THE COMPOSITION OF GOLD NANOPARTICLE CONJUGATES WITH IMMUNOGLOBULINS AT PHYSICAL ADSORPTION OR COVALENT BINDING

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Abstract

A quantitative comparison was made of the efficiency of conjugation of immunoglobulins G and gold nanoparticles obtained by the Turkevich-Frens method. The conjugates were synthesized by physical adsorption and covalent immobilization using orthopyridyl disulfide polyethylene glycol succinimidyl ester (OPSS-PEG-NHS). The study is conducted using an original method of determining the composition of protein nanoconjugates based on measuring the intrinsic fluorescence of tryptophan in the protein. The percentage of bound immunoglobulins was higher when using OPSS-PEG-NHS: 43% versus 22% for adsorption conjugation at an added IgG concentration of 2.3 μg/ml. After increasing the IgG concentration to 4.6 μg/ml, the percentage of binding immunoglobulins was close for the two methods of the conjugation: 26% and 22%, respectively, which may indicate the filling of the surface of the particles.

Keywords: Gold nanoparticles, conjugates, tryptophan fluorescence, immunoglobulin

1. INTRODUCTION

Conjugates of gold nanoparticles (GNPs) with bioreceptor molecules (e.g., antibodies) have found use as applications for solving many biochemical and bioanalytical tasks [1,2]. The main method of obtaining GNPs is the citrate reduction of hydrochloric acid (Turkevich-Frens method) [3,4]. This technique has several advantages: a simple and reproducible synthesis, the ability to obtain particles in a wide range of sizes (10-150 nm), and the stability of the colloid due to the stabilization of particles by citrate ions. Citrate ions can be easily replaced by other molecules; therefore, such stabilization does not limit the possibility of surface modification.

For the functionalization of GNPs by the biomolecules, two main approaches are used: physical adsorption and covalent binding [5-8]. Physical adsorption is the most widespread technique as a result of its methodological simplicity and minimal impact on the structure and properties of the adsorbed molecules [9]. The main disadvantage of noncovalent immobilization is the possible partial desorption of protein molecules and, consequently, a decrease in the functional activity of the conjugate [10,11].

Despite many methods having been proposed for studying the composition of nanoconjugates (see the review in [12]), the number of molecules bound to the GNP remains debatable. This is a consequence of significant differences in methods and conditions for the preparation of conjugates.

A comparative study was conducted on the composition of GNPs' conjugates with antibodies obtained in two ways: direct adsorption and covalent binding using orthopyridyl disulfide polyethylene glycol succinimidyl ester (OPSS-PEG-NHS). The quantitative composition of the conjugates was determined by fluorescence spectroscopy [13]. This method is based on determining the intrinsic fluorescence of tryptophan in the protein and allows for evaluation of the composition of protein conjugates without the need for additional labels.

2. EXPERIMENTAL

The present study used monoclonal anti-troponin I antibodies, IC4 clone (“Bialexa”, Russia). OPSS-PEG-NHS (M_W = 5,000) and thiolated PEG (PEG-SH, M_W = 5,000) were obtained from Creative PEG Works (Chapel Hill, NC, USA).

2.1. Synthesis of GNPs

GNPs were synthesized according to the Frens method [3] with modifications [14]. First, 1.0 ml of a 1 % aqueous solution of hydrochloric acid was added to 97.5 ml of deionized water and brought to a boil. Then, 1.5 ml of a 1% aqueous sodium citrate solution was added and stirring. The mixture was boiled for 25 min, cooled, and stored at 4-6 °C. The spectra of GNPs’ preparations were recorded using a Biochrom Libra S60 spectrophotometer (Biochrom, UK).

2.2. Synthesis of antibody-GNP conjugates by adsorption immobilization

In accordance with the procedure described previously [15], GNP solutions (D_{520} = 1.0, pH 9.0) were added to the solutions of antibodies at concentrations of 4.6 and 2.3 μg/ml. Antibody concentrations were chosen on the basis of theoretical calculations of the amounts of antibodies forming a monolayer coating on the surface of the GNPs with different diameters. The mixtures were incubated for 30 min at room temperature and stirred, after which an aqueous PEG-SH solution was added to a final concentration of 0.25 %. GNP-IgG conjugates were separated from unbound antibodies by centrifugation for 10 min at 12,000 g and 4 °C using an Allegra 64R centrifuge (Beckman Coulter, USA). The precipitate was suspended in a 20 mM Tris-HCl buffer, pH 7.6, containing 1.0 % BSA, 1.0 % sucrose, 0.1 % Tween 20, and 0.1 % sodium azide.

2.3. Synthesis of antibody-GNP conjugates by covalent immobilization

First, OPSS-PEG-NHS cross-linker was reacted overnight with IC4 at a 10:1 molar ratio in 100 mM sodium bicarbonate (pH 8.5). Modified antibodies were purified by gel filtration. Then, OPSS-PEG-NHS-treated antibodies were added to GNPs for 2 h to obtain the conjugates (final concentrations of OPSS-PEG-NHS-treated antibodies were 4.6 and 2.3 μg/ml). In the next step, 20 μL of 1 mM PEG-SH was added to additionally stabilize the nanoparticles. Finally, the conjugates were centrifuged at 12,000 g for 10 min.

2.4. Determination of the composition of nanoconjugates

The supernatant after centrifugation of the conjugates was divided into two parts. The first part, in a volume of 200 μl, was added to the microplate wells (Nunc MaxiSorp white microplates; Roskilde, Denmark). IC4 antibodies were added to the second part to a final concentration of 6 μg/ml, and 200 μl of the obtained solutions was transferred to the microplate wells and used as a calibration solution. The fluorescence was measured on a microplate reader (Perkin Elmer En Spire 2300, Waltham, MA) with excitation and emission wavelengths of 280 nm and 350 nm, respectively.

The protein concentration in the supernatant (in μg/ml) was determined by the following formula:

$$6 * N / (C - N),$$

where N is the fluorescence in the supernatant, C is the fluorescence in the calibration solution, and 6 is the concentration of added antibodies in the calibration solution in μg/ml.

The difference between the initially added protein concentration and its concentration in the supernatant gives the concentration of the protein conjugated with GNPs.

3. RESULTS AND DISCUSSION

3.1. Characteristic of GNPs

Dimensional characteristics of the GNPs' preparation were obtained by transmission electron microscopy. The average diameter of the particles was 33.7 nm. Particle concentration was determined by ICP-MS according to the method described by Byzova et al. [16]. According to the data obtained, $1.3 \cdot 10^{11}$ GNPs were contained in 1 ml of the solution.

IgGs were added to GNPs in two concentrations, based on the calculation of 25 nm^2 and 50 nm^2 of the particle surface area per one IgG molecule. The calculated amounts of GNPs and immunoglobulins are summarized in **Table 1**.

Table 1 Quantitative parameters of GNPs and added IgG during the conjugation

Diameter, nm	Surface area, nm^2	GNPs / ml	IgG per particle (1)*	IgG per particle (2)*	[IgG] (1)*, µg/ml	[IgG] (2)*, µg/ml
33.7	3566	$1.3 \cdot 10^{11}$	72	143	2.3	4.6

* (1) at the rate of 1 IgG molecule on 50 nm^2 of the GNP surface; (2) at the rate of 1 IgG molecule on 25 nm^2 of the GNP surface.

3.2. Determination of the composition of nanoconjugates

The intrinsic fluorescence of a protein is a convenient tool for determining its concentration. Tryptophan is the most intensely fluorescent amino acid [17]. However, its fluorescence strongly depends on its molecular environment, including the ion composition of the solution [13]. This does not allow the use of calibration solutions that differ from the test sample in ionic composition. To account for this, the addition of a known amount of protein directly in the analyzed sample is proposed. In this case, the difference in fluorescence in the sample with and without an additive allows for estimation of the signal-to-protein ratio and, thus, calculation of the protein concentration in the sample. For this, it is necessary that in the entire range of measured concentrations, strict proportionality of the signal and concentration is observed. Proportionality was verified by measuring the fluorescence of IgG solutions in the supernatants after centrifugation of GNPs.

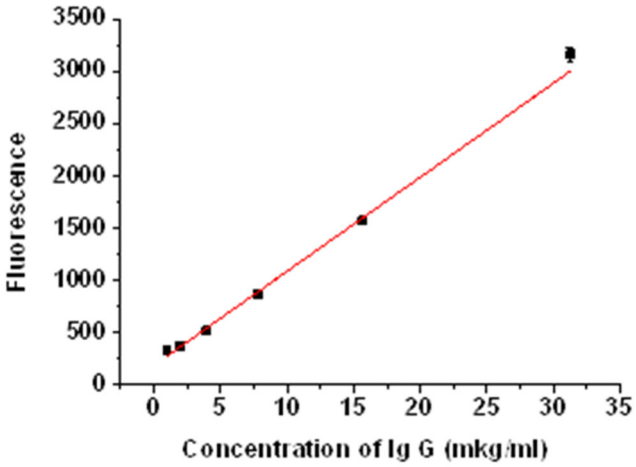

Figure 1 Fluorescence in solutions containing known IgG concentrations

The dependence of the fluorescence intensity at 350 nm on the concentration of added protein was determined in the calibration solutions and in the test samples. In the concentration range of 0-30 µg/mL, dependence of the intensity of the protein fluorescence on the concentration in the calibration solution was linear (R was >0.99; **Figure 1**).

Next, fluorescence was measured in the samples obtained after centrifugation of the conjugates of GNPs with IgG and calibration solutions. The calibration solutions for each sample were aliquots of the corresponding

sample with added IgG at a final concentration of 6 µg/ml. The volume of an aliquot after the addition of IgG changed by no more than 2.5 %; therefore, the change in the concentrations of the components of the sample can be neglected.

The obtained fluorescence values for 4 samples and 4 corresponding calibration solutions are presented in **Figure 2**.

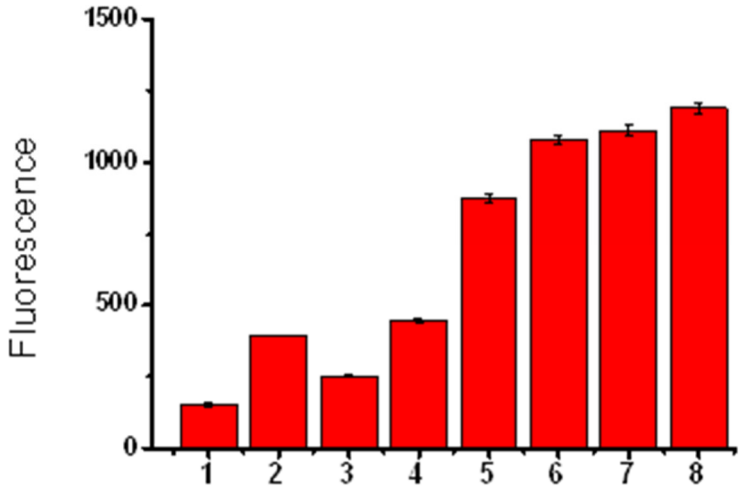

Figure 2 Fluorescence in supernatants of conjugate samples (1-4) and calibration solutions (5-8). The supernatants of the GNPs conjugates: 1-2.3 µg/ml of IgG, covalent conjugation; 2-4.6 µg/ml of IgG, covalent conjugation; 3-2.3 µg/ml of IgG, adsorption conjugation; 4-4.6 µg/ml of IgG, adsorption conjugation.

Based on the obtained fluorescence values, the protein concentrations in the supernatants and in the conjugates were calculated (**Table 2**).

Table 2 Quantitative parameters of GNPs and added IgG during conjugation

Sample	IgG concentration in the supernatant	IgG concentration in the conjugate	% of binding
GNPs + 2.3 µg/ml of IgG, covalent conjugation	1.3	1.0	43
GNPs + 4.6 µg/ml of IgG, covalent conjugation	3.4	1.2	26
GNPs + 2.3 µg/ml of IgG, adsorption conjugation	1.8	0.5	22
GNPs + 4.6 µg/ml of IgG, adsorption conjugation	3.6	1.0	22

The presented data indicate a more efficient binding of IgG during covalent immobilization by OPSS-PEG-NHS than with adsorption. However, for a denser fit, the advantage of the covalent method is negligible. Probably, in this case, steric difficulties for contact of IgG with the surface became a limiting factor.

4. CONCLUSION

Covalent immobilization of IgG appears to be a more effective way of functionalizing GNPs than physical adsorption. Despite the complication of the conjugation procedure, covalent immobilization allows the achievement of a higher coverage of the particle surface by IgG. In addition, a higher strength of interaction suggests the greater stability of covalent nanoconjugates during their storage.

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