Synthesis of the Most Effective Streptavidin Conjugates with Small Gold Nanoparticles for Indirect Labeling in Lateral Flow Assay

Alexey V. Samokhvalov1,5, Shyatesa C. Razo1,2, Irina V. Safenkova1, Elvira S. Slutskaya1, Svetlana M. Pridvorova4, Anatoly V. Zherdev1, Boris B. Dzantiev1*

1A.N. Bach Institute of Biochemistry, Research Center of Biotechnology of the Russian Academy of Sciences, Leninsky prospect 33, Moscow 119071, Russia.
2Agricultural-Technological Institute, Peoples’ Friendship University of Russia, Mikluho-Maklaya street 8/2, Moscow 117198, Russia.

*Correspondence
Orcid: 0000-0002-3621-4321

Abstract

The use of gold nanoparticles (GNPs) with a small size (diameter <10 nm) for the immobilization of biomolecules requires the small area of each nanoparticle and the large curvature of the surface to be taken into account. The aim of this study is to determine the optimal conditions for the biomolecules’ immobilization using small GNPs synthesized in different ways. The GNPs were synthesized by the two most typical methods: using either sodium borohydride or a combination of sodium citrate and tannic acid as reducing agents. The average diameters were determined to be equal to 4.4 ± 0.6 and 6.9 ± 1.5 nm by transmission electron microscopy, respectively. Based on these nanoparticles, 24 conjugates were synthesized and characterized at different pH (6.5, 7.4, and 9.0) and different concentrations of immobilized protein (streptavidin with concentrations of 20, 40, 60, and 80 μg/mL). Depending on the synthesis conditions, polydispersity indexes and formed precipitates varied, and the hydrodynamic diameters of the conjugates varied from 1570 to 15 nm. For both of the GNPs preparations, the conjugates that were most monodisperse, stable, and without aggregate formation were obtained at pH 9.0 with streptavidin concentrations of 40 (GNPs by sodium citrate/tannic acid) and 80 μg/mL (sodium borohydride). The binding properties of the all streptavidin–GNP conjugates synthesized at pH 9.0 were tested using lateral flow tests. All conjugates showed high binding properties up to 0.004 optical densities for conjugate λmax. This study has great potential to improve the synthesis of the conjugates with small GNPs for immunoassay applications.

Keywords: conjugates with gold nanoparticles; gold nanoparticles; lateral flow tests; streptavidin; small nanoparticles.

INTRODUCTION

Gold nanoparticles (GNPs) and their conjugates are some of the most studied nanomaterials, with promising applications in many immunoanalytical systems for medicine and agriculture [1, 2]. As in most of these applications, the monodispersity of GNPs is a desirable feature; moreover, synthesis should result in controlled NP size, distribution, shape, and stability. To date, chemical reduction is still the most common strategy for the synthesis of GNPs, most probably because of the simplicity of the method and apparatus required [3, 4]. A variety of reducing (for example, borohydrides, sodium citrate, aminoboranes, hydrazine, and formaldehyde) and stabilizing agents (hydrazine, ascorbic acid, cetyltrimethylammonium bromide, poly(vinyl alcohol), poly(vinylpyrrolidone), poly(ethylene glycol), sodium citrate, and tannic acid) can be used to synthesize GNPs [5–10]. Related to this, the second important point is the coating of nanoparticles with stabilizing molecules that further influence the immobilization of target biomolecules (antibodies, enzymes, streptavidin, etc.). The method of GNP synthesis affects the surface features, which can affect the immobilization of biomolecules [11, 12]. For small GNPs (diameter <10 nm), these are particularly relevant issues related to the small area of each nanoparticle and the large curvature of the surface [13, 14]. In these terms, uniform, spherical, small GNPs can only be obtained using a limited number of methods. Under these conditions, the number of syntheses of small GNPs deserving attention is limited.

One of the most common syntheses for obtaining monodisperse and stable small GNPs (5–10 nm diameter) is a chemical synthesis with tannic acid and sodium citrate as both reducing and stabilizing agents [15, 16]. The combined use of sodium citrate and tannic acid enables control over the nucleation, growth, and stabilization processes, which leads to reproducible monodisperse GNPs. Ranoszczek-Soliwoda et al. showed that the formation of a sodium citrate–tannic acid complex acts as a reducing agent in the synthesis of NPs [17]. As a result, NPs are stabilized by the oxidation products of the complex [17]. The second typical synthesis for obtaining small GNPs is based on sodium borohydride reduction [18–20]. Although the preparation of GNPs by both chemical reductions can probably make a stable and reproducible colloid containing spherical GNPs, the surface coverage of the nanoparticles is different. This factor is of great importance for synthesizing conjugate biomolecules immobilized on the GNP surface by nondirectional and noncovalent physical adsorption [12, 21]. It is well known that the main active forces of noncovalent immobilization are donor–acceptor interactions involving SH-
groups of molecules, hydrophobic interactions, and Coulomb interactions between \( \text{NH}_2 \) groups of lysine and ions on the surfaces of GNPs [22, 23].

In this paper, we present the comparison of the conditions for the synthesis of GNP conjugates synthesized by reduction with the sodium citrate/tannic acid and the sodium borohydride methods. We synthesized the GNP conjugates under different conditions, and experimentally defined that the pH affects the characteristics of the resulting conjugates in different ways. For immobilization to the GNP surface, we chose streptavidin, which forms a high-affinity strong noncovalent interaction with biotin [24, 25]. We used a lateral flow assay (LFA), the most common method using GNPs as a colored label, to find the effective streptavidin–GNP conjugates for improving the sensitivity of the assay. Streptavidin–GNP conjugates can be used to detect, localize, or quantify the binding of biotinylated molecules in the test zone of lateral flow tests. The streptavidin–GNP conjugate is a very effective tool as a signal amplifier and for increasing sensitivity, particularly for the indirect labeling in LFA [26–28]. Targeting molecules need not be directly modified with GNPs, only biotinylated so that they are able to interact with streptavidin–GNP conjugates. The formation of the GNP–streptavidin complexes with biotinylated antibodies in the test zone of the lateral flow test strips leads to an increase in the number of bound GNP labels and improves the sensitivity of LFA.

**MATERIALS AND METHODS**

### Reagents

Polyclonal antibodies (fraction of IgG) specific to plant virus were used for biotinylation and testing streptavidin–GNP conjugates. Streptavidin from *Streptomyces avidinii* was purchased from Imtech (Russia). Tetrachloroauric acid, tannic acid, and sodium borohydride were purchased from Fluka (Taufkirchen, Germany). Tris(hydroxymethyl)aminomethane, nitric acid (70% w/w, purified by redistillation), bovine serum albumin (BSA), biotin amidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester, and sodium citrate were purchased from Sigma-Aldrich (USA). The Au standard for inductively coupled plasma mass spectrometry (ICP-MS) (999 ± 2 mg/mL, in 5% HCl [w/w]) came from Fluka, Switzerland; ICP-MS internal standards came from Bruker Daltonics Chemical Analysis, USA; hydrochloric acid (38% w/w, ultrapure) came from Reactiv-component, Russia. All acids, alkali, salts, and solvents were purchased from Khimmed (Moscow, Russia). The compounds used in buffer solutions were of analytical or chemical grade. Solutions were prepared using water deionized by a Milli-Q system produced by MilliPore (USA). Lateral flow test strips were fabricated using plastic supports with a nitrocellulose membrane (CNPC 12) and absorbent pads (AP045) produced by Advanced Microdevices (India).

### Synthesis of gold nanoparticles using sodium citrate/tannic acid

GNPs were prepared according to the protocol described by Hermanson [29] with modification. For the synthesis of 100 μL of GNPs, 1 μL of 1% aqueous solution of tetrachloroauric acid was added to 79 μL of deionized water, then heated to 60 °C. Simultaneously, aqueous solutions of 1% sodium citrate (4 mL), 1% tannic acid (0.5 mL), 25 mM potassium carbonate (0.5 mL), and deionized water (15 mL) were mixed and also heated to 60 °C. Then, two solutions at 60 °C were mixed and refluxed with slight stirring until the tetrachloroauric acid had been completely reduced and a stable colloidal solution had formed. The solution was then cooled to room temperature.

### Synthesis of gold nanoparticles using sodium borohydride

GNPs were prepared according to the protocol described by Dykman [30] with modification. Aqueous solutions of 30 mM EDTA (0.5 mL) and potassium carbonate (0.2 mL) were added to an Erlenmeyer flask with deionized water (48.7 mL). The mixture was cooled to 4 °C and all further processes were carried out at this temperature. Then, 1% (w/v) aqueous solution of tetrachloroauric acid (0.5 mL) was added with intense stirring. Following this, 0.5% (w/v) aqueous solution of sodium borohydride (125 μL) was quickly added to the flask. The reaction mixture was incubated for the night at 4–6 °C with intense stirring.

### Synthesis of gold nanoparticle conjugates

Synthesis of streptavidin–GNP conjugates was carried out as described by Hermanson [29] with modification. The streptavidin conjugates were synthesized with both kinds of GNPs at pH 6.5, 7.4, and 9.0. To adjust the pH of GNPs, 0.2 M potassium carbonate solution or 0.02 M HCl were used. To determine the minimal stabilizing concentration of the streptavidin for the conjugation (floculation method), the obtained GNP solution (1.0 mL portions) was added to solutions (0.1 mL) of streptavidin in concentrations from 1 to 250 μg/mL. The mixture was incubated at room temperature for 10 min. Then we added a 10% NaCl solution (0.1 mL) to each sample, stirred the mixtures for 10 min, and measured OD at 580 nm, plotting its dependence in the presence of 10% NaCl against the streptavidin concentration (floculation curves). The concentration corresponding to the beginning of the plateau in the flocculation curve was determined, and a concentration exceeding this value by 10–15% was chosen for streptavidin conjugation.

To synthesize streptavidin–GNP conjugates, streptavidin was added to GNPs (at pH 6.5, 7.4, and 9.0) to the final concentration of 20, 40, 60, and 80 μg/mL. The mixture was incubated without stirring at 20–22 °C for 30 min. Then, BSA was added to each sample to the final concentration of 100 μg/mL. The separation of unbound streptavidin was carried out.
using Beckman Coulter Optima L-100XP ultracentrifuge (USA) at 45,000 g for 25 min. After the removal of the supernatant, the precipitate was resuspended in 10 mM Tris-HCl buffer, pH 8.5. The spectra of the GNPs and their conjugates were recorded with a Biochrom Libra S60 double beam spectrophotometer (Biochrom, UK).

Transmission electron microscopy
The size and shape of GNPs were determined using transmission electron microscopy (TEM) (Jeol CX-100 electron microscope, Japan; accelerating voltage 80 kV; magnification of 3,300,000–25,000,000x). The GNPs samples for TEM measurements were dropped onto carbon-coated copper grids (300 mesh, Pelco International, USA) coated with polyvinyl formal support film. The obtained images of GNPs and their conjugates were digitalized with Image Tool software (University of Texas Health Science Center in San Antonio, USA). To approximate TEM data about the distribution of GNPs’ diameters, one-peak Gauss approximation was used. The fitting was calculated by OriginPro 9.0 software (Origin Lab, USA).

Dynamic light scattering and electrophoretic light scattering
All experiments were performed with a Zetasizer Nano ZSP (Malvern Instruments, UK), which features a 4 mW He–Ne laser (633 nm). The temperature was stabilized at 25°C, and the scattering angle was 173°. All measurements were performed at least 80 times.

A disposable cell (DTS0012; Malvern Instruments, UK) was used for dynamic light scattering (DLS) measurements. The hydrodynamic sizes of GNPs and streptavidin–GNP conjugates were measured by DLS. The polydispersity index (PdI) was calculated as: (standard deviation/mean value)².

For determining the streptavidin isoelectric point (pI), zeta potentials of the streptavidin were measured at different pH (4.0, 5.0, 6.0, and 7.0) using electrophoretic light scattering (ELS) according to the Malvern Instruments recommendation [31]. For ELS measurements, samples were loaded into a disposable clear zeta cell (DTS1060; Malvern Instruments, UK). Statistical processing was performed using Malvern software ver. 7.11.

Measurements of Au by inductively coupled plasma mass spectrometry
To estimate of GNPs and conjugate amounts in the solutions, Au concentrations were obtained by ICP-MS. The ICP-MS measurements were carried out with a quadrupole ICP-MS instrument Aurora M90 (Bruker Corp., USA) equipped with a MicroMist low-flow nebulizer. A series of Au standard solutions (0.1–5.0 ppb in 1% HCl [v/v]) were prepared before each experiment. Scandium was used as the internal standard, eliminating the fluctuations coming from the measuring conditions. All samples were prepared in triplicate. Quantum software (Bruker Corp., version v.3.1) was used for data collection and processing. The measurements and calculations were carried out as described previously by Byzova et al. [31, 32].

The ICP-MS measurements were carried out on equipment at the Shared Access Equipment Centre “Industrial Biotechnology” of the Federal Research Center “Fundamentals of Biotechnology” of the Russian Academy of Sciences (Moscow, Russia).

Biotinylation of antibodies
For testing streptavidin–GNP conjugates, polyclonal antibodies were biotinylated as described by Hermanson [29]. The antibodies were dialyzed against a 1,000-fold volume of 50 mM potassium phosphate, pH 7.4, 0.14 M NaCl (PBS), for at least 14 hours at 4 °C. Then, biotin amidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester (10 mg/mL in dimethyl sulfoxide) was added to the antibodies in a 20:1 molar ratio. The mixture was incubated for 1 hour at room temperature under intensive stirring and then dialyzed against PBS overnight.

Preparation of lateral flow test strips
A binding zone was formed on the nitrocellulose membrane CNPC 12 using biotinylated antibodies. We dispensed biotinylated antibodies (1 mg/mL) at 0.15 µL per mm membrane width in PBS containing 5% glycerol [33] using IsoFlow dispenser (Imagene Technology, USA). The nitrocellulose membranes were dried at 37 °C for 8 h. Following this, we attached adsorbed pads AP045 to plastic supports with the nitrocellulose membranes. After assembling the membranes, we used an Index Cutter-1 (Gibbstown, NJ, USA) to cut multimembrane composites into 3.5 mm-wide strips and used an FR-900 continuous band sealer (Wenzhou Dingli Packing Machinery, China) to seal the strips hermetically into bags composed of laminated aluminum foil with silica gel as a desiccant. We carried out the cutting and packing at 20–22 °C in a separate room with a relative humidity of no more than 30%.

Lateral flow assay and data processing
The synthesized streptavidin–GNP conjugates were adjusted to the same optical density at λmax using 10 mM Tris-HCl buffer, pH 8.5. Then, PBS with 0.05% Triton X-100 (PBST) was added to final optical density (λmax) of conjugates corresponding 0.2, 0.07, 0.02, and 0.004. The test strips were dipped into the obtained conjugate solutions. Ten minutes after
beginning the assay, the result was visually checked, a digital image of the test strips was obtained with a CanoScan LiDE 90 scanner, and the integrated intensities of the color in the binding zones were calculated as described previously by Safenkova et al. [34].

RESULTS AND DISCUSSION

Characterization of gold nanoparticles

GNPs synthesized by reduction with sodium citrate/tannic acid (SC–TA) were of an average diameter of 4.4 ± 0.6 nm (n = 133), and the form factor (ratio of maximum and minimum axes) was 1.1 ± 0.05 (Figure 1-A) by TEM. GNPs synthesized by reduction with sodium borohydride (BH) were of an average diameter of 6.9 ± 1.5 nm (n = 205), and the form factor was 1.1 ± 0.1 (Figure 1-B) by TEM. Thus, the obtained sizes were close, taking into account standard deviations. The TEM provided data regarding the true dimensions of GNP cores, facilitating their characterization. However, differences between the two methods of synthesis were determined, among other things, by the surface features of the nanoparticles, which are better characterized by the DLS.

The colloidal state and the hydrodynamic size of GNPs in the case of all samples were measured and calculated using DLS (Figure 2). According to DLS, the synthesized GNPs were monodispersed and their average diameter was 8.8 ± 1.8 and 5.7 ± 1.1 nm. Because the hydrated shell around the GNP contributed to the data of DLS measurements, the average hydrodynamic diameter of GNPs SC–TA was larger than GNPs BH. For both GNPs, polydispersities were closer: a PdI of 0.248 for GNPs SC–TA and 0.217 for GNPs BH. Here and further to evaluate the PdI values, we propose using the manufacturer’s recommendation [35], which indicates a PdI smaller than 0.05 (rarely seen) for highly monodisperse standards, and a PdI greater than 0.7 for samples with a very broad size distribution.

To further assess the amounts of GNPs and their conjugates and to evaluate the changes occurring during the synthesis of conjugates, GNP solutions were characterized with respect to their absorbance by UV–VIS spectrophotometry. UV–Vis spectroscopy is an simple method for detecting GNPs because GNPs exhibit a characteristic absorption peak at about 520 nm, which is attributed to surface plasmon excitation due to the combined vibration of free electrons in resonance with the light wave [36]. The absorption band maximum for GNPs SC–TA and GNPs BH was observed at λ = 521 nm and λ = 507 nm, respectively. Figure 3 shows the UV–Vis absorption spectra of GNPs synthesized using a mixture of both SC–TA and BH.

Figure 1: TEM images and size distribution histograms of GNPs synthesized using a mixture of sodium citrate and tannic acid (A) and sodium borohydride (B).
Figure 2: DLS size distribution histograms of GNPs synthesized using a mixture of sodium citrate and tannic acid (A) and sodium borohydride (B).

Figure 3: UV–Vis spectra of GNPs. Curves 1 and 2 represent the spectra of GNPs synthesized using a mixture of sodium citrate and tannic acid, and sodium borohydride, respectively.
Table 1: The overall results for the syntheses of GNPs using a combination of sodium citrate/tannic acid, and sodium borohydride.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>TEM diameter, nm</th>
<th>Form factor</th>
<th>DLS diameter, nm</th>
<th>PdI</th>
<th>λmax, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium citrate / tannic acid mixture</td>
<td>4.4 ± 0.6</td>
<td>1.1 ± 0.05</td>
<td>8.8 ± 1.8</td>
<td>0.248</td>
<td>521</td>
</tr>
<tr>
<td>Sodium borohydride</td>
<td>6.9 ± 1.5</td>
<td>1.1 ± 0.10</td>
<td>5.7 ± 1.1</td>
<td>0.217</td>
<td>507</td>
</tr>
</tbody>
</table>

Choice of conditions for conjugate synthesis

The main factors for effective conjugation by nondirectional and noncovalent immobilization by physical adsorption are the conjugation pH and concentration of biomolecules (streptavidin). Native streptavidin from *Streptomyces avidinii* used for conjugates’ syntheses has no carbohydrate and has an acidic isoelectric point [24]. The exact value of pI was found to be equal to 5.2 by ELS measuring zeta potentials of the streptavidin at different pH. Due to the symmetric tetrameric structure of the streptavidin [24], the orientation of streptavidin upon immobilization is minimally responsible for the efficiency of the biotin-binding. These circumstances determine the choice of the pH range. The classical recommendation regarding the functionalization of GNPs argues that the pH of the medium should be at least 0.5 units higher than the pI of the immobilized protein [37], at which point the transition of the protein from the dissolved to the aggregated state is energetically favorable. Therefore, the conjugation was performed at three pH values (6.5, 7.4, and 9.0).

The choice of streptavidin concentration was associated with full coverage of the GNP surface. For the starting point, we used a typical flocculation method determining the minimal stabilizing concentration of streptavidin (see Materials and methods, section 2.4). This means that the addition of 10% NaCl solution results in changes in the electric double layer of GNP and a shift in the equilibrium between the electrostatic repulsion and attraction of particles [38] and, correspondingly, the flocculation of the GNPs and the change in the solution color. The aggregation of GNPs in the presence of excess salt (10% NaCl) will stop if the GNP surface is covered with streptavidin in large part. An absorption change at the wavelength of 580 nm (Figure 4) indirectly indicates the presence of aggregates in the preparation of GNPs. However, the typical flocculation view was observed only for GNPs BH (Figure 4-B). For GNPs SC–TA, no change in color or corresponding change of OD at 580 nm were observed (Figure 4-A). The reason is probably associated with good stabilization of the GNPs’ surface by the complex of oxidized products of SC–TA [17]. Obviously, the flocculation method is not very informative for GNPs synthesized with SC–TA.

Figure 4: Flocculation curves of streptavidin’s immobilization on the GNP’s surface. Dependencies of the absorbance at 580 nm in the presence of excess salt (10% NaCl) for GNPs synthesized using a mixture of sodium citrate and tannic acid (A), and sodium borohydride (B) after the addition of different concentrations of the streptavidin. Curves 1, 2, and 3 represent the plots for the used pH 6.5 (A), 7.4 (B), and 9.0 (C), respectively.

The range of concentration dependence, starting from a concentration of 15 μg/mL (point on plateau curve) for GNPs BH at pH 9.0 (see Figure 4-B), corresponds to the stabilization of the GNP by streptavidin. With the addition of excess salt, conjugates synthesized at lower streptavidin concentrations...
were not stable. At the same time, the outlet to the plateau did not occur at pH 6.5 and 7.4 (see Figure 4-B). Taking into account the diameter of streptavidin (5 nm [39, 40]) and the average size of the nanoparticles determined by TEM (see section 3.1), approximately two to three molecules of streptavidin can be placed on one nanoparticle. This means that at 100% streptavidin binding to the GNP surface, all GNPs in the solution would be streptavidin covered even at 15 μg/mL. However, the percentage of streptavidin bonded to the surface of GNP depends on the equilibrium constants and curves in Figure 4-B, confirming the pH effect on the immobilization. For this reason, we synthesized conjugates with GNP BH at pH 6.5, 7.4, and 9.0 using streptavidin with concentrations more than that of the flocculation point (20, 40, 60, and 80 μg/mL). For GNP SC–TA, the same synthesis conditions were chosen.

Characterization of streptavidin–gold nanoparticle conjugates

Each of the 24 conjugates (12 GNP SC–TA and 12 GNP BH) were characterized before ultracentrifugation by dynamic light scattering. A summary of the data is shown in Table 2. For GNP SC–TA, precipitate formation and high PdI were observed for all streptavidin concentrations at pH 6.5 (see Table 2). At pH 7.4 these effects were less pronounced, and at high concentrations of streptavidin, the precipitate did not fall out, and the agglomerates formed became smaller. At pH 9.0, large aggregates were absent, and with an increase in streptavidin concentration from 20 to 80 μg/mL, a small decrease in hydrodynamic diameters from 25 (98.3%) to 15.6 nm (99.1%) occurred. The same effect was observed for GNP BH (see Table 2). Some difference in the synthesis of conjugates appeared at pH 7.4, at which conjugates with GNP BH had a lower polydispersity. The resulting distributions of the hydrodynamic diameters for streptavidin–GNP SC–TA (Figure 5-A, B, C) and streptavidin–GNP BH (Figure 6-A, B, C) well confirm the values presented in Table 2. The formation of aggregates under different conditions was visualized using TEM. Figure 7-A, B and C present the typical images of conjugate aggregates with different sizes, and Figure 7-D presents the streptavidin–GNP conjugate of the monodisperse state according DLS.

<table>
<thead>
<tr>
<th>Conditions for conjugate synthesis</th>
<th>Streptavidin concentration, µg/mL</th>
<th>DLS diameter (% volume), nm</th>
<th>PdI</th>
<th>Precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium citrate / tannic acid mixture pH 6.5</td>
<td>20</td>
<td>1570 (100%)</td>
<td>0.316</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>1990 (37.4%)</td>
<td>0.507</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1727 (26.3%)</td>
<td>0.422</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>44.5 (77.3%)</td>
<td>0.269</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>283.9 (100%)</td>
<td>0.254</td>
<td>yes</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>40</td>
<td>143.5 (63.8%)</td>
<td>0.197</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>30.6 (82%)</td>
<td>0.268</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>28.9 (93.1%)</td>
<td>0.296</td>
<td>no</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>20</td>
<td>25 (98.3%)</td>
<td>0.207</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>17.8 (87.8%)</td>
<td>0.306</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>16.5 (98%)</td>
<td>0.308</td>
<td>no</td>
</tr>
<tr>
<td>Sodium borohydride pH 6.5</td>
<td>20</td>
<td>39 (87.7%)</td>
<td>0.242</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>1305 (100%)</td>
<td>0.442</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1001 (72.6%)</td>
<td>0.437</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>26.2 (98.5%)</td>
<td>0.263</td>
<td>no</td>
</tr>
<tr>
<td>pH</td>
<td>Time (min)</td>
<td>Hydrodynamic Diameter (nm)</td>
<td>Polydispersity Index</td>
<td>Sedimentation</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>-----------------------------</td>
<td>----------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>20</td>
<td>23.2 (82.4%) 9.7 (17.6%)</td>
<td>0.276</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>57.7 (93.2%) 16.4 (70.7%)</td>
<td>0.266</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>46.5 (94%) 16.4 (70.7%)</td>
<td>0.246</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>26.2 (98.5%) 16.4 (70.7%)</td>
<td>0.263</td>
<td>no</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>20</td>
<td>8.3 (28%) 16.4 (70.7%)</td>
<td>0.366</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>21.2 (96.8%) 16.4 (70.7%)</td>
<td>0.285</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>18.3 (97.4%) 16.4 (70.7%)</td>
<td>0.34</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>16.9 (99.1%) 16.4 (70.7%)</td>
<td>0.303</td>
<td>no</td>
</tr>
</tbody>
</table>

**Figure 5:** DLS data of the distribution of hydrodynamic diameters of streptavidin–GNP conjugates (with GNP SC–TA) before ultracentrifugation at pH 6.5 (A), 7.4 (B), and 9.0 (C) and after ultracentrifugation at pH 9.0 (D).
Figure 6. DLS data of the distribution of hydrodynamic diameters of streptavidin–GNP conjugates (with GNP BH) before ultracentrifugation at pH 6.5 (A), 7.4 (B), and 9.0 (C) and after ultracentrifugation at pH 7.4 (D), 9.0 (E).
For ultracentrifugation, all conjugates of streptavidin–GNP SC–TA at pH 9.0 and all streptavidin–GNP BH conjugates at pH 7.4 and 9.0 were chosen to separate unbound streptavidin molecules. After ultracentrifugation, the distribution of the hydrodynamic diameters of streptavidin–GNP conjugates showed more polydispersity for pH 7.4 (see Figure 6-D). Therefore, the conjugates that were most monodisperse, stable, and without aggregate formation were obtained at pH 9.0 for both of the GNP preparations. Conjugates with streptavidin concentrations of 40 (GNP SC–TA) and 80 μg/mL (GNP BH) have the narrowest distributions (see Figures 5-D and 6-E, respectively).

**Biotin binding properties of streptavidin–gold nanoparticle conjugates in lateral flow assay**

The conjugates (pH 9.0) synthesized at different streptavidin concentrations were compared according to biotin binding properties in LFA. In LFA with GNP label, the detectable signal is the color in the binding zone; it is affected by the affinity of the conjugate and the optical properties of the conjugate with GNP. To correctly compare conjugate binding properties, optical properties were studied with the absorption spectra of the conjugates (Figure 8). As can be seen in Figure 8, the maxima of the absorption bands were close between different conjugates and shifted to longer wavelengths: \( \lambda = 519–523 \text{ nm} \) for GNP SC–TA, 513–516 nm for GNP BH. However, the peaks of the conjugates differed. Using the ICP-MS (see Materials and methods, section 2.7), we confirmed that the optical densities of the conjugates correlate with the Au concentration (Table 3), which means that differences in peaks are most likely associated with losses of the conjugate during the ultracentrifugation.

![Figure 7: TEM images of streptavidin–GNP conjugates before ultracentrifugation](image-url)
Table 3: Primary data of ICP-MS measurements carried out with a quadrupole ICP-MS instrument Aurora M90 (Bruker Corp., USA).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>OD (λ&lt;sub&gt;max&lt;/sub&gt;)</th>
<th>Au concentration, µg/mL</th>
<th>GNP concentration, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNPs with sodium citrate / tannic acid mixture</td>
<td>0.83</td>
<td>14.2</td>
<td>28</td>
</tr>
<tr>
<td>GNPs sodium borohydride</td>
<td>0.67</td>
<td>11.5</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Streptavidin–GNP conjugate at pH 9.0</th>
<th>Parameters</th>
<th>OD (λ&lt;sub&gt;max&lt;/sub&gt;)</th>
<th>Au concentration, µg/mL</th>
<th>GNP concentration, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing agent</td>
<td>Streptavidin concentration, µg/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium citrate / tannic acid mixture</td>
<td>20</td>
<td>0.65</td>
<td>8.8</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.53</td>
<td>8.2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.63</td>
<td>9.0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>0.50</td>
<td>7.9</td>
<td>15</td>
</tr>
<tr>
<td>Sodium borohydride</td>
<td>20</td>
<td>0.44</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.35</td>
<td>6.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.35</td>
<td>5.9</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>0.49</td>
<td>7.5</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 8. Absorption spectra of streptavidin–GNP conjugates after ultracentrifugation at pH 9.0.

All conjugates showed staining in the binding zones and high binding properties up to 0.004 optical densities at λ<sub>max</sub> (Figure 9). Differences in the color intensity in the binding zones were very weak at high conjugate concentrations and slightly more intense for 40 and 60 µg/mL of streptavidin at low conjugate concentrations (0.02, 0.004 of optical density) for GNP SC–TA (see Figure 9-F, E). However, at large concentrations (0.2, 0.07 of optical density), the binding zones at 20 and 80 µg/mL of streptavidin were blurred and wide for both kinds of GNP (see Figure 9-A, B). Therefore, the most effective streptavidin–GNP conjugates for LFA with indirect labeling were synthesized using pH 9.0 and 40–60 µg/mL of streptavidin.
CONCLUSION

The results confirm that through the use of both reagents—SC–TA and BH—is it possible to obtain monodisperse, spherical GNPs. The optimal conditions of the conjugate synthesis did not differ for small GNPs synthesized by different methods. The paper shows that the flocculation method is not informative for the choice of the synthesis conditions for GNP SC–TA. In carrying out a flocculation experiment, the nanoparticles obtained by reduction with BH showed stability at three different pH (6.5, 7.4, 9.0), but the characteristics of the conjugates by the DLS method showed significant differences. For the synthesis of monodisperse and stable conjugates of
small GPNs (irrespective of the synthesis method) with streptavidin (pI = 5.2), a pH 9.0 is optimal. TEM and DLS data confirmed the presence of aggregates for both types of GNP in the synthesis under conditions of pH 6.5 and 7.4. The binding properties of the all streptavidin–GNP conjugates synthesized at pH 9.0 were high. In LFA, conjugates showed binding up to 0.004 optical densities (λmax). Thus, for LFA with indirect labeling and small GNP, we recommend synthesizing the streptavidin–GNP conjugates using pH 9.0 and 40–60 µg/mL of streptavidin.

ACKNOWLEDGMENTS
This work was financially supported by the Ministry of Education and Science of the Russian Federation within the framework of the Federal Targeted Program “Research and Development in Priority Areas of Science and Technology Complex of Russia for 2014–2020” (Agreement from September 26, 2017, no. 14.607.21.0184, unique project identifier no. RFMEFI60717X0184).

REFERENCES


