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Original article

OPTIMIZATION OF PARAMETERS OF SALINE SODIUM CITRATE BUFFER FOR STABILITY OF COLLOIDAL GOLD NANOPARTICLES USED IN DNA HYBRIDIZATION BIOSENSOR

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Background. The use of optical biosensors based on surface plasmon resonance (SPR) spectrometry have long been established as a viable alternative to the traditional molecular biological methods, such as immunostaining or ELISA. Its capacity to perform real-time quantitative measurements is complemented with the possibility for the enhancement of the sensor signal with the use of optically active colloidal nanoparticles. On the other hand, few such nanoparticle-containing DNA biosensors have been developed, owing to poor colloidal stability of nanoparticles in chemical conditions suitable for hybridization of nucleic acid sequences.
Objective. This study investigates the possibility of using gold nanoparticles (AuNPs) modified with thiolated oligonucleotides, 6-mercapto-1-hexanol, and lipoic acid as part of a hybridization system for biosensor detection of DNA sequences in order to improve its main analytical characteristics.
Methods. A study of the colloidal stability of nanoparticles in SSC (saline sodium citrate) media of different multiplicity before and after modification was carried out in order to select the best environment for the operation of the biosensor system. Aggregation of modified AuNPs was facilitated by their centrifugation, after which pelleted nanoparticles were resuspended and investigated by spectrophotometry. The conclusions regarding the colloidal stability of AuNPs were based on the drop in concentration of colloidal AuNPs after each centrifugation.
Results. The possibility of using the studied NPs in biosensor analysis was shown, and 0.1×SSC buffer solution was determined to be the optimal medium for their operational stability. Approbation of the nanoparticle-containing DNA hybridization biosensor based on SPR spectrometry was carried out in 2.0×SSC medium.
Conclusions. The prospective use of the studied NPs as components of DNA hybridization systems for the detection of genetic markers has been proven.

Keywords: surface plasmon resonance; DNA hybridization biosensors; gold nanoparticles; Philadelphia chromosome.

Introduction

Due to their cheapness, low toxicity and the possibility of chemical modifications of their surface, gold nanoparticles have become the objects of many scientific studies in the field of biosensors [1, 2]. Main reasons for the use of nanoparticles are their unique optical properties compared to macroscopic materials and the ability to control and fine-tune their properties such as surface-to-volume ratio according to the needs of a specific application [3]. On the other hand, nanoparticles may be prone to nonspecific aggregation on sensor surface in chemical conditions favorable for affinity interactions between biomolecules, which negatively affects the efficiency of the biosensor [4–6]. Aggregation (clustering) of metal nanoparticles is caused by van der Waals attractive forces arising due to the spontaneous delocalization of electrons of atoms of the surface of the nanoparticle which leads to the occurrence of a dipole moment in such atoms and the cascading polarization of neighboring atoms at a distance of 0.2 to 0.5 nm [7]. The weak electrostatic attraction that occurs in such conditions between the approaching nanoparticles leads to their irreversible convergence with a change in their chemical and physical properties. Ways for preventing the processes of aggregation of nanoparticles include modification of their surface with a dense layer of uncharged polymers, the change in conformation of which during the approach of two nanoparticles is energetically disadvantageous (steric stabilization). Charged molecules immobilized on the surface of nanoparticles can act as another

source of repulsive forces between them (electrostatic stabilization). The most interesting and promising approach is the combination of these two methods which is called electrosteric stabilization of nanoparticles [5].

Various nanoparticles, including noble metal nanoparticles, carbon and semiconductor nanomaterials, metal oxide nanostructures, and hybrid nanostructures are currently being used in immunosensors and immunoassays [8]. The available data on the use of colloidal nanoparticles in molecular biological methods reflects the successful implementation of the idea of electrosteric stabilization of NPs modified by antibodies, while in DNA hybridization sensors, where affinity interactions between oligonucleotides occur along the entire length of these macromolecules, excessive repulsion between them inevitably leads to the decrease of sensitivity of such analytical devices [5, 9, 10]. The development of a nanoparticle-containing DNA biosensor capable of analying biological samples for the presence of certain genetic markers requires its preliminary calibration in stable chemical conditions that are maximally favorable for hybridization between probe and target oligonucleotides. A medium widely used in hybridization assays is sodium saline citrate buffer solution or SSC (15 mM sodium citrate, 150 mM NaCl, pH 7.0). The presence of a large number of Na^+ ions in its composition contributes to the neutralization of the negative charge of DNA, which simplifies the approach of its individual sequences in the process of forming a double helix [11–13].

Previous studies have shown that the optimal option for colloidal stabilization of DNA-bound gold nanoparticles is the assembly of a dense monolayer of blocking molecules, over which the ligands – DNA probe sequences – are freely placed [6]. In this work, it was decided to use such nanoparticles in the SSC environment as molecular labels capable of multiplying the signal of the SPR biosensor [14].

Based on the above, the aim of this work was to determine the colloidal stability of gold nanoparticles modified with oligonucleotides and blocking molecules in SSC media of different multiplicity.

The proposed technology will allow the possibility of development of novel DNA biosensors for the purpose of monitoring of a wide range of DNA biomarkers. Such biosensors can be applicable in the fields of microbiology, virology, food safety, detection of bioweapons and other problems of biosafety.

Materials and Methods

Reagents

In this research we used urea, KH_2PO_4, SSC, lipoic acid (LA) and 6-mercapto-1-hexanol (MCH) (Fluka, Switzerland), oligonucleotides (Metabion, Germany); all reagents were of *Pro Analysi* grade. All solutions were prepared using Milli-Q deionized water.

For modification of the surface of AuNPs, a single-chain oligonucleotide **SH-DP**, functionalized at the 5'-end with a thiol group through a hexamethylene spacer (SH-$(CH_2)_6$-TTT TTT TTT GGC TGA GTG GAC GAT GA), was used. Its sequence corresponds to a fragment of the e13a2 BCR-ABL hybrid gene of the Philadelphia chromosome. With the aim of approbation of the modified AuNPs as a part of DNA hybridization biosensor two other thiolated oligonucleotides were selected for immobilization on SPR sensor surface. The first one of them, **SH-IP** (SH-$(CH_2)_6$-TCA TCG TCC ACT CAG CCA), has sequence that is complementary to **SH-DP**. Nonspecific interactions between modified AuNPs and biosensor surface were monitored with immobilized probe oligonucleotide **mod-Ph** (SH-$(CH_2)_6$-GCT GAA GGG CTT TTG AAC TCT GCT), which is noncomplementary to **SH-DP**.

Synthesis of AuNPs

AuNPs were synthesized by method of Turkevich *et al.* with the substitution of $HAuCl_4$ for $AuCl_3$ [15]. First, 10 mL of 1 mM $AuCl_3$ were heated to a boiling point with constant stirring. Then 1 ml of 1% trisodium citrate was added. Heating and stirring were continued for 10 minutes until the color of the colloid stopped changing with time. After the reduction of all gold ions, the color of the solution became deep red, after which the solution was cooled to room temperature. The obtained AuNPs were expected to appear as practically monodisperse spheres with diameter of 10 to 15 nm and a concentration of 1.5×10^{-8} mol/l and stabilized with electrostatically bound citrate ions. AuNPs studied in this work were characterized with a light absorption maximum at 520 nm [16–19].

After the synthesis, the diameter of the obtained AuNPs was estimated using a photon correlation spectroscopy system of the Malvern Zetasizer Nano series. The concentration of colloidal AuNPs was determined using a NanoDrop 2000 spectrophotometer according to the Bouguer–Lambert–Beer law.

Modification of AuNPs with oligonucleotides and surface blocking molecules

Immobilization of molecules on gold sensor surface was achieved by the method of covalent binding of thiolated oligonucleotides and surface blocking molecules as detailed by Matsishin *et al.* [12]. The high affinity of sulphur to Au atoms allows to form a stable bond between them [20].

The electrosteric stabilization of AuNPs can be achieved in two steps: immobilization of long negatively charged thiolated oligonucleotides and immobilization of short neutral molecules. As it was shown previously [21], it was insufficient to only use 6-mercapto-1-hexanol as a surface blocking molecule, therefore an additional charged molecule – lipoic acid – was used as such.

Immobilization of thiolated oligonucleotides and the above blocking molecules on AuNPs was executed by incubating 2.5 nM AuNPs in a solution of 750 nM SH-DP oligonucleotides for 12 hours in 0.1×SSC medium (1.5 mM sodium citrate, 15 mM NaCl, pH 7.0) at room temperature, after which a solution of 2 μM MCH and 2 μM LA was added to these AuNPs and a second incubation was carried out for an hour under the same conditions. Next, the AuNPs were transferred to the studied medium by two 30-minute centrifugations at a relative centrifugal force of 13.226 relative units, followed by discarding the supernatant. A schematic representation of AuNPs before and after immobilization of oligonucleotides and blocking molecules is shown in Fig. 1. The concentration of thiolated oligonucleotides was selected based on the calculation of the ratio to the concentration of AuNPs at the level of 300:1 [22].

Biosensor measurement system design

Oligonucleotide hybridization processes were studied using a SPR spectrometer "Plasmon-6" in Kretschmann optical configuration (Fig. 2) with

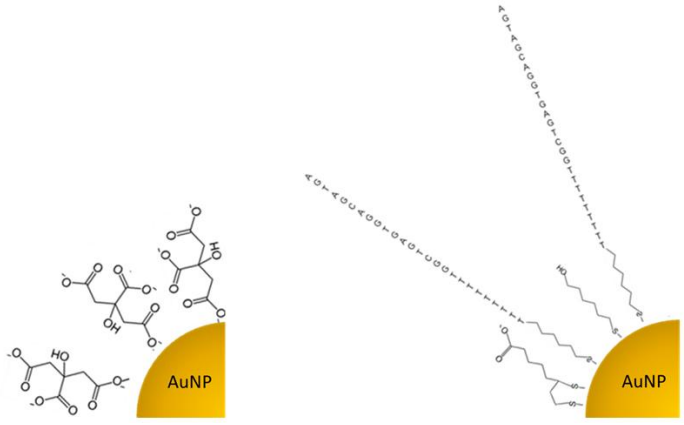

Figure 1: Schematic of the investigated AuNPs stabilized by citrate ions (a) and molecules containing a thiol group (b)

Figure 2: SPR spectrometer "Plasmon-6", which includes an optical unit containing laser and photodiode (a), measuring flow cell in the Kretschmann configuration (b) and a holder for the test sample (c)

angular modulation of the sensor signal developed at the V.E. Lashkaryov Institute of Semiconductor Physics of the National Academy of Sciences of Ukraine. The sensor surface of this device is a layer of gold with a thickness of 50 nm on a plate (chip) made of optical glass; the volume of the measuring flow cell is 30 μL. The operation surface of a sensitive element in the SPR spectrometer consists of a gold layer with the thickness of 50 ± 2 nm deposited on a substrate made of optical glass F1 [23]. Before the experiments, the surface of the gold-coated glass plate was cleaned with freshly prepared Piranha solution (3:1 mixture of concentrated H_2SO_4 and 30% H_2O_2) at room temperature for 3 minutes, then washed thoroughly with Milli-Q deionized water, then air-dried.

The cleaned chip was mounted on the spectrometer prism using an immersion fluid having the same refractive index as the prism and the chip. The flow rate (40 μl/min) through the measuring flow cell was controlled by a peristaltic pump (Ismatec, USA) [14]. The SPR signal was processed using the Biosuplar software developed by Mivitec GmbH (http://www.biosuplar.de/software.htm).

Statistical methods

Results obtained over the course of the study are represented with mean values and standard deviations.

Results

The graph below shows the distribution of AuNPs by diameter in percentage (Fig. 3). From it, their average diameter was determined at the level of 16.77 nm.

It is known that non-aggregated AuNPs have the highest optical density at a wavelength of 520 nm, that is, they absorb light in the blue spectrum and are permeable to waves corresponding to the red color [15]. In turn, aggregated gold nanoparticles have a lower optical density at a wavelength of 520 nm and a higher one at 650 nm, which is perceived by the human eye as an acquisition of a blue hue (Fig. 4).

The initial concentration of AuNPs was established through the Bouguer–Lambert–Beer law, which describes the relationship between the concentration of AuNPs and their optical density:

$$A = \varepsilon CL,$$

where A is the optical density, ε is the molar extinction coefficient, C is the concentration, and L is the path length of the beam in the spectrophotometer. Under the conditions of $L = 0.1$ cm the value of A was equal to 0.38525. To calculate the molar extinction coefficient of AuNPs with a mean diameter of 16.77 nm, the method of Liu et al. was utilized [24]. The application of the authors' calcu-

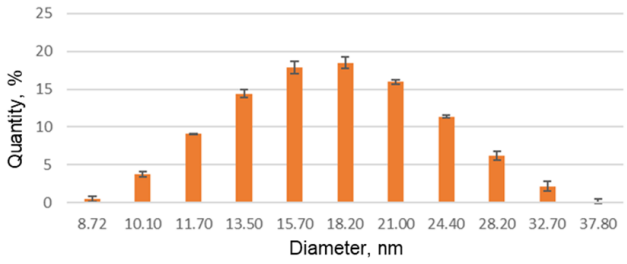

Figure 3: Distribution of citrate-bound spherical gold nanoparticles by diameter. Data was obtained by the method of photon correlation spectroscopy

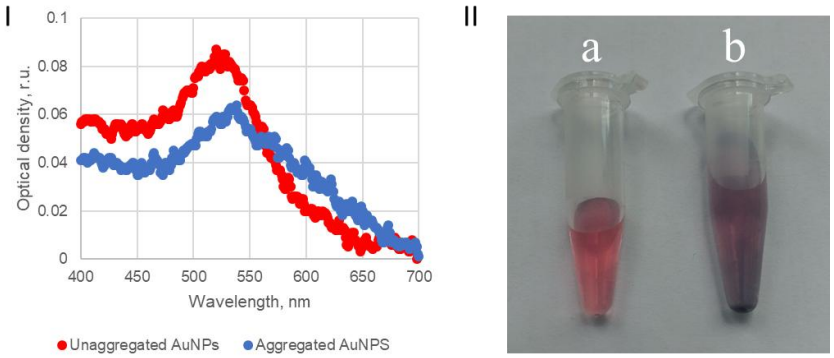

Figure 4: Absorption spectra (I) and an ordinary photo (II) of unaggregated (a) and partially aggregated (b) AuNPs

lations made it possible to establish that such coefficient was equal to 5.74×108 r.u.×mol^{-1}×cm^{-1}. Insertion of these values into the Bouguer–Lambert–Beer law allowed us to determine that the concentration of as-synthesized AuNPs was equal to 6.7×10^{-9} mol/l or 6.7 nM.

The colloid obtained as a result of the synthesis, as well as its dilutions in Milli-q ultrapure water, were used to plot a calibration curve for determination of concentration of AuNPs from their optical density (Fig. 5).

The next stage of our work was to find out the parameters of buffer solutions for the best functioning and storage of modified AuNPs. In order to select the best environment for operational stability of modified gold nanoparticles, a number of experiments was conducted. Their aim was to stimulate the aggregation of AuNPs by reducing the distance between them through successive centrifugations. After each such centrifugation, the AuNPs samples were resuspended with subsequent measurements of their optical density. Their concentration, calculated according to the Bouguer–Lambert–Beer law, was compared to the initial concentration, after which a conclusion was made about the number of AuNPs that underwent irreversible aggregation and did not undergo resuspention.

First, the above described experiment was carried out for gold nanoparticles modified only with blocking molecules without their modification with oligonucleotides. Four buffer media were chosen for the experiments: Milli-Q deionized water, 0.1×SSC, 0.2×SSC, and 2.0×SSC (Fig. 6).

Below is a graph of the results of studies of the dependence of the optical density on the number of centrifugations for gold nanoparticles already modified with oligonucleotides and blocking molecules (Fig. 7). Buffer media were the same as used previously: Milli-Q deionized water, 0.1×SSC, 0.2×SSC and 2.0×SSC.

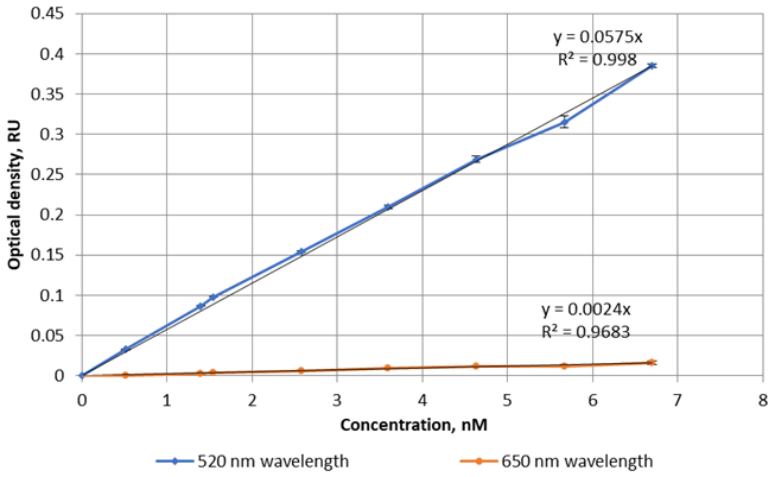

Figure 5: Calibration curves of the dependence of the optical density of the sample at different wavelengths on the concentration of the studied AuNPs. The path length of light in spectrophotometer was equal to 0.1 cm

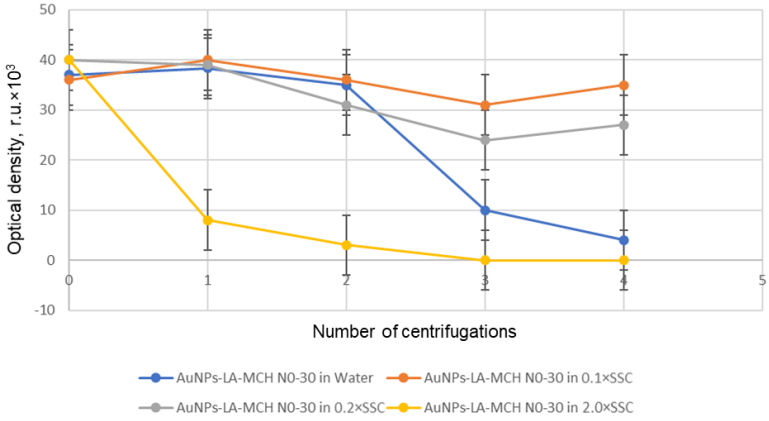

Figure 6: The effect of centrifugation in water and SSC media of different multiplicity on colloidal stability of AuNPs modified with blocking molecules

In order to confirm the possibility of successful use of modified gold nanoparticles as part of a DNA hybridization biosensor, the results of experiments on non-complementary and complementary binding of AuNPs to the surface of the SPR biosensor were obtained as shown in Fig. 8. Analyzing the received biosensor response, we can observe a significant amplification of the SPR signal after injection of AuNPs modified with complementary oligonucleotides, as well as the possibility of regeneration of the biosensor surface by desorption of AuNPs after the experiment.

Discussion

The main purpose of this work is the reduction of the detection limit of SPR biosensors by increasing the specific signal of such biosensors with added modified AuNPs. For this, it was necessary to comprehensively investigate the conditions and procedures for modifying AuNPs with oligonucleotides and blocking molecules, as well as to choose optimal conditions for operational stability of already modified AuNPs.

The average diameter of modified gold nanoparticles should be equal to 10–19 nm. The use of AuNPs of this size is dictated by two factors: on the one hand, the result of the synthesis of AuNPs with an average diameter of 20 nm or more by the Turkevich method is high polydispersity of such nanoparticles [15]. On the other hand, AuNPs preparations with an average diameter of less than 10 nm include fractions of nanoparticles with a diameter of up to 4 nm, which have cytotoxic and genotoxic properties [25]. The AuNPs synthesized for this research were characterized with diameters from 8.72 to 37.80 nm with an average value of 16.77 nm and an average concentration of 6.7 nM. This contrasts with the expected values of average diameter (10 to 15 nm) and concentration (15 nM).

The reasons for this disparity may be rooted at the imperfection of the AuNPs synthesis setup. Firstly, the reaction mixture was not placed under reflux for the sake of simplicity of the procedure.

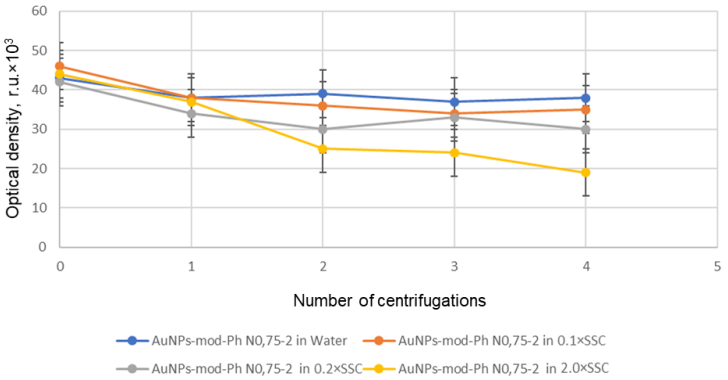

Figure 7: The effect of centrifugation in water and SSC media of different multiplicity on colloidal stability of AuNPs modified with oligonucleotides and blocking molecules

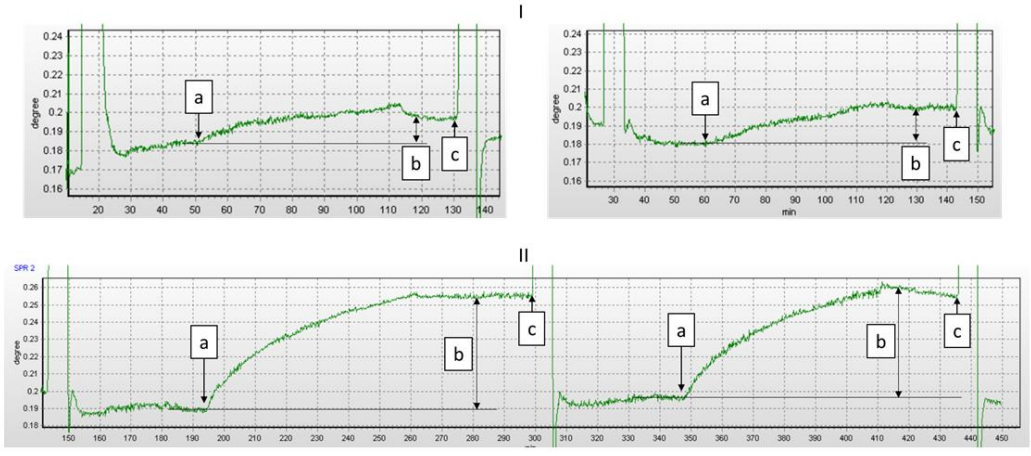

Figure 8: Typical responses of the SPR biosensor to AuNPs modified with oligonucleotides, non-complementary (I) and complementary (II) to the bioselective element, and surface blocking molecules. The hybridization experiment steps were as follows: injection of modified AuNPs into the measuring flow cell (a), washing of measuring flow cell with 2.0ЧSSC buffer solution and determination of the sensor response (b), regeneration of the bioselective element with 8 M Urea (c). *X*-axis – time (min), *Y*-axis – resonance angle (degrees)

Secondly, the Au source was $AuCL_3$ and not $HAuCl_4$ as per original protocol [15]. Finally, the absence of spectrophotometric control of optical density of the reaction mixture resulted in the impossibility of accurate recognition of the moment in time when all Au atoms became incorporated in the nanoparticles. This meant that the heating may have been sustained for longer than necessary, resulting in undesirable processes in the colloid.

Another limitation of this work was the expensiveness of reagents, specifically AuNPs and thiolated oligonucleotides. It was the reason that facilitated the choice of NanoDrop 2000 for spectrophotometric analyses, as it allowed to determine the optical density of studied samples without the use of cuvettes, only requiring a 2 μL aliquot for each measurement. The downside of this choice, however, was the inability to analyse the optical density of samples of fully aggregated AuNPs which only contained precipitated nanoparticles.

One of the most important characteristics of the initial AuNPs, which should be observed during their further modification, is the ratio of the optical density at 520 and 650 nm, and it should be ≥20. It can be seen from the calibration curve that there is a linear relationship between optical density and concentration. Furthermore, with the increase in concentration of AuNPs, the ratio of optical densities at 520 and 650 nm wavelengths is preserved (see Fig. 5).

From the graph describing the effect of centrifugation in water and SSC media of different multiplicity on colloidal stability of AuNPs modified with blocking molecules (6-mercapto-1-hexanol and lipoic acid), it can be seen that in chemical media such as deionized water and 2.0×SSC, gold nanoparticles modified only with mercaptohexanol and lipoic acid aggregate and precipitate. In a concentrated SSC solution, such a reaction occurs due to too high an ionic strength. In deionized water, the nature of this process can be explained by the hydrophobic interaction between nonpolar blocking molecules on the AuNPs surface.

As a resut of the study of the effect of centrifugation in water and SSC media of different multiplicity on colloidal stability of AuNPs modified with oligonucleotides and blocking molecules, it was found that the aggregation of nanoparticles was not as active as in the previous experiment. This was due to the fact that thiolated oligonucleotides and surface blocking molecules completely covered the AuNPs, creating both electrostatic and steric hindrances to aggregation, which made them more stable in the SSC environment. Therefore, after analyzing the results on the graph, it was established that the best conditions for usage of AuNPs modified in this way appear both in deionized water and in SSC solutions of low multiplicity, especially 0.1×SSC.

The results of the SPR experiments indicate a three-fold discrepancy between the sensor signals of complementary and noncomplementary AuNPs, which can be attributed to the energy of hybridization between the oligonucleotides immobilized on the operation surface of SPR biosensor and the oligonucleotides immobilized on the surface of AuNPs.

Conclusions

The paper investigated the effect of parameters of the working buffer solution on the stability of colloidal gold nanoparticles, which should be used as part of a DNA hybridization biosensor.

1. The parameters of synthesized AuNPs were acceptable from the point of view of biological safety, but different from theoretically predicted. In further studies, special attention should be paid to the synthesis process of colloidal AuNPs.

2. After plotting the calibration curves of optical density dependence on the concentration of gold nanoparticles under study, it was found that the ratio of the optical density of non-aggregated AuNPs at 520 and 650 nm wavelengths was ≥20, which is an indicator of a low level of aggregation of nanoparticles.

3. It was determined that the aggregation of gold nanoparticles modified with thiolated oligonucleotides and blocking molecules occurs more slowly than of gold nanoparticles modified only with blocking molecules. This is due to the fact that thiolated oligonucleotides and blocking molecules completely cover AuNPs, creating both electrostatic and steric hindrances to their aggregation.

4. It was shown that the investigated AuNPs can be used as part of a DNA hybridization biosensor in a 2.0×SSC medium. For their long-term storage, the optimal medium was 0.1×SSC.

Interests disclosure

Authors declare that there is no conflict among the contributing authors related to the financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

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ОПТИМІЗАЦІЯ ПАРАМЕТРІВ ЦИТРАТНО-СОЛЬОВОГО НАТРІЄВОГО БУФЕРА ДЛЯ СТАБІЛЬНОСТІ НАНОЧАСТИНОК КОЛОЇДНОГО ЗОЛОТА, ЩО ВИКОРИСТОВУЮТЬСЯ В ГІБРИДИЗАЦІЙНОМУ ДНК-БІОСЕНСОРІ

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Проблематика. Оптичні біосенсори на основі спектрометрії поверхневого плазмонного резонансу давно зарекомендували себе альтернативою традиційним молекулярно-біологічним методам, таким як імуноферментні аналізи. Їх здатність виконувати кількісні вимірювання в реальному часі доповнюється можливістю посилення сигналу датчика за допомогою оптично активних колоїдних наночастинок. З іншого боку, до сьогодні була розроблена мала кількість ДНК-біосенсорів, що містять наночастинки, оскільки наночастинкам властива низька колоїдна стабільність у хімічних умовах, придатних для гібридизації послідовностей нуклеїнових кислот.

Мета. У роботі досліджується можливість застосування наночастинок золота (НЧЗ), модифікованих тіольованими олігонуклеотидами, меркаптогексанолом і ліпоєвою кислотою в складі гібридизаційної системи для біосенсорного детектування ДНК-послідовностей із метою покращення її основних аналітичних характеристик.

Методика реалізації. Було проведено дослідження колоїдної стабільності наночастинок до та після модифікації в середовищах SSC (saline sodium citrate) різної кратності з метою підбору найкращого середовища для роботи біосенсорної системи. Агрегація модифікованих НЧЗ стимулювалася центрифугуванням, після чого осаджені наночастинки ресуспендували та досліджували за допомогою спектрофотометрії. Висновки щодо колоїдної стабільності НЧЗ ґрунтувалися на величині зниження концентрації колоїдних НЧЗ після кожного центрифугування.

Результати. Показано можливість використання досліджуваних НЧЗ у біосенсорному аналізі, а також визначено оптимальні умови їх навколишнього середовища (0,1×SSC). Проведено апробацію НЧЗ-вмісного гібридизаційного ДНК-біосенсора на основі спектрометрії ППР у середовищі 2.0×SSC.

Висновки. Доведено перспективність використання НЧЗ як компонентів ДНК-гібридизаційних систем для виявлення генетичних маркерів.

Ключові слова: поверхневий плазмонний резонанс; гібридизаційні ДНК-біосенсори; наночастинки золота; філадельфійська хромосома.