International Journal of Applied Engineering Research ISSN 0973-4562 Volume 12, Number 24 (2017) pp. 15464-15469

Study of the Composition of Gold Nanoparticle—Immunoglobulins G Conjugates Using Dissociating Lanthanide Label

D.V. Sotnikov*, A.Sh. Radchenko, A.V. Zherdev and B.B. Dzantiev

A.N. Bach Institute of Biochemistry,
Federal Research Center «Fundamentals of Biotechnology» of the Russian Academy of Sciences,
Leninsky prospect 33, Moscow 119071, Russia.
** Corresponding author*

Abstract

The content of immunoglobulins G in their conjugates with gold nanoparticles (25 nm in diameter) was characterized by fluorescence spectroscopy using europium as the marker and the N1-(p-isothiocyanatobenzyl)-diethylenetriamine-N1,N2,N3,N4-tetraacetate as the chelator. Europium dissociated from the its complex with the chelator in an acid medium, and then, after centrifugation and separation of gold nanoparticles, the fluorescence of the europium with β-diketone-tri-n-octylphosphine in a micellar aqueous solution was measured. We demonstrated that the removal of gold nanoparticles, which are fluorescence quenchers, provides a highly sensitive detection and a correct evaluation of the conjugates' composition. The concentration dependency of the sorption of antibodies on the surface of gold nanoparticles is nonlinear: at small IgG concentrations, a large part of the molecules are sorbed onto the particles, and at high levels only about 20% of the added IgG is adsorbed. The dependence of the number of bound antibodies does not reach the plateau after reaching the limit for monolayer binding, which may indicate the formation of additional layers on the gold nanoparticles' surface.

Keywords: gold nanoparticles, immunoglobulins, fluorescent dissociating label, europium

INTRODUCTION

To date, labelled antibodies are one of the most commonly used detection agents in analytical systems, and coloured particles, primarily gold nanoparticles (GNPs), are the dominant type of labels for the antibodies [1]. The labelling with GNPs has undeniable advantages because of the special plasmon properties that enhance the recorded absorption of light [2]. The stability of the GNPs and the simplicity of their functionalization are other important factors for their choice.

The effectiveness of the use of GNP-antibody conjugates depends to a large extent on the composition of these preparations. A number of studies have demonstrated that varying this composition can significantly affect the affinity of the interaction with the analyte and the minimum detectable concentrations [3-6]. Preparations of conjugates of different composition can differ in these characteristics by 1–3 orders of magnitude [5]. In analytical systems that perform competitive immunodetection of low-molecular weight analytes, there is a certain optimal antibody:GNP ratio, which simultaneously provides effective competition and formation of a sufficient number of complexes for reliable registration [7]. In noncompetitive systems of immunodetection of high-molecular compounds, it is potentially preferable to achieve the maximal antibody:GNP ratio. However, the excessive consumption of antibodies not accompanied by a gain in analytical characteristics is not justified, and the saturation of both high- and low-affinity binding sites on the surface of the particle leads to dissociation of the antibodies during the assay and a decrease in the assay results [8].

Despite the relevance of information on the composition of GNP-antibody conjugates, in most cases, researchers continue to be guided by empirical recommendations, for example, by selecting the composition of the conjugates based on data on their flocculation in solutions with high ionic strength [9-10]. Studies of the concentration dependencies for antibodies binding to GNPs showed that this interaction is sensitive to immobilization conditions and differs for various antibody preparations [11-13]. Many of the approaches used to characterize the composition of the conjugates are methodically complex and time-consuming (e.g., the use of isotope labels) or are based on modification of antibodies that change their adsorption and antigen-binding properties [14].

In this paper, we demonstrate the possibility of using europium ion, a fluorescent label attached to antibodies by chelator, for these purposes. Analytical applications of this reagent are popular due to easy release from its complex and the possibility of sensitive detection [15]. However, the europium label is not studied as a means to characterize processes of protein adsorption on the surface of nanoparticles. It should be noted that the optical properties of the GNPs cause fluorescence quenching [16], and therefore the work with fluorescent labels in the characterization of GNP conjugates requires additional methodological solutions.

EXPERIMENTAL

Materials

We used monoclonal immunoglobulin G (IgG) from Imtek (Russia), bovine serum albumin (BSA) from MP Biomedicals (USA), and chloroauric acid from Fluka (Germany). All salts used were analytical or reagent grade. All solutions were prepared using deionized water with a resistance of 18.2 MV/cm at 25 °C that was obtained by a Simplicity system from Millipore (USA).

Antibody labelling with a fluorescent label [17]

Chelate derivative of europium (Chel-Eu), namely N1-(p-isothiocyanatobenzyl)-diethylenetriamine-N1,N2,N3,N4-tetraacetate of Eu^{3+} from PerkinElmer (Finland) was used for labelling the antibodies. An IgG solution (2 mg/mL) in 0.2 M carbonate buffer, pH 8.5, was mixed with 0.2 mL of a 7.5 mM aqueous solution of Chel-Eu and incubated overnight at +4 °C.

The modified antibodies were purified by gel filtration using a 1.5x28 cm column with Sephadex G-50 in 50 mM Tris-HCl with 0.15 M NaCl and 0.05% NaN_3, pH 7.75. The optical density of the obtained fractions at 280 nm was measured by Shimadzu 1202 (Japan) spectrophotometer.

To determine the amount of Chel-Eu bound to antibodies, their modified preparation was diluted in DELFIA® Enhancement Solution (PerkinElmer, Finland), incubated for 2 min, and then time-resolved fluorescence was measured by Wallac 1234 Fluorometer (Perkin Elmer, Finland) under the following parameters: delay time 0.1 ms, flash frequency 1 ms, signal read time 0.4 ms, excitation at 340 nm, emission at 615 nm.

Preparation of gold nanoparticles by the Frens method [18]

To obtain GNPs with an average diameter of 25 nm, 2.95 mL of 0.34% chloroauric acid was added to 97.5 mL of boiling deionized water. The mixture was boiled for 2 min and stirred. Then, 1.5 mL of a 1% solution of sodium citrate was added, stirred, boiled for 30 min, and then cooled to room temperature. The obtained preparation was stored at 4 °C.

Determination of the GNPs dimensions by transmission electron microscopy

GNP preparations were applied to hexagonal copper grids (200 mesh) coated with Formvar film. The JEM-100 CX/SEG (Jeol, Japan) transmission electron microscope was used for the analysis. Images of the particles were processed by the Image Tool program, measuring the largest and smallest particle diameters.

Preparation of the GNPs conjugates with IgG [19]

We mixed 4 mL of GNP solution (adjusted to pH = 9.0 with 0.1 M K_2CO_3) with IgG reaching their final concentrations 40, 30, 20, 15, 10, 5, and 1 mkg/mL. The mixture was incubated for 30 min while it was stirred at room temperature. Then 250 mkL of 10% BSA were added and incubated for 15 min at room temperature while being stirred. The solution was centrifuged for 30 min at 4 °C and 10,000 g. The supernatant was decanted and the precipitate was adjusted to 1 mL by 10 mM Tris-HCl, pH 9.0, with 1% BSA. Then the procedure of centrifugation, decanting, and dilution was repeated 2 more times.

Dissociative lanthanide fluoroimmune analysis (DELFIA) of conjugates using fluorescence enhancement

IgG preparations and their conjugates with GNPs were mixed with 2-naphthyltrifluoroacetone and a fluorescence-enhancing micellar solution, pH 3.2, containing trioctylphosphine oxide and Triton X-100.

IgG was diluted in this enhancing solution to 200 mkL, incubated for 10 min, and fluorescence was then measured. The amount of the europium label in the resulting preparations was calculated from the comparison of their fluorescence and fluorescence of calibration solution of native europium.

Then the enhancing solution (500 mkL) was added to the conjugate (100 mkL), mixed, and centrifuged for 20 min at 4 °C at 10,000 g; 200 mkL of supernatant was taken to measure the fluorescence and calculate contents of the europium label and labelled IgG.

RESULTS AND DISCUSSION

Evaluation of the size and homogeneity of the GNPs

The sizes of the obtained GNPs were characterized using the transmission electron microscopy. The data of the micrograph analysis are given in Table 1. The average particle diameter was 25 nm with a variation of 20% typical for this method of synthesis.

Table 1: Characteristics of the dimensions of the GNPs (n = 86).

Length of major axis, nm	25.7±4.8
Length of minor axis, nm	23.8±4.3
Average diameter, nm	24.8±4.8
Ellipticity	1.15

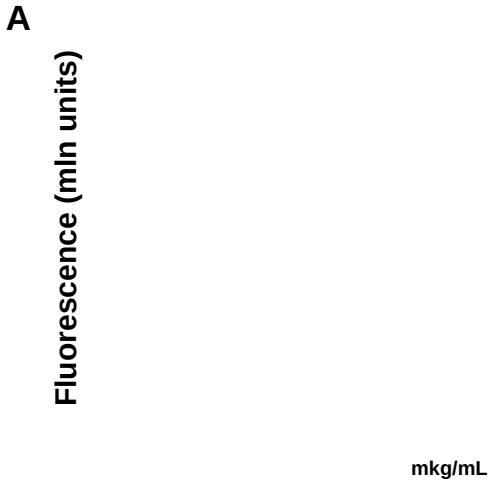
Antibodies labelling by Chel-Eu

After conjugation with Chel-Eu and purification, the concentration of IgG was determined by absorption spectroscopy. This value was equal to 0.79 mg/mL. The

content of the conjugated label, calculated from its fluorescence, was 2.8 mkM. Based on these values, we have a molar ratio of Eu^{3+}:IgG in the conjugate equal to 0.57, which indicates a weak modification of the native structure of immunoglobulins.

Obtaining calibration curve for the determination of labelled antibodies concentration the europium fluorescence

To obtain the calibration curve, solutions of labelled IgG with concentrations of 100, 50, 25, 12.5, 6.25, 3.1, and 1.5 mkg/mL in the enhancement solution were used. The registered fluorescence values minus the background signal are shown in Fig. 1.



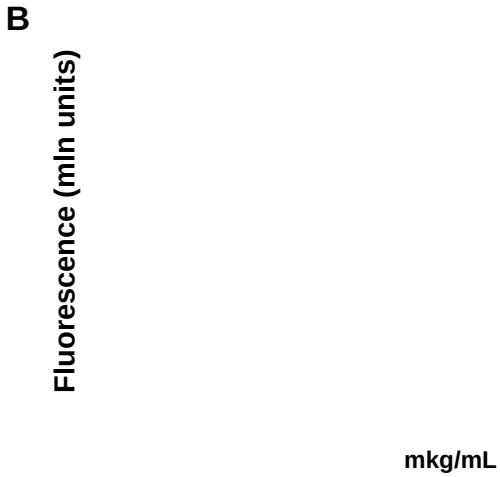


Figure 1: A. Fluorescence dependence on the concentration of the IgG-Chel-Eu conjugate. B. The linear portion of the dependence shown in Fig. A.

The dependence is linear up to IgG concentration of 6.25 mkg/mL (Fig. 1B); the R-factor of linearization is 0.99989. The linearizing dependence $y = a + b \cdot x$ is characterized by the values $a = (4.8 \pm 4.1) \cdot 10^4$; $b = (1.931 \pm 0.012) \cdot 10^9$. For further calculations of IgG concentrations, a linear part of the calibration curve was used.

Determination of the amount of antibodies in the GNP-IgG conjugate

The conjugates synthesized and purified by triplicate centrifugation were incubated in an extracting solution to release Eu^{3+}, then fluorescence was measured in solution. Based on the fluorescence data and the calibration curve, the concentration of labelled IgG was determined. The concentration of bound IgG in the starting solution for the conjugation was then calculated, taking into account the concentration-dilution factors. The obtained values are given in Table 2.

Table 2: Determination of the IgG amount in GNPs conjugates with different loading during conjugation.

C (IgG-Chel-Eu) upon conjugation, mkg/mL	Fluorescence, million units	C (IgG-Chel-Eu), mkg/mL	% of bound IgG-Chel-Eu from their initial amount	Number of IgG on one GNP
1	3.12	1.0	100.0	12,7
5	5.99	2.1	41.0	26,6
10	11.9	4.1	40.9	51,9
15	15.2	5.2	34.9	65,9
20	17.9	6.2	30.8	78,5
30	22.1	7.6	25.4	96,3
40	23.9	8.2	20.6	103,9

* The accuracy of the determination is 2%.

Table 3: Determination of the amount of antibodies in the supernatant after centrifugation of GNP conjugates with different antibody loads.

C (IgG-Chel-Eu) upon conjugation, mkg/mL	Fluorescence, million units	C (IgG-Chel-Eu) in the supernatant with 4-fold dilution, mkg/mL	% of free IgG-Chel-Eu from their initial concentration
1	– *	0	0
5	0.4	0.7	14.7
10	2.9	5.9	59.1
15	4.5	9.1	60.9
20	6.2	12.8	63.8
30	9.8	20.2	67.2
40	12.8	26.4	66.0

* The fluorescence in the solution is below the standard deviation of the background fluorescence.

Fluorescence measurements with a dissociating label exclude the influence of GNPs, which are powerful fluorescence modulators that do not allow detecting fluorescence directly from the surface of the conjugate.

As can be seen from the presented data, the percentage of bound IgG decreases with an increase in the concentration of added IgG. The resulting concentration dependence (Fig. 2) shows a gradual decrease in the slope of the curve and hence saturation of the surface. However, complete saturation is not observed, even at a concentration of 40 mkg/mL.

Figure 2: Dependence of the concentration of IgG-Chel-Eu, bound by GNPs, on the initially added concentration of IgG-Chel-Eu in the course of the synthesis.

Calculation of the antibody:GNP ratio

The surface area of one GNP with a diameter of 25 nm is $2 \cdot 10^3$ nm^2. At saturating amounts of antibodies in the case of monolayer formation, the orientation of the Fc fragment to the direction of the particle surface is most probable [20]. The area of the base of the Fc fragment is 26 nm^2. If we do not take into account steric hindrance caused by the Fab fragments, then up to 76 molecules of IgG can fit on the surface of a single GNP with a diameter of 25 nm.

However, the experimental data presented in Table 3 (last column) demonstrate the sorption of more IgG. Given that we did not reach saturation of the surface (see Fig. 2), the formation of polylayer structures can be assumed. Such structure accords to data in previous works [21-23].

Determination of free IgG concentration in the supernatant after the first centrifugation of the conjugate

The technique used in the work allows us to characterize the composition of conjugates in two ways: by measuring the concentration of the bound protein and by measuring the protein concentration in the supernatant and further calculation of material balance. The comparison of these data gives possibility to evaluate the accuracy of the measurements.

The measured fluorescence values for supernatants and the calculated concentrations of free IgG are presented in Table 3.

It can be seen that at concentrations of up to 5 mkg/mL, almost all IgG are adsorbed on GNP surface. Then, as the surface becomes saturated, fewer and fewer immunoglobulins are absorbed and the dependence of free IgG concentration on the added IgG concentration becomes almost linear (Fig. 3).

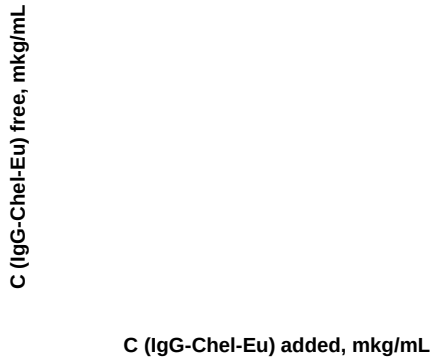


Figure 3: Dependence of the IgG-Chel-Eu concentration in the supernatant after the first centrifugation of the conjugate, from the initial concentration of the IgG-Chel-Eu during the synthesis.

To confirm the obtained data about the composition of conjugates, we compared the results for bound and unbound IgG. The curves in Fig. 4 show a good conformity of these values. When the percentages of immunoglobulins in the conjugate and in the supernatant are summed for cases of initially added IgG 1, 5, 10, 15, 20, 30, and 40 mkg/mL, the following results are obtained: 100.0, 55.8, 100.0, 95.8, 94.6, 92.6, and 86.6%, respectively. With the exception of the second sample (probably ejection), the loss was varied from 0 to 13.4%, which can be related to IgG lost as a result of supernatant separation and measurement errors. As a whole, both kinds of measurements demonstrate good complementarity and confirm the efficiency of the proposed approach.

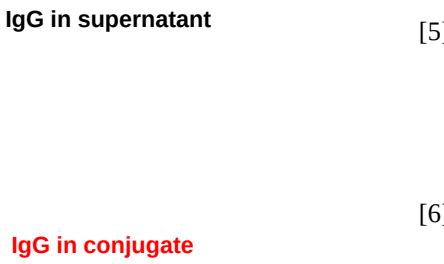


Figure 4: Percentage of IgG-Chel-Eu distributed between the conjugate and the supernatant.

CONCLUSION

The proposed procedure allows reliable determination of the conjugates' composition. Due to the ability to measure the concentrations of both bound and unbound proteins, it also makes it possible to control the accuracy of the measurements. On the example of conjugates of gold nanoparticles and IgG (pH of conjugation 9.0), high reliability of the presented technique is shown. The possibility of multilayer structures formation on the surface of nanoparticles with 25 nm in diameter is established.

ACKNOWLEDGMENTS

This study was financially supported by the Ministry of Science and Education of the Russian Federation (grant agreement No. 14.613.21.0080 on 22.11.2017, unique identifier RFMEFI61317X0080).

REFERENCES

[1] Goryacheva, I. Y., 2016, "Formats of rapid immunotests—Current-day formats, perspectives, pros and cons," Compr. Anal. Chem., 72, pp. 33–78.

[2] Amendola, V., Pilot, R., Frasconi, M., Maragò, O. M., and Iatì, M. A., 2017, "Surface plasmon resonance in gold nanoparticles: a review," J. Phys. Condens. Matter., 29(20), 203002.

[3] Li, M. H., Choi, S. K., Leroueil, P. R., and Baker, J. R. Jr., 2014, "Evaluating binding avidities of populations of heterogeneous multivalent ligand-functionalized nanoparticles," ACS Nano, 8(6), pp. 5600–5609.

[4] Wang, W. J., Voigt, A., Wolff, M. W., Reichl, U., and Sundmacher, K., 2016, "Binding kinetics and multi-bond: Finding correlations by synthesizing interactions between ligand-coated bionanoparticles and receptor surfaces", Anal. Biochem., 505, pp. 8-17.

[5] Safenkova, I. V., Zherdev, A. V., and Dzantiev, B. B., 2010, "Correlation between the composition of multivalent antibody conjugates with colloidal gold nanoparticles and their affinity," J. Immunol. Methods, 357(1), pp. 17-25.

[6] Thyparambil, A. A., Wei, Y., and Latour, R. A., 2015, "Experimental characterization of adsorbed protein orientation, conformation, and bioactivity," Biointerphases, 10(1), 019002.

[7] Zvereva, E. A., Byzova, N. A., Sveshnikov, P. G., Zherdev, A. V., and Dzantiev, B. B., 2015, "Cut-off on demand: Adjustment of the threshold level of an immunochromatographic assay for chloramphenicol," Anal. Methods, 7(15), pp. 6378-6384.

[8] Byzova, N. A., Safenkova, I. V., Slutskaya, E. S., Zherdev, A. V., and Dzantiev, B. B., 2017, "Less is more: A comparison of antibody–gold nanoparticle conjugates of different ratios," Bioconjugate Chem., 28(11), pp. 2737-2746.

[9] Wiriyachaiporn, N., Maneeprakorn, W., Apiwat, C., and Dharakul, T., 2014, "Dual-layered and double-targeted nanogold based lateral flow immunoassay for influenza virus," Microchim. Acta, 182(1-2), pp. 85-93.

[10] Bell, N. C., Minelli, C., and Shard, A. G., 2013, "Quantitation of IgG protein adsorption to gold nanoparticles using particle size measurement," Anal. Methods, 5(18), pp. 4591-4601.

[11] Mi, L. J., Zhang, X., Yang, W. C., Wang, L. H., Huang, Q., Fan, C. H., and Hu, J., 2009, "Artificial nano-bio-complexes: Effects of nanomaterials on biomolecular reactions and applications in biosensing and detection," J. Nanosci. Nanotechnol., 9(4), pp. 2247-2255.

[12] Ansari, S. A., and Husain, Q., 2012, "Potential applications of enzymes immobilized on/in nano materials: A review," Biotechnol. Adv., 30(3), pp. 512-523.

[13] Lacerda, S. H. D. P., Park, J. J., Meuse, C., Pristinski, D., Becker, M. L., Karim, A., and Douglas, J. F., 2010, "Interaction of gold nanoparticles with common human blood proteins" ACS Nano, 4(1), pp. 365-379.

[14] Sapsford, K. E., Tyner, K. M., Dair, B. J., Deschamps, J. R., and Medintz, I. L., 2011, "Analyzing nanomaterial bioconjugates: a review of current and emerging purification and characterization techniques," Anal. Chem., 83(12), pp. 4453-4488.

[15] Yuan, J., and Wang, G., 2005, "Lanthanide complex-based fluorescence label for time-resolved fluorescence bioassay," J. Fluorescence, 15(4), pp. 559-568.

[16] Kang, K. A., Wang, J., Jasinski, J. B., and Achilefu, S., 2011, "Fluorescence manipulation by gold nanoparticles: from complete quenching to extensive enhancement," J. Nanobiotechnology, 9(1), 16.

[17] http://www.perkinelmer.com/CMSResources/Images/44-73411man_1244-302.pdf

[18] Frens, G., 1973, "Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions," Nature (London), Phys. Sci., 241(105), pp. 20–22.

[19] Safenkova, I., Zherdev, A., and Dzantiev, B., 2012, "Factors influencing the detection limit of the lateral-flow sandwich immunoassay: a case study with potato virus X," Anal. Bioanal. Chem., 403(6), pp. 1595-1605.

[20] Jurgens, L., Nichti, A., and Werner, U., 1999, "Electron density imaging of protein films on gold-particle surfaces with transmission electron microscopy," Cytometry Part A, 37(2), pp. 87-92.

[21] Monopoli, M. P., Walczyk, D., Campbell, A., Elia, G., Lynch, I., Bombelli, F. B., and Dawson, K. A., 2011, "Physical-chemical aspects of protein corona: Relevance to in vitro and in vivo biological impacts of nanoparticles," J. Am. Chem. Soc, 133(8), pp. 2525-2534.

[22] Docter, D., Westmeier, D., Markiewicz, M., Stolte, S., Knauer, S. K., and Stauber, R. H., 2015, "The nanoparticle biomolecule corona: Lessons learned – challenge accepted?" Chem. Soc. Rev., 44(17), pp. 6094-6121.

[23] Pearson, R. M., Juettner, V. V., and Hong, S., 2014, "Biomolecular corona on nanoparticles: A survey of recent literature and its implications in targeted drug delivery," Front. Chem., 2, 108.

LIST OF SYMBOLS AND ABBREVIATIONS

BSA – bovine serum albumin

Chel-Eu – chelate derivative of europium

DELFIA – dissociative lanthanide fluoroimmune analysis

GNP – gold nanoparticle

GNP-IgG – conjugate of GNP and IgG

IgG – immunoglobulin G

IgG-Chel-Eu – conjugate of IgG and Chel-Eu

°C

C

Eu^{3+}

g

mg/mL

min

mL

nm

mM

ms

MV/cm

mkg/mL

mkL

n

nm^2

pH