Journal of Chromatography A, 1477 (2016) 56–63

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Complex analysis of concentrated antibody-gold nanoparticle conjugates' mixtures using asymmetric flow field-flow fractionation

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ARTICLE INFO

Article history:
Received 5 August 2016
Received in revised form
17 November 2016
Accepted 21 November 2016
Available online 22 November 2016

Keywords:
Aggregation
Asymmetric flow field flow fractionation
AF4
Gold nanoparticles
Antibody-gold nanoparticle conjugates
Analytical solution with stabilizers

ABSTRACT

Conjugates of gold nanoparticles (GNPs) with antibodies are powerful analytical tools. It is crucial to know the conjugates' state in both the concentrated and mixed solutions used in analytical systems. Herein, we have applied asymmetrical flow field-flow fractionation (AF4) to identify the conjugates' state. The influence of a conjugate's composition and concentration on aggregation was studied in a true analytical solution (a concentrated mixture with stabilizing components). GNPs with an average diameter of 15.3 ± 1.2 nm were conjugated by adsorption with eight antibodies of different specificities. We found that, while the GNPs have a zeta potential of −31.6 mV, the conjugates have zeta potentials ranging from −5.8 to −11.2 mV. Increased concentrations (up to 184 nM, $OD_{520} = 80$) of the mixed conjugate (mixture of eight conjugates) did not change the form of fractograms, and the peak areas' dependence on concentration was strongly linear (R^2 values of 0.99919 and 0.99845 for absorption signal and light scattering, respectively). Based on the gyration (R_g) and hydrodynamic (R_h) radii measured during fractionation, we found that the nanoparticles were divided into two populations: (1) those with constant radii ($R_g = 9.9 \pm 0.9$ nm; $R_h = 14.3 \pm 0.5$ nm); and (2) those with increased radii from 9.9 to 24.4 nm for R_g and from 14.3 to 28.1 nm for R_h. These results confirm that the aggregate state of the concentrated and mixed conjugates' preparations is the same as that of diluted preparations and that AF4 efficiently characterizes the conjugates' state in a true analytical solution.

1. Introduction

Conjugates of gold nanoparticles (GNPs) with antibodies are powerful tools for analytical purposes [1–3]. Rapid immunoassay formats such as lateral flow immunoassay [4–6] and microfluidic immunoassay [7,8] use these conjugates in their concentrated forms to achieve significant binding and high signals with minimal interaction time. A high concentration of nanoparticles implies that the effective volume fraction (φ_e, total volume of particles divided by dispersion volume) of the concentration exceeds the Einstein limit, which is 0.01 [9,10]. The use of such concentrations in kinetic processes could significantly improve the detection limit of assays. Additionally, in concentrated solutions, the probability that particles will coagulate may increase significantly, as shown in [9,11–13]. To assess the stability of colloidal preparations, nanoparticles' concentrations should be determined, especially when the nanoparticles have low surface potential. Thus, a high concentration of conjugates is a risk factor that may lead to coagulation and aggregation. Generally, conjugate solutions ready for use in analytical systems contain many components (detergent, protein stabilizers, carbohydrate stabilizers, etc.) that are designed to prevent aggregation [14]. However, we cannot consider all factors and mathematically predict the aggregation conditions for a multicomponent mixture. There is additional uncertainty for mixtures of different conjugates. Such mixtures are commonly used in multiplex bioanalytical systems [15–17].

Understanding the aggregate state of GNPs and their conjugates is crucial for many analytical and biomedical activities [18–20]. For predictable use in analytical systems, conjugates should be close to a monodispersed state, that is, they should contain few or no aggregates. The peak size distribution and percentage of polydispersity should be as low as possible when considering the suspension stability of the conjugates. An increase in the percentage of polydispersity with increasing concentration indicates that aggregates have formed. However, the conjugates' state in concentrated solutions remains unknown.

This study aims to investigate conjugates' composition, concentration, and influence on nonspecific binding, that is, the binding of a GNP conjugate with an antibody to something other than the target antigen. The study may be performed using different

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http://dx.doi.org/10.1016/j.chroma.2016.11.040
0021-9673/

methodologies. The most commonly used methods to measure the aggregate state of nanoparticles and their conjugates are light scattering with quantification of zeta potentials, UV/VIS absorption spectroscopy, and transmission electron microscopy [21,22]. However, light scattering cannot be used in this study as it identifies average size values, not individual quantitative data for differently shaped and sized particles. To accurately estimate the composition of the concentrated conjugate mixture, its components should be separated. Analytical and physical characterization techniques, including separation of samples in the first stage and analysis of fractions in the following stages (e.g., size exclusion chromatography, hydrodynamic chromatography, and field-flow fractionation) are the best ways to achieve this [22,23]. Many studies have used asymmetric flow field-flow fractionation (AF4) coupled with online detectors to analyze nanoparticles due to its high separation power, broad size separation range, and compatibility with different matrices [24,25].

AF4, as an effective separation and detection platform for quantitatively determining the size of particles, has been used for measuring GNPs [26–29], finite-sized GNP clusters [30], GNP mixtures [24], engineered nanoparticles in the presence of natural nanoparticles and in different matrices [31–34], functional nanoparticles [35], and the effectiveness of conjugation between proteins and nanoparticles [36,37]. However, no studies have examined antibody–GNP conjugates that are synthesized for analytical purposes and included in multicompound solutions (concentrated mixtures with stabilizing components) for storage and further use. In addition, no studies have determined the stability of concentrated and mixed conjugates using AF4. Considering the features of AF4 and advancements in the abovementioned studies, which allow nanoparticles and their conjugates to be characterized as dilute suspensions ($\varphi_e \leq 0.01$), we believe that AF4 will be an effective way to measure the conjugates under investigation. This study aims to determine the applicability of AF4 as a tool for estimating the stability of concentrated and mixed antibody–GNP conjugates.

We used AF4 to investigate the conjugates of GNPs and eight polyclonal antibodies specific to different plant pathogens: potato virus X, potato virus M, potato virus A, potato virus S, ordinary-type potato virus Y, necrotic-type potato virus Y, potato leaf roll virus, and *Clavibacter michiganensis* subsp. *sepedonicus*. These pathogens are extremely important potato disease-causing agents that require timely detection to reduce economic losses in the agricultural sector [38]. Although all eight antibodies belong to the same protein, immunoglobulin G (IgG), their biochemical properties may differ because of the immunizing antigens in the amino acid sequences in the variable regions of IgG. In this study, we discuss the structural characteristics of single and mixed GNP–antibody conjugates that are appropriate model for characterizing concentrated and mixed antibody–GNP conjugates using AF4.

2. Materials and methods

2.1. Materials and reagents

Polyclonal rabbit antibodies against potato virus X (Ab1), potato virus M (Ab2), potato virus A (Ab3), potato virus S (Ab4), ordinary-type potato virus Y (Ab5), necrotic-type potato virus Y (Ab6), potato leaf roll virus (Ab7), and *Clavibacter michiganensis* subsp. *sepedonicus* (Ab8) were provided by Y.A. Varitsev (A.G. Lorch All-Russian Potato Research Institute, Kraskovo-1, Moscow Region, Russia). This study used gold standard for inductively coupled plasma mass spectrometry (ICP–MS) (999 ± 2 mg/mL in 5% w/w HCl, Fluka, Switzerland); inductively Coupled Plasma Mass Spectrometry (ICP–MS) internal standards of Li^6, Sc, Y, In, and Tb (100 ± 0.5 μg/mL in 2% HNO_3, Bruker Daltonics Chemical Analysis, USA); hydrochloric acid (38%, ultrapure, Reactiv-component, Russia), nitric acid (70% w/w, purified by redistillation), tris(hydroxymethyl)aminomethane (Tris), Tween-20, sodium azide, and sucrose (Sigma-Aldrich, USA); chloroauric acid (Fluka, Germany); bovine serum albumin (BSA) and sodium citrate (MP Biomedicals, UK); glycerol, sodium chloride, and potassium carbonate (DiaM, Russia); and potassium dihydrogen phosphate and potassium hydroxide (Khimmed, Russia). All chemicals were analytical or chemical grade. Ultrapure Milli-Q water ($18.2\ M\Omega \times cm$) was obtained using a Simplicity® Water Purification System (Millipore, Milford, MA, USA) and used in all experiments.

2.2. Synthesis of gold nanoparticles

One milliliter of 1% $HAuCl_4$ was added to 95 mL of deionized water. The solution was heated to its boiling point, and 4 mL of 1% sodium citrate was added with agitation [39]. The mixture was boiled for 25 min and then cooled, and the GNP preparation was stored at 4–6 °C.

2.3. Synthesis of antibody–GNP conjugates

Antibody–GNP conjugates were synthesized according to method modified by Safenkova et al. [40]. The antibodies were dialyzed against a 1000-fold volume of 10 mM Tris-HCl, which has a pH of 9.0, at 4 °C for 2–3 h. The pH of the GNP preparation was adjusted to 9.5 with 0.2 M K_2CO_3. Antibodies at concentrations of 20 μg/mL were added to the GNPs. The mixture was stirred at room temperature for 1 h, and BSA was added to achieve a final concentration of 0.25%. GNP-conjugated antibodies were separated from unbound reagents by centrifugation at 20,000*g* for 30 min, followed by resuspension in phosphate buffered saline (PBS) (50 mM of potassium phosphate buffer with a pH of 7.4 and 0.1 M NaCl) containing 0.25% BSA, 0.25% (v/v) Tween-20, and 1% sucrose (conjugate buffer; CB). For long-term storage, NaN_3 was added to achieve a final concentration of 0.02%. The spectra of the GNPs and their conjugates were recorded using a Biochrom Libra S60 Double Beam Spectrophotometer (Biochrom, UK). To produce the mixed antibody–GNP conjugate, equal amounts of all the conjugates (GNP–Ab1, GNP–Ab2, GNP–Ab3, GNP–Ab4, GNP–Ab5, GNP–Ab6, GNP–Ab7, and GNP–Ab8) were mixed in a bottle.

2.4. Transmission electron microscopy (TEM)

Preparations of the GNPs and their conjugates were applied to 300 mesh grids (Pelco International, USA) coated with a support film of poly(vinyl formal) dissolved in chloroform. Images were obtained using a JEM CX-100 electron microscope (JEOL, Japan) operating at 80 kV. The digital images were analyzed with ImageTool (UTHSCSA, USA).

2.5. Estimation of GNPs and conjugate concentrations

The concentration of Au in the solutions was determined by ICP-MS. These measurements were conducted with an Aurora M90 quadrupole ICP-MS instrument (Bruker Corp., USA) equipped with a MicroMist low-flow nebulizer. A series of gold standard solutions (0.1–5 ppb in HCl 1% (v/v)) were prepared before each experiment. Scandium was used as an internal standard. The optimal operating parameters are presented in Table S1 in the Supplementary data. All samples were prepared in triplicate. Quantum software (Bruker Corp., version v.3.1 b1433) was used for data collection and processing.