating QD:Ab bioconjugates was based on the modification of the COOH groups of the outer polymeric layer of the water-solubilized core-shell QDs with EDC chemistry for subsequent attachment of the amine groups from the antibodies [26]. In our case, the rat monoclonal IgG-2a antibody was selected as model.

The bioconjugation reaction was carried out at room temperature with constant stirring for 2 h. The QDs:Ab molar ratios used in this study were varied from 0.5:1 to 3:1. Final molar concentration of the antibody was kept constant (0.5 μM). In order to ensure the success in the bioconjugation, a 1500 molar excess of EDC was always added. For that purpose 0.05 M solution of EDC in