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SIGNAL UPSCALING WITH THE SEQUENTIAL ADDITION OF REAGENTS FOR ENHANCEMENT OF DETECTION SENSITIVITY

Master's thesis

Applied Measurement Science (30 EAPs)

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ABBREVIATIONS

AuNPs	gold nanoparticles
BSA	bovine serum albumin
DBCO	dibenzocyclooctyne
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
FITC	fluorescein isothiocyanate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LFIA	lateral flow immunoassay
PBS	phosphate-buffered saline
SPAAC	strain-promoted azide-alkyne cycloaddition

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1. INTRODUCTION

The detection of low-abundance molecules has been an ongoing challenge in many analytical applications. A vast number of molecules need to be detected at very low levels (*e.g.*, estradiol in the blood plasma of children). Moreover, the target analyte is often in a complex matrix which further complicates the sensitive detection of the analyte. Highly sensitive and selective instrumentation-based analytical techniques must be applied in these cases. However, these techniques have several drawbacks, such as high cost, complexity, and labor intensity. Their use is often far from meeting the practical requirements.

Bioanalytical methods, such as immunoassays, are a widely used group of analytical methods. Due to their high selectivity, sensitivity, and practical utilization, these methods are applied for the analysis of a wide variety of analytes in different samples. However, often in the case of low abundance molecules, the sensitivity of the methods is lacking.

The long-term goal of this project is to increase the sensitivity of bioanalytical methods but simultaneously maintain simplicity and low cost of analysis (*e.g.,* in the case of lateral flow immunoassays). In this thesis, a microplate format was used as a simplified model system enabling parallel measurement of up to 96 samples. Moreover, this thesis describes two different approaches for achieving high sensitivity. In both cases, the goal is to enhance the sensitivity by sequentially adding more and more "layers" of interacting compounds (*e.g.,* gold nanoparticles (AuNPs)) that are captured and therefore give additional strength to the signal. The first approach streptavidin-biotin binding to capture "layers" of AuNPs: streptavidin and biotin-labeled AuNPs are added to the test in turns which leads to more AuNPs being captured each time. In the second approach, click chemistry is used for binding the AuNPs. Click chemistry is a set of biocompatible reactions, and the approach used in this work is based on the strain-promoted azide-alkyne cycloaddition (SPAAC) reaction. AuNPs with azide and with alkyne labeling are added to the test in turns, leading to more and more AuNPs collecting in the test.

2. LITERATURE REVIEW

2.1. Immunoassays

Immunoassays (IA) have the advantage of highly selective immunological recognition [1]. In IA, antibodies and antigens bind selectively even in complex matrices such as biological fluids, and this effect can be observed using a label attached to either the antigen or the antibody [2].

Besides selectivity, high sensitivity is often also necessary to develop an IA. IA are performed in competitive or sandwich formats (Figure 1). Both formats use a label to create a signal that can be used to quantify the concentration of the analyte. Gold nanoparticles (AuNPs), silver nanoparticles, enzymes and others can be used to obtain signals [1]. The intensity of the output signal depends on the sensitivity of the IA and analyte concentration [3]. High sensitivity is necessary to measure low concentrations of analyte. Therefore, one of the primary goals in developing unconventional immunosensing methods is to find new approaches to increase sensitivity [1].

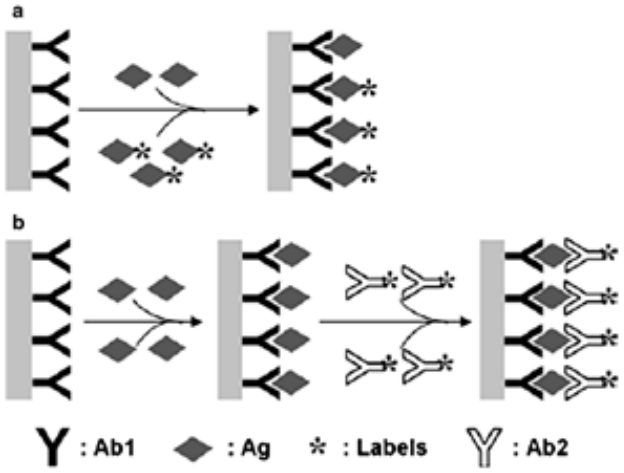


Figure 1. Schematic illustration of competitive immunoassay (a) and sandwich immunoassay (b) [1].

Several types of IA exist, most of which are performed in microwell-plates (*e.g.*, fluorescence immunoassay, enzyme-linked immunosorbent assay, radioimmunoassay, etc.). An alternative to well-plates is offered by the lateral flow immunoassays (LFIA) which utilize nitrocellulose and other porous materials [4].

2.1.1. Lateral flow immunoassay (LFIA)

Compared to other IA, lateral flow immunoassays (LFIA) are simple, low cost, compact and have high analysis speed, and are therefore suitable for point-of-care analysis [5]. In the case of sandwich format LFIA, the analyte first interacts with labeled detection antibodies, moves through the membrane with liquid flow created by capillary forces, and is captured in the test zone due to binding to the capture antibodies. Gold nanoparticles (AuNPs) have traditionally served as labels, and LFIA readout has been accomplished by visually examining or measuring the colour intensity of the lines with a scanner [6]. However, LFIA are plagued with sensitivity issues which significantly restrict its applicability [7].

2.2. Gold nanoparticles (AuNPs)

Gold nanoparticles (AuNPs) have unique properties such as controllable size (5-400 nm), chemical, optical, and catalytic properties. AuNP produce plasmon resonance effects which lead to high molar extinction coefficients (up to $9.74 \cdot 10^{10}$ M^{-1} cm^{-1}) [8]. AuNP surface chemistry can be easily modified by covalent binding of molecules via a thiol functional group to the surface of the gold. This allows binding a wide range of molecular probes to the AuNP for detection of chemical and biological targets. Each of these AuNP characteristics has fueled significant efforts to create a new generation of sensing strategies with improved sensitivity, specificity, and stability [9].

AuNPs are commonly used in electroanalysis, biosensors, and colorimetric detection, along with many other applications. AuNPs absorb and scatter light and with increasing size of AuNPs, the wavelength of absorbed light increases (*i.e.*, 100 nm AuNPs have absorption maximum at 572 nm). Moreover, the color change caused by AuNP aggregation or corrosion can be used to monitor the colorimetric detection of target analytes with AuNPs [10].

2.3. Signal amplification approaches

Colorimetric assays have gained popularity due to their cost-effectiveness, simplicity, and technical convenience. The colorimetric response is easy to observe with the naked eye, and no sophisticated instrumentation is needed. However, colorimetric assays again often lack high sensitivity [11]. For this reason, wide range of signal amplification approaches have been proposed. These approaches can be divided into three categories: aggregation-based approaches, catalytic approaches (*e.g.*, by using enzymes) and growth-based approaches (*e.g.*, silver enhancement) [9]. In this thesis, novel

aggregation-based approaches are proposed and therefore these approaches are discussed more thoroughly.

Colorimetric assays using aggregation of AuNPs can be based on two different effects: (1) aggregation of AuNP results in color change due to change in the surface plasmon resonance when AuNP are aggregated [9], and (2) greater number of AuNP lead to higher absorbance values [12]. The first aggregation approach can be further divided into labeled and label-free systems. In the labeled method, AuNPs are directly attached to ligands such as DNA, antibodies, and peptides through chemical linkage. The label-free colorimetric methods are regulated by electrostatic stabilization; a repulsive electric layer is generated from the AuNPs' surface charges. Presence of the analyte leads to neutralization of surface charges which results in the aggregation of AuNPs, and therefore color change from red to purple [9]. Several articles have also demonstrated the second aggregation approach for amplification of signal [13–17]. In this case, streptavidin-biotin binding has been widely used to amplify the signal in LFIA due to its high specificity and affinity [18]. The complex conjugate can be created by binding AuNP-labeled biotinylated antibodies with streptavidin. Streptavidin acts as an effective cross-linker between the individual gold nanoparticles. As a result, aggregates of nanoparticles containing adsorbed specific antibodies form, and specific immunochemical interactions on the test line of the analytical membrane in the LFIA should amplify the detected signal due to the increased number of bound AuNPs [13]. However, in these cases, the aggregate is assembled prior to addition of the AuNPs to the test, which limits the amount of AuNPs in the aggregate because excessively large aggregates cannot be eluted on the LFIA membrane.

2.4. Aim of the study

This thesis aims to develop novel signal amplification methods that use sequential addition of reagents so that more and more signal-generating labels can be collected and therefore enhanced signal is achieved. For this purpose, two approaches have been employed:

- streptavidin-biotin approach, based on non-covalent interaction of biotin and streptavidin (Figure 2);
- click chemistry approach, based on the strain-promoted azide-alkyne cycloaddition (SPAAC) reaction (Figure 3).

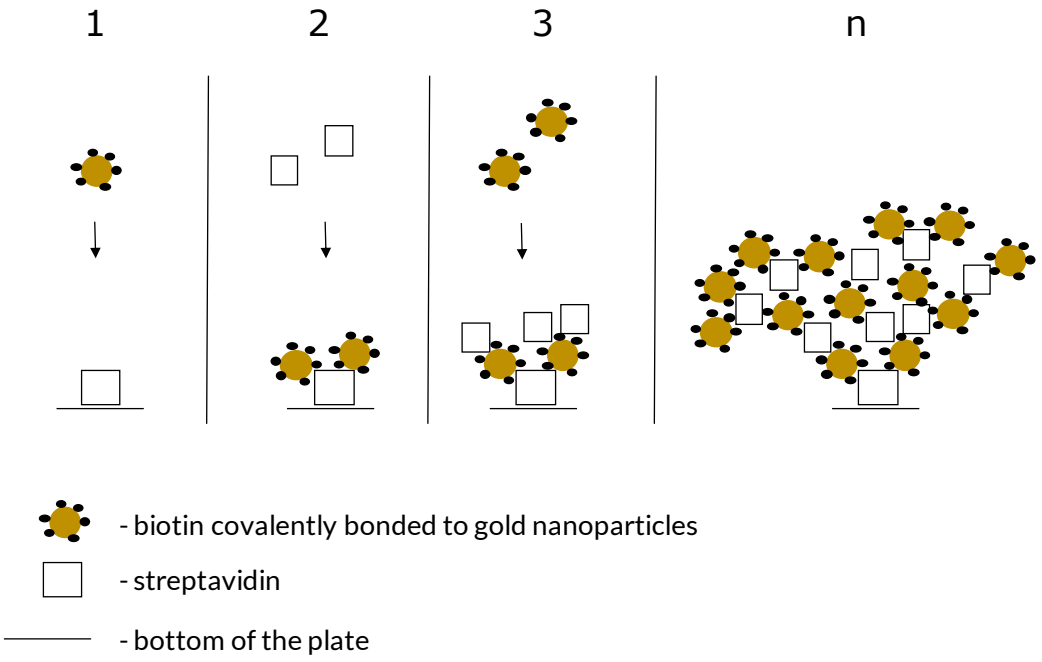


Figure 2. Schematic representation of the streptavidin-biotin approach.

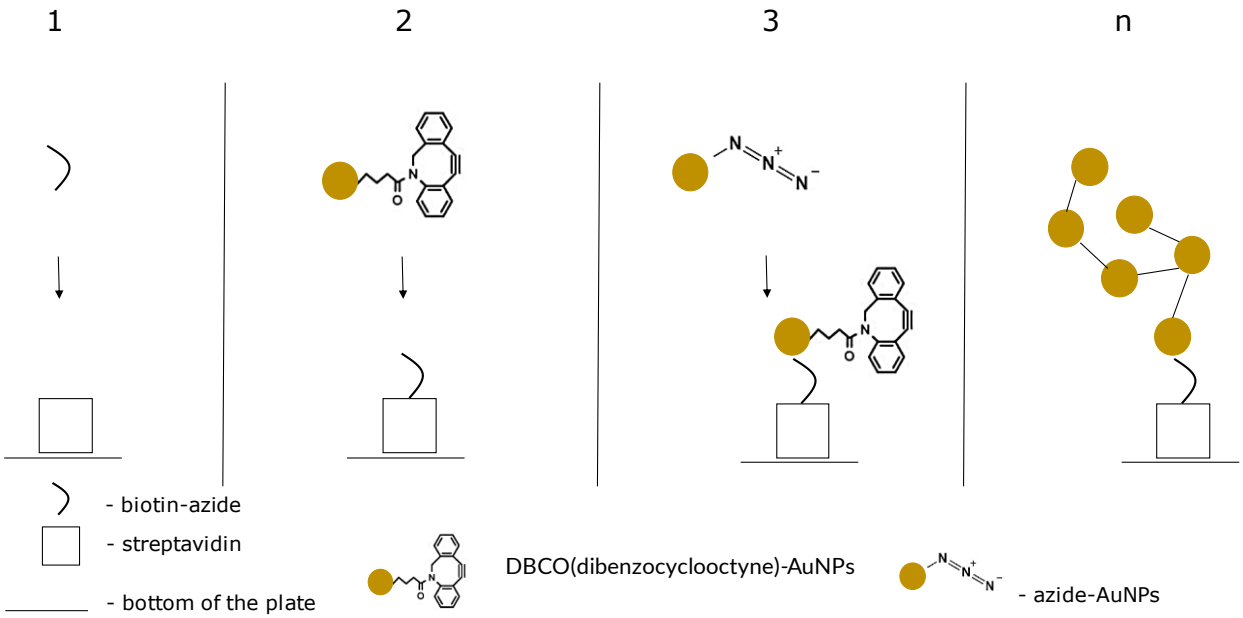


Figure 3. Schematic representation of the click chemistry approach.

The goal is to assess if signal upscaling is achieved by studying these approaches. It is hypothesized that the sequential addition of reagents will allow higher signal amplification than previously discussed aggregation approaches because a larger amount of AuNPs can be added and bound. Due

to the large amount of AuNPs collected, it is expected that absorbance values increase substantially. Moreover, this amplification approach is expected to lead to a low background due to the specificity of the binding mechanisms used. Although the end goal is the application of these approaches to LFIA, in this thesis, a simplified model system using a microtiter plate was applied to test the feasibility of the chosen approaches.

2.5. The streptavidin-biotin interaction

Ligand binding involves the formation of non-covalent bonds between the protein (*e.g.*, streptavidin) and the ligand (*e.g.*, biotin) at specific interacting surfaces [19]. Because of their highly selective and stable interaction with biotin, streptavidin and its homologs are commonly used in biochemical applications. Other factors leading to the popularity of the streptavidin-biotin system include the protein's stability and the variety of chemical and enzymatic biotinylation (attaching biotin covalently to a molecule of interest) methods available for use with various experimental designs [20].

The streptavidin-biotin interaction is known as one of the most robust non-covalent interactions in nature due to the high binding affinity of streptavidin for biotin with a dissociation constant (K_d) of 10^{-14} M. Streptavidin can bind four biotin molecules [21]. The crystal structure of streptavidin complexed with biotin shows that binding is mediated by a complex network of hydrogen bonds and van der Waals interactions [22], [23]. The protein is also highly thermostable (T_m 112 °C for streptavidin-biotin) and resistant to extreme pH, denaturing agents, and enzymatic degradation, which are important characteristics for use in a variety of experimental conditions [20].

2.6. Click chemistry

Click chemistry is a group of reactions between small molecules used in many areas of modern chemistry, such as bioconjugation, drug discovery, and materials science [24]. K. B. Sharpless first introduced the idea behind click chemistry in 2001. Click reactions, by definition, are reactions that have high efficiency, specificity, and the reagents are stable [25], [26]. The goal of Kolb *et al.* was to create a growing set of selective, robust, and modular "units" (*i.e.*, functionalized molecules) that work reliably in both small and large-scale applications [25].

The ability to achieve high selectivity by modifying a molecule within a complex sample is beneficial for research in a wide range of chemical and biological systems. As a result of the immense variety of functionality present in biological systems, side reactions and nonspecific labeling have long been a significant barrier to the selective derivatization of biomolecules. Several chemical reactions orthogonal to functional groups found in biological systems have transfigured this field by exhibiting selectivity in labeling biological targets within complex samples [27]. Moreover, by definition, bonds formed in click chemistry reactions are stable (*e.g.*, cannot get hydrolyzed or enzymatically degraded) under biologically relevant conditions [25].

The azide group is used in many click reactions because it has a high intrinsic reactivity but is (1) quite selective in how it reacts, (2) stable in water, and (3) unreactive in natural samples. Moreover, the azide moiety is advantageous due to its small size, making its introduction into a biomolecule target only a minor structural modification [27]. The azide functional group selectively reacts with phosphines or activated alkynes [28].

The initially utilized click chemistry reactions, azide-alkyne cycloaddition and copper-free azide-alkyne cycloaddition reactions, are shown in Figure 4. Other reactions that have been employed as click chemistry reactions are Staudinger ligation, the reaction of a thiol with a maleimide functionality, the Diels-Alder cycloaddition, the thiol-ene reaction [27].

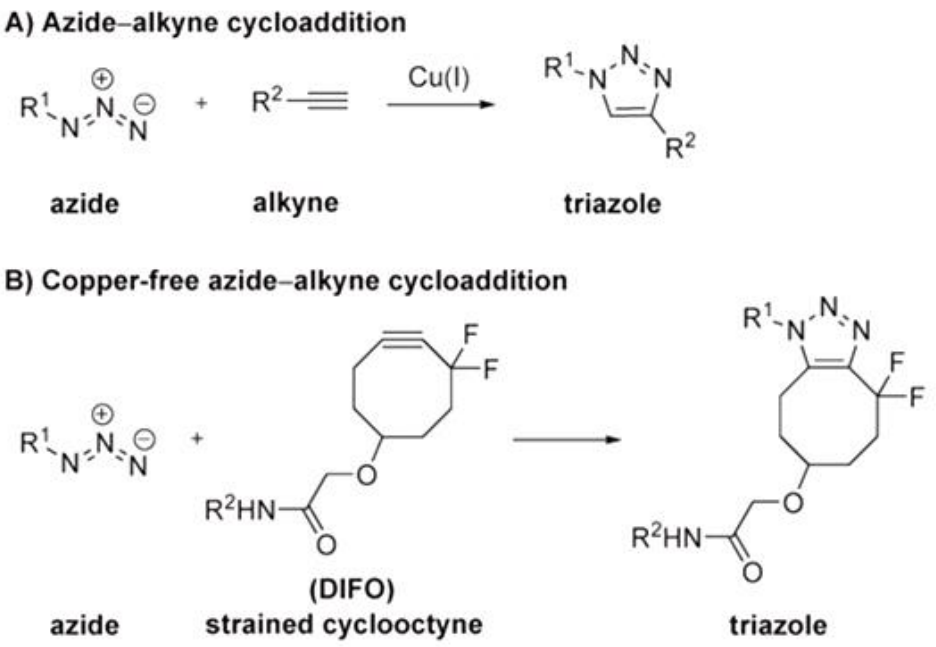


Figure 4. Bioorthogonal reactions generally applied for labeling of biological molecules. (A) azide-alkyne cycloaddition. (B) copper-free azide-alkyne cycloaddition [27].

2.6.1. Azide-alkyne cycloaddition click reactions

Discovered by Huisgen in 1963, The Huisgen 1,3-dipolar cycloaddition reaction, also known as the azide-alkyne cycloaddition, is the most well-known click reaction that combines two unsaturated reactants (azides and alkynes). This reaction is popular due to the fact that it meets the click chemistry criteria for mild reaction conditions, high yield and selectivity. The reaction catalyzed by Cu(I) species supplied from elemental copper simplifies the experimental procedure - only a tiny piece of copper metal (wire or turning) is added to the reaction mixture, which is then shaken or stirred for 12–48 hours. In the presence of catalytic amounts of Cu(I) species the reaction rate can increase up to 7 orders of magnitude [29]. Though the Cu-based procedure requires prolonged reaction times when performed at room temperature, it typically provides very pure triazole products with low Cu contamination. Likewise, the reaction can be carried out at elevated temperatures using microwave irradiation, which reduces the reaction time to 10–30 minutes [30]. In the absence of a catalyst, the initial Huisgen 1,3-cycloaddition reaction (the reaction of unactivated alkynes and azides) proceeds slowly and usually involves high temperatures or pressures [28].

Nevertheless, this reaction is not suitable for use with biological components (*e.g.*, proteins) due to the reactivity of the catalysts towards those. Furthermore, the use of catalysts complicates the kinetics of the immobilization process, demands polar solvents, and can change surface properties. In addition, copper can interfere with a variety of electrical and optoelectronic phenomena [31].

Over the last decade, interest in developing new click reactions that do not rely on metallic catalysts has increased. The strain-promoted 1,3-dipolar cycloaddition of azides and cyclooctyne (SPAAC) derivatives occurs in the absence of auxiliary reagents such as copper even under physiological conditions [32]. The SPAAC reactions have second-order reaction rate constants (approximately 0.1 M^{-1} s^{-1}) in aqueous conditions [33]. This metal-free click chemistry reaction has already been used for protein labeling and purification, insertion of fluorescent tags into living cells and organisms, and modification of luminescent quantum dots. However, one of the limitations of the cyclooctyne reactant is that it is bulky and hydrophobic [34].

The structure of the azide and cyclooctyne substrates has a strong influence on the efficiency of the SPAAC reaction between azides and cyclooctynes, whereas the solvent choice has less influence. The selection of solvents for the click reactions is based on substrate solubility. Some of the commonly used solvents are dimethyl sulfoxide (DMSO), dimethylformamide and acetonitrile [32]. Several authors have observed that SPAAC proceeds significantly faster in solvent with more water in it. Increasing temperature (*i.e.*, 4-37°C) also increases the dibenzocyclooctyne (DBCO (see structure in Figure 3), a commonly used reactive cyclooctyne derivatives) and azide reaction rate. Typical reaction times are less than 12 hours, but incubating for longer periods of time can improve yield [35].

3. EXPERIMENTAL

3.1. Chemicals and materials

3.1.1. Streptavidin-biotin approach

In the experiments using the streptavidin-biotin approach for amplifying the signal, the following chemicals and materials were used: phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS) was used as a buffering solution (pH 7.5) (Corning), streptavidin (EMD Millipore), D-(+)-Biotin (Alfa Aesar), biotin functionalized gold nanoparticles (biotin-AuNPs) (Cytodiagnostics), Milli-Q water (produced with Millipore Milli-Q Advantaged A10 system), surfactant Triton X-100 (Scharlau), protein low-binding microcentrifuge tubes (Corning, Costar), 15 mL and 50 mL polypropylene (PP) centrifuge tubes (Capp, Nerbe), 96-well streptavidin coated plates (Kaivogen), low-binding pipette tips (Corning).

3.1.2. Biotin-FITC experiment

In the biotin-FITC experiment, the following chemicals and materials were used: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Calbiochem), sodium chloride (NaCl) (Lachner), Tween 20 (Sigma-Aldrich), dithiothreitol (DTT) (Fisher), 384-well low volume black polystyrene plate with protein non-binding surface and round bottom (Corning), 96-well clear PP plate with non-treated surface (Nunc), ClipTip racked pipette tips (1-30 µL and 2-125 µL volume; Thermo Fisher Scientific), biotin-4-fluorescein isothiocyanate conjugate (biotin-FITC) – synthesized by Dr. Kaido Viht (purity over 95% by HPLC-UV), control streptavidin (Amresco).

3.1.3. Click chemistry approach

In the experiments using the click chemistry approach, the following chemicals and materials were used: biotin-PEG3-azide (Sigma-Aldrich), azide-functionalized gold nanoparticles (azide-AuNPs) (Nanopartz), dibenzocyclooctyne-functionalized gold nanoparticles (DBCO-AuNPs) (Nanopartz), dimethyl sulfoxide (DMSO) (Lach-Ner), bovine serum albumin (BSA) (Sigma-Aldrich), and other laboratory plasticware and chemicals as mentioned in Section 3.1.1.

3.2. Instruments

Instruments used in this thesis are presented in Table 1**.**

Table 1. List of instruments

Instrument	**Company**	**Country**
Shakers (incubators)	BMG LABTECH	Germany
Rocking shaker	ELMI	U.S.A.
Mini centrifuge	ELMI	U.S.A.
Vortex mixer	Heidolph	Germany
Ultrasonic bath	Grant Instruments	United Kingdom
Analytical balance	RADWAG	Poland
Cytation™ 5 Cell Imaging Multi-Mode Reader	BioTek	U.S.A.
PHERAstar plate reader	BMG LABTECH	Germany
Automated multichannel pipette (1-30 µL and 2-125 µL volume)	Thermo Fisher Scientific E1-ClipTip	U.S.A.

3.3. Software

Fiji ImageJ software was used for analyzing the microscope images captured by the Cytation 5 cell imaging reader. Data analyses for the biotin-FITC experiments were conducted in GraphPad Prism software.

3.4. Preparation of standard solutions

3.4.1. Streptavidin-biotin approach

For preparing streptavidin stock solution, 1 mg of streptavidin was dissolved in 1 mL of Milli-Q water. From this stock with 1 mg/mL concentration, 50 µL aliquots were deposited into several microcentrifuge tubes and stored at -20 °C for later use. Biotin stock solution was prepared by weighing 0.02 g of biotin (MW 244.31 g/mol) and dissolving it in 100 mL of Milli-Q water. To prepare the necessary amount of biotin-AuNP solution, biotin-AuNPs stock was diluted 30-fold in PBS solution. The 0.1% Triton X-100 detergent solution used for washing steps in the experiments was prepared by adding 250 µL of 20% Triton X-100 to 50 mL of PBS. For certain incubation steps, 0.01% Triton X-100 was made by 10-fold dilution of the 0.1% Triton X-100 detergent solution in PBS.

3.4.2. Click chemistry approach

6 mg of biotin-azide (MW 615.79 Da) was weighed and dissolved in 1 mL of DMSO to prepare a stock solution of 0.01 M. The stock was then aliquoted into microcentrifuge tubes and stored at -20 °C for further experiments. The stock was diluted to 10 µM in PBS for the experiments. Solutions of azide-AuNPs and DBCO-AuNPs ($4.17 \cdot 10^{11}$ and $4.37 \cdot 10^{11}$ particles/mL, respectively) were made by diluting the corresponding stocks 65-fold in PBS. 0.1% and 0.01% Triton X-100 detergent solutions were prepared as explained in Section 3.4.1.

3.5. Methods

This part will introduce the method applied for (1) optimization of the concentration of biotin-AuNPs, and (2) the main methods used in this thesis work.

3.5.1. Optimization of AuNPs concentration

Stock solution of biotin-AuNPs ($1.92 \cdot 10^{11}$ particles/mL) was diluted to determine the optimal concentration: first, a 10-fold dilution of the stock was prepared, and thereafter 2-fold serial dilutions were made. In total, 8 dilutions were prepared in PBS. 150 µL of each dilution was pipetted into the wells of a 96-well streptavidin coated plate. Each concentration of the serial dilutions was run in duplicate. The plate was incubated for 1 h at 30 °C. After incubation, wells were washed 3 times with 0.1% Triton X-100 and left in PBS. Measurements of absorbance were performed with Cytation 5 microplate reader at 572 nm.

3.5.2. Streptavidin-biotin approach

Experiments were conducted in the 96-well streptavidin coated plates. A total of six experiments were carried out using the streptavidin-biotin approach. 5 dilutions of streptavidin solution were prepared over the range of 23-364 nM from the stock (1 mg/mL) in PBS. The volume used in the wells was 150 µL. The layout of the plate used in the experiment is presented in Figure 5. The procedure used in the experiments is described below:

- Step 1: Pre-wash the wells with PBS for 10 min.
- Step 2: Dilute the biotin-AuNPs stock 80-fold in PBS and add to all the wells, then incubate the plate in the shaker for 1 h at 25 °C.

- Step 3: After incubation, wash the wells 3 times with 0.1% Triton X-100 solution and with Milli-Q water at the end to clear the wells from foam.
- Step 4: Add 5 dilutions of streptavidin solution to the separate wells from higher concentration to lower (Figure 5), and incubate the plate for 1 h hour at 25 °C.
- Step 5: Repeat Step 3 and add biotin-AuNPs to all the wells, incubate the plate in the shaker for 1 h 25 °C.
- Step 6: Repeat Step 3 and leave the wells in rows A, B and C in PBS.
- Step 7: Add 5 dilutions of streptavidin solution to the wells in rows D and E, and incubate the plate for 1 h hour at 25 °C.
- Step 8: Repeat Step 3 and add biotin-AuNPs to the wells in rows D and E.

Finally, after the last washing, wells are left in PBS for overnight incubation at 4 °C. On the next day, the plate is pre-equilibrated at room temperature, and absorbance is read using Cytation 5 microplate reader at 570 nm. PBS is used instead of streptavidin solution in control wells to compare if added biotin-AuNPs bind to the streptavidin at the bottom of the plate, and therefore leads to increase in intensity.

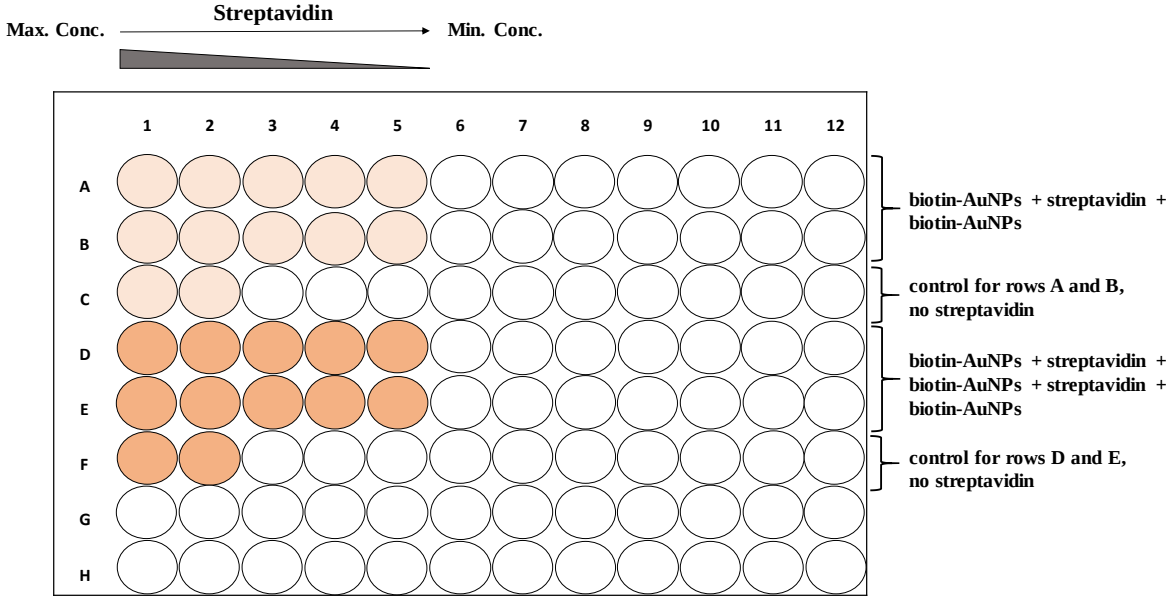


Figure 5. Experimental design of the 96-well plate for the streptavidin-biotin approach.

3.5.3. Biotin-FITC experiment

The goal of this experiment was to test if streptavidin was biologically active following storage at -20 °C. For this purpose, two streptavidin samples were compared, of which one was an active control (based on Dr. Kaido Viht's previous experiments) and the second was used in this thesis. Overall, three independent experiments were done based on the previously reported experimental approach [36] validated by Dr. Kaido Viht (unpublished data). For checking the activity of the streptavidin, a fluorescence quenching assay using biotin-FITC was performed. The experiment was done on the 384-well plate.

Buffer solution used in this experiment was prepared as follows: 10 mL of 50 mM HEPES + 150 mM NaCl (pH 7.4), supplemented with 0.005% Tween-20 and 5 mM of DTT. Concentration of biotin-FITC stock was checked by UV-Vis spectrophotometer. On a 96-well plate, 2-fold serial dilutions of biotin-FITC (over the range of 0.98 - 2000 nM) and 2-fold serial dilutions of streptavidin samples (in the range of 6-100 nM) were prepared in the buffer solution. Dilutions of biotin-FITC (10 µL) and streptavidin samples (10 µL) were transferred from the 96-well plate to the 384-well plate using an automated multichannel pipette. The final volume was 20 µL. The plate was incubated in the shaker for 20 min at 30 °C. Fluorescence intensity was measured with a PHERAstar microplate reader using filter block FI 603A (excitation at 485 (12) nm and emission at 520 (515-545) nm).

3.5.4. Click chemistry approach

In the click chemistry approach, two AuNPs (one labeled with DBCO and one with azide), are conjugated through the covalent bond formed during DBCO-azide reaction. Altogether, 7 experiments were performed using the click chemistry approach, where several parameters were varied (Section 4.2). The design used in the experiments is shown in Figure 6.

- Step 1: Pre-wash the wells with PBS for 10 min.
- Step 2: Add biotin-azide to all the wells, excluding control wells, then incubate the plate in the shaker for 15 minutes. The incubation temperature was set to 30 °C.
- Step 3: After incubation, wash the wells twice with 0.01% Triton X-100 solution.
- Step 4: Add DBCO-AuNPs to the wells and incubate the plate for 2 hours.
- Step 5: Wash the wells twice and add azide-AuNPs, and incubate for 2 hours.

Steps 4 and 5 were repeated for further sequential addition (Figure 6).

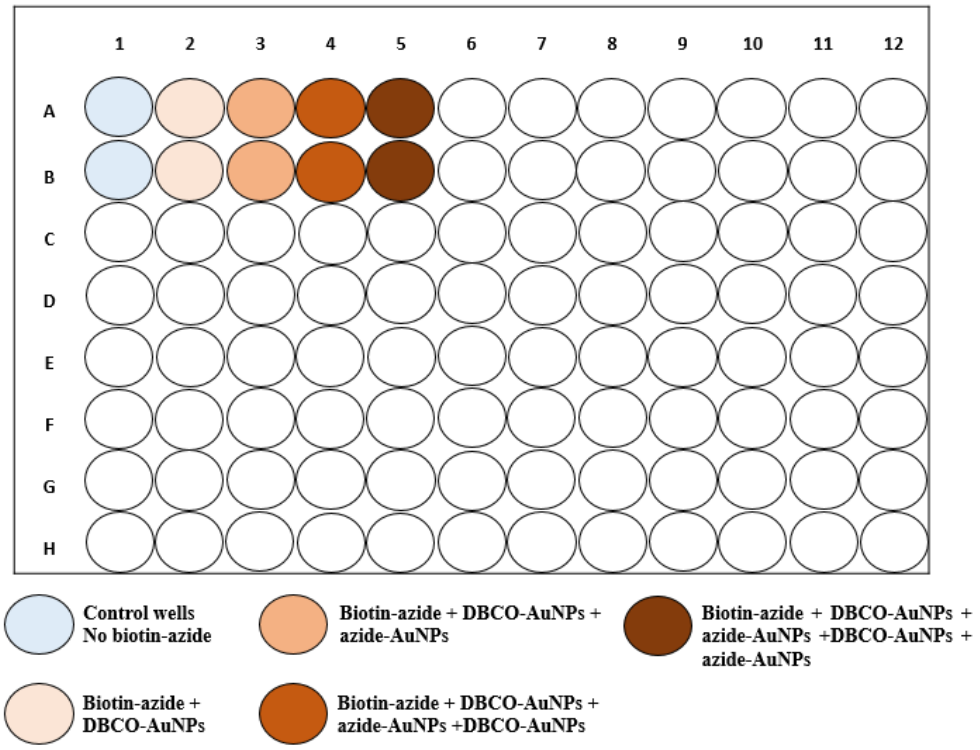


Figure 6. Experimental design used in the click chemistry approach.

4. RESULTS AND DISCUSSION

4.1. Streptavidin-biotin approach

4.1.1. Optimization of AuNPs concentration

A standard curve was constructed to identify the linear working range. The goal was to establish the working measurement range for signal amplification experiments so that the difference between the initial and higher intensity values could be accordingly measured. On the other hand, it was intended to avoid the excessive consumption of AuNP reagents. Therefore, a high enough amount of AuNPs was used to get a measurable signal and low enough to spare the reagent. Also, it was important to maintain a low AuNP concentration to avoid the possibility that biotin-AuNPs would compete for the binding sites on the streptavidin. The concentration range examined was $1.50 \cdot 10^{8}$ - $1.92 \cdot 10^{10}$ particles/mL. The results of the experiment are shown in Figure 7.

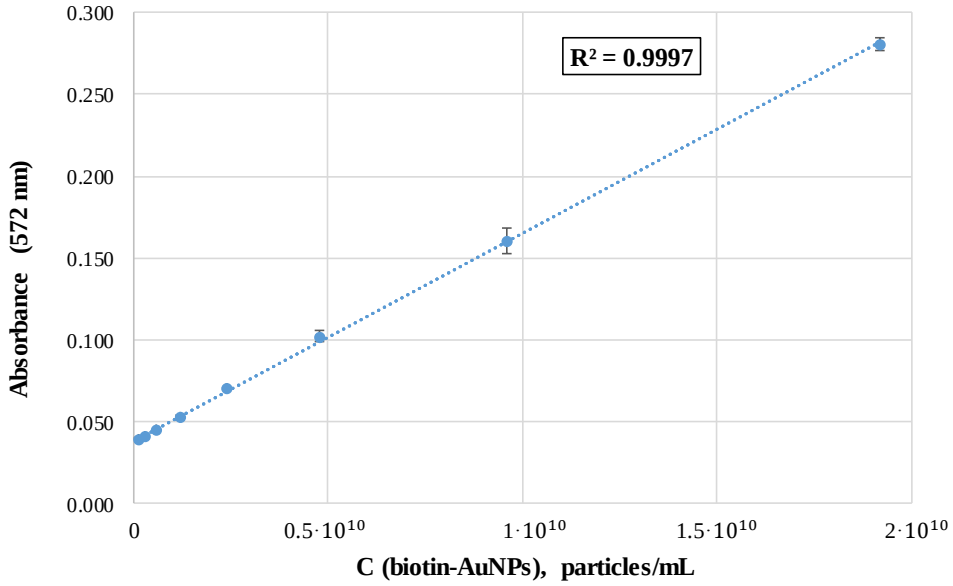


Figure 7. A standard curve plot of the serial dilution of biotin-AuNPs. Error bars correspond to the standard deviations calculated from two replicates.

Based on this experiment, concentrations of biotin-AuNPs to be used in the next experiments were chosen. Concentration range used was $2.4 \cdot 10^{9}$-$6.4 \cdot 10^{9}$ particles/mL (0.064-0.120 AU) which was within the linear range. Absorbance spectra of highest concentration of biotin-AuNPs were recorded, and absorption maximum was in the proximity of the ultimate maximum (572 nm) (Figure 8).

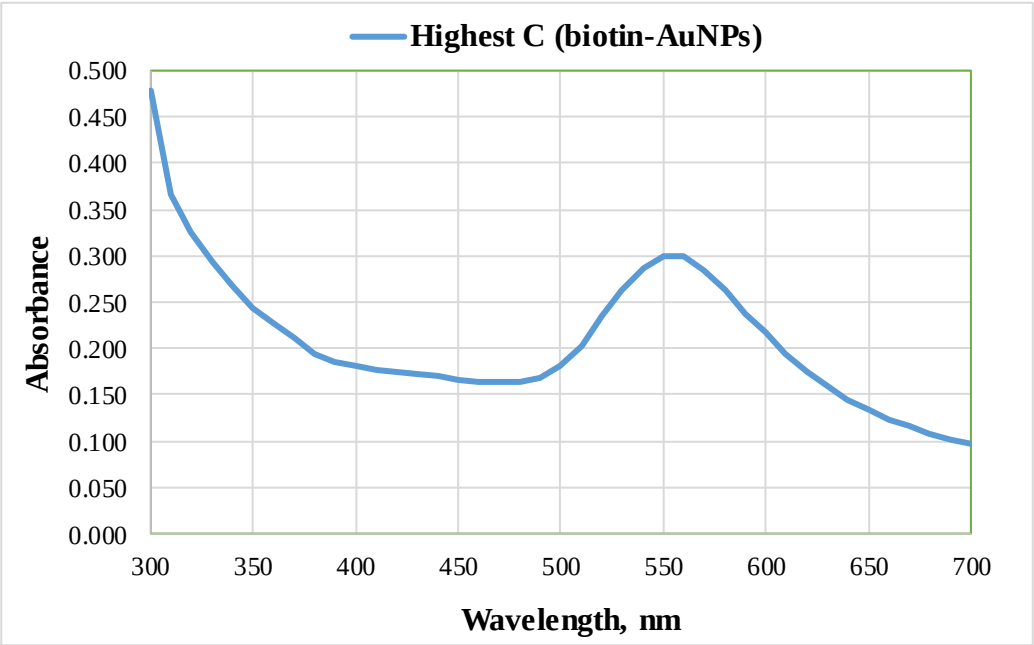


Figure 8. Absorbance spectra of highest concentration of biotin-AuNPs ($1.92 \cdot 10^{10}$ particles/mL).

4.1.2. Experiment 1

5 streptavidin dilutions and control wells with no streptavidin were used in this experiment. Amplification was monitored after 2 and 3 sequential additions (as described in Section 3.5.2). Results of the experiment can be seen in Figure 9. Mean values and standard deviations of 2 replicates are shown in the graph.

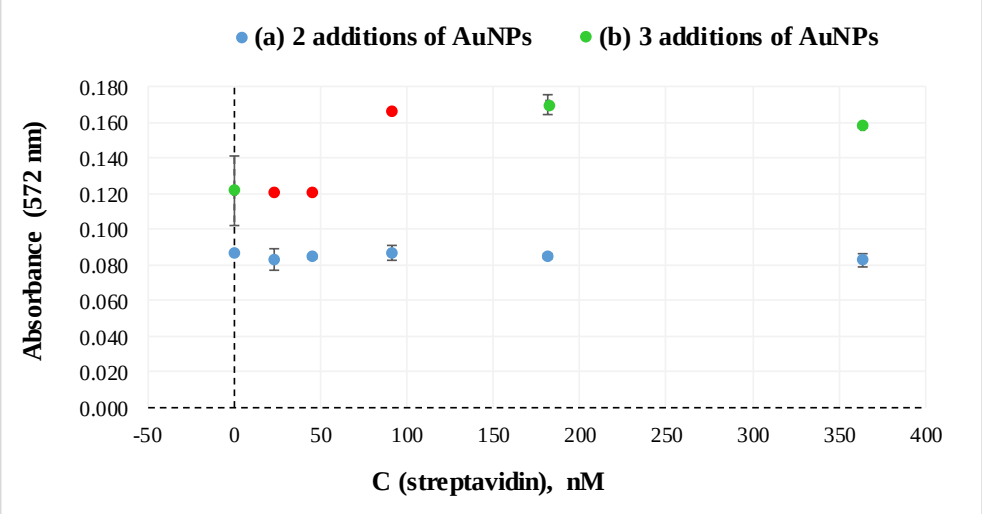


Figure 9. (a) 2 additions of AuNPs (rows A-B on the well plate) and control without streptavidin (row C on the well plate), (b) 3 additions of AuNPs (rows D-E on the well plate) and control without streptavidin (row F on the well plate). Red-colored points in the graph are used to indicate the absence of the replicate.

From the graph (Figure 9), it was observed that in the case of 3 additions of biotin-AuNPs, absorbance values were higher compared to 2 additions of biotin-AuNPs. However, significant differences between the data points obtained with and without streptavidin addition (controls) cannot be claimed. For better comparison of surface homogeneity, bright-field images of the wells were captured using Cytation 5 microscope (Figure 10).

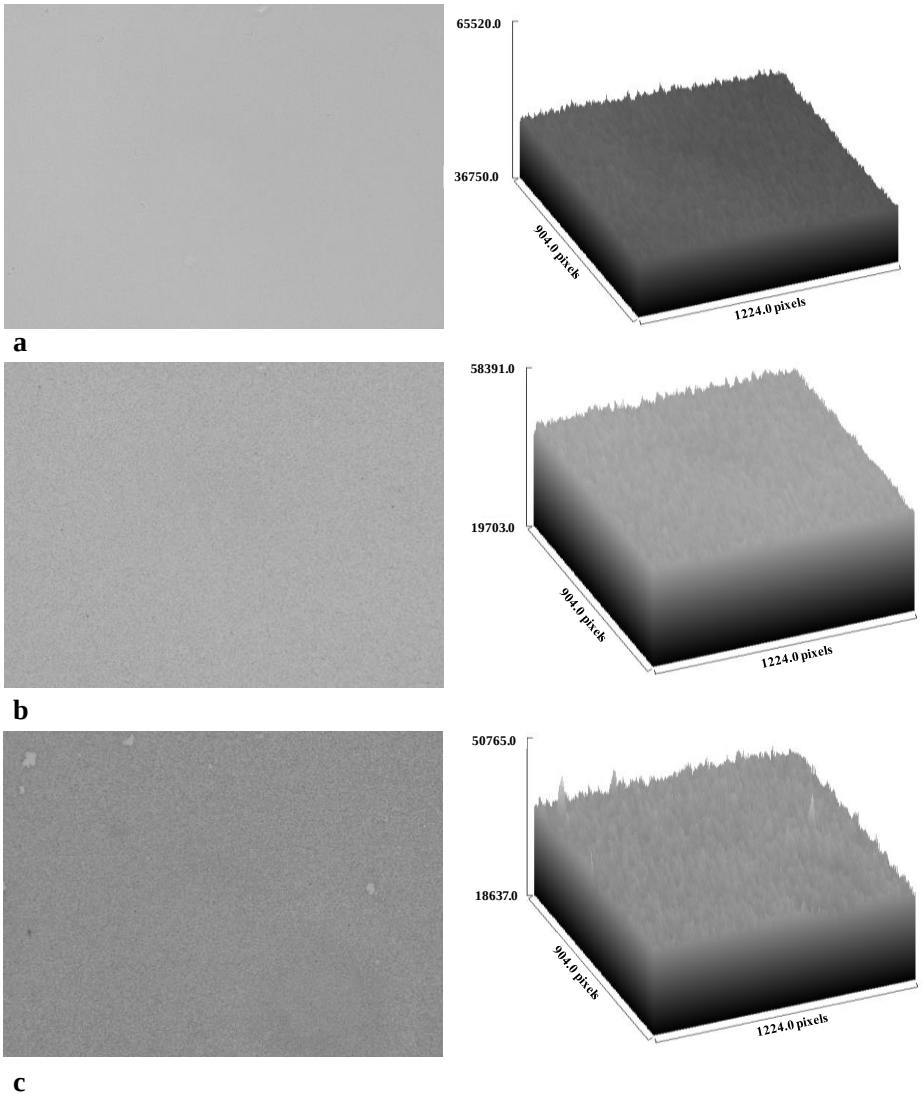


Figure 10. Microscopy images (left) and surface plots (right) of the wells (20× objective) in case of (a) empty well (only PBS added), (b) 2 additions of biotin-AuNPs (well A3), C (streptavidin) = 91 nM, (c) 3 additions of biotin-AuNPs (well D3), C (streptavidin) = 91 nM. Please note that the Z-scale values of surface plots (right) are different.

The bright-field microscopy visualizes the amount of the light passed through the sample at different locations: darker regions indicate local absorption or scattering of light. Visual comparison between the images shows the change in brightness, *i.e.*, the surface gets darker (from (a) to (c) in Figure 10). This is due to several additions of biotin-AuNPs, indicating that with increasing concentration, more biotin-AuNPs bind to the surface.

In conclusion, based on the results of absorbance and microscopy, it can be concluded that the approach did not work as hypothesized and further parameter changes were made to test this amplification approach further.

4.1.3. Experiment 2: effect of increased concentration of biotin-AuNPs on the speed and yield of reaction

It was assumed that an insignificant difference between signals of wells with and without added streptavidin could be because the concentration of added biotin-AuNPs was too low. Therefore, for the next experiment, the dilution of biotin-AuNPs was decreased from 80-fold to 30-fold. Other aspects of the experimental setup were kept constant. The results are presented in Figure 11.

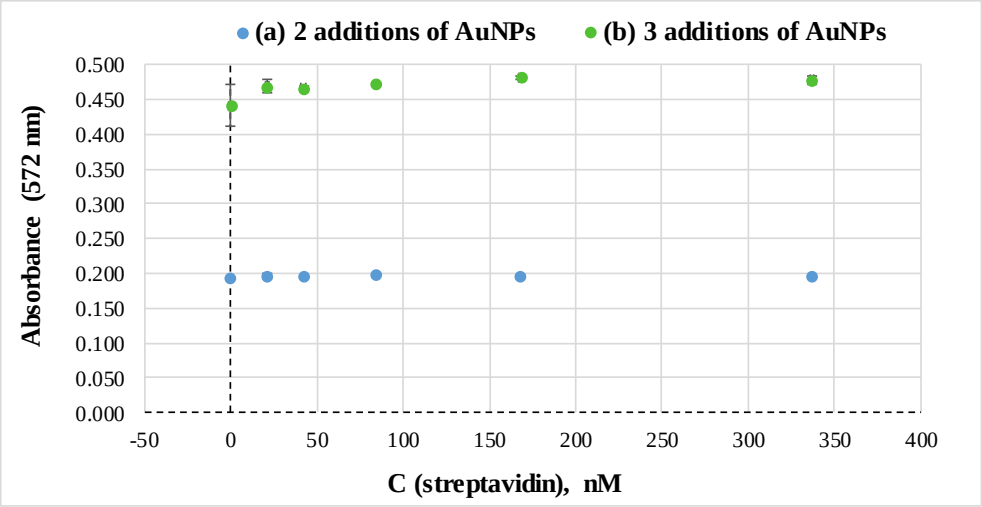


Figure 11. Results of the experiment with the increased concentration of biotin-AuNPs (30-fold dilution). (a) with 2 additions of AuNPs and zero streptavidin control, (b) with 3 additions of AuNPs and zero streptavidin control.

As a result of increasing the concentration of biotin-AuNPs, it was observed that the absorbance values increased. However, these values do not indicate a substantial increase between data points in case of control wells (no streptavidin) compared to the wells with added streptavidin, so that the difference cannot be considered significant.

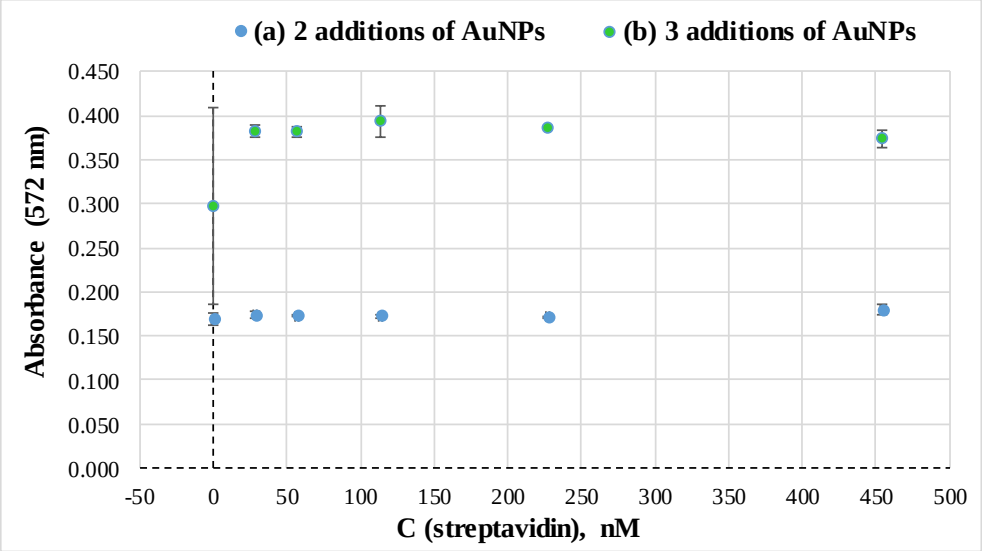
4.1.4. Experiment 3: effect of blocking non-saturated binding sites on the surface

Based on the conditions used in Experiments 1 and 2, the amounts of streptavidin at the bottom of the plate, amount of biotin-AuNPs, and streptavidin added to a well were calculated (Table 2).

Table 2. Calculations made for interpretation of results in Experiment 2

Reagent	**Amount / pmol**
Streptavidin binding sites available at the bottom of the well	19
Biotin-AuNPs added in one addition to a single well (30X dilution)	0.0016
Streptavidin binding sites in one addition to a single well (C = 337 nM)	51
Biotin immobilized onto the AuNPs (in 150 µL of 30X dilution)	3005

It was found that the amount of streptavidin covalently bound to the bottom of the plate was much higher than added biotin-AuNPs to the wells. Therefore, it was presumed that the sites of immobilized streptavidin could not be saturated by a single addition of AuNPs, and thus the added streptavidin bound to the added biotin-AuNPs would not give any significant signal increase. Consequently, to block the unsaturated sites on streptavidin at the bottom of the well, a biotin stock solution (819 µM) was added 3 times after the first incubation with biotin-AuNPs (55X dilution) and incubated for 10 minutes before streptavidin addition. It is expected that the biotin blocks the excess streptavidin binding sites at the bottom of the plate, and therefore increase in the signal can only be caused by biotin-AuNP binding to streptavidin bound to the first addition of biotin-AuNP. An identical experiment layout (Figure 5) was used for further experiments. Results of the experiment are presented in Figure 12.



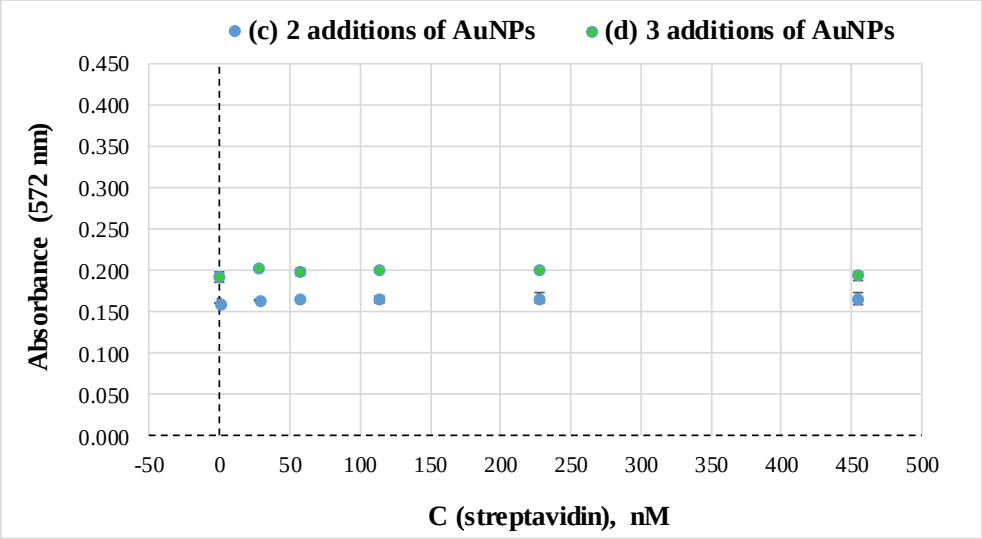


Figure 12. Results of the experiment with and without biotin blocking. 55X dilution of biotin-AuNPs was used. (a) 2 additions of biotin-AuNPs without biotin blocking, (b) 3 additions of biotin-AuNPs without biotin blocking, (c) 2 additions of biotin-AuNPs with biotin blocking, (d) 3 additions of biotin-AuNPs with biotin blocking.

From the results (Figure 12), it was observed that the absorbance values in the wells with biotin blocking after 3rd addition of biotin-AuNPs (c) were approximately 50% lower compared to the wells with 3 additions of biotin-AuNPs where no biotin blocking (a) was used. It was hence confirmed that biotin blocking had the desired effect: biotin-AuNPs were blocked from binding to the bottom, leading to lower signals. Still, it can also be seen from Figure 12 (c, d) that even with

biotin blocking a significant increase in the signal was not achieved with sequential addition of AuNPs (*i.e.*, comparing the control well with the test wells).

4.1.5. Experiment 4: effect of decreased incubation time

Another experiment with biotin blocking was performed where the incubation time of all the reagents was decreased from 1 h to 10 min. The hypothesis was that prolonged incubation could lead to the biotin-AuNPs dissociation from the streptavidin at the bottom of the plate and binding with the added streptavidin while incubating.

Decreasing incubation time did not lead to the difference in the signals between 3 additions of biotin-AuNPs with and without biotin blocking (Figure 13). It can also be seen that absorbance values in the control well and test wells do not differ in both cases (a and b).

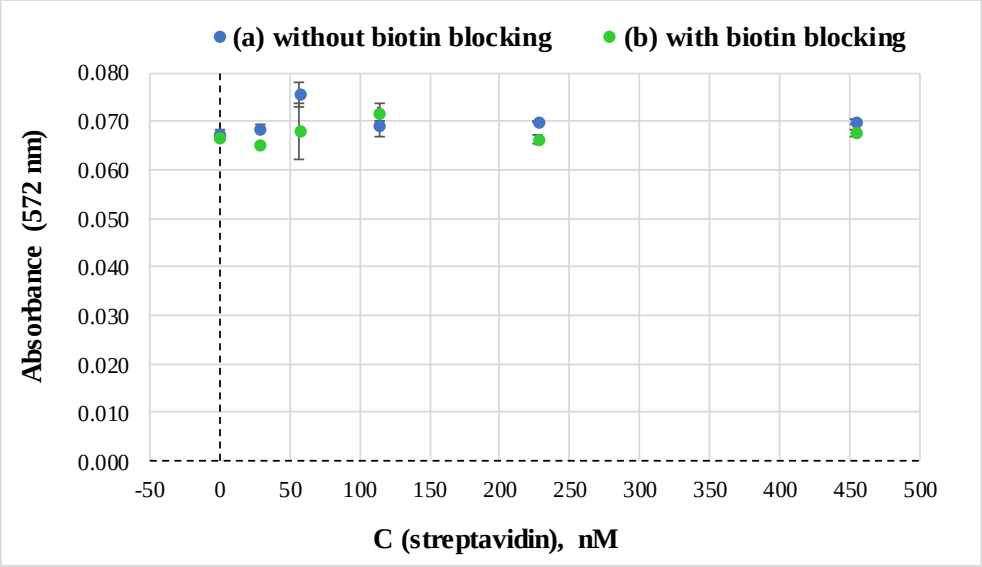


Figure 13. Results of the experiment where incubation time was decreased from 1 h to 10 min. (a) 3 additions of biotin-AuNPs (55X dilution) without biotin blocking, (b) 3 additions of biotin-AuNPs (55X dilution) with biotin blocking.

4.1.6. Experiments 5 and 6: effect of detergent concentration in the washing solution and effect of pre-mixing of reagents

As it was hypothesized that elevated concentration of the detergent can interfere with biological interactions between streptavidin and biotin, 0.01% Triton-X detergent solution instead of 0.1% was used in this experiment. Moreover, two cases were examined here: (1) adding reagents to the wells sequentially (as usual) and (2) mixing and incubating streptavidin with biotin-AuNPs in

microcentrifuge tubes before adding to the wells. The latter was carried out to test whether streptavidin-biotin reaction could be accelerated in solution compared to the reaction on the surface. Incubation time was 1 h. The results for both cases can be observed in the graph (Figure 14).

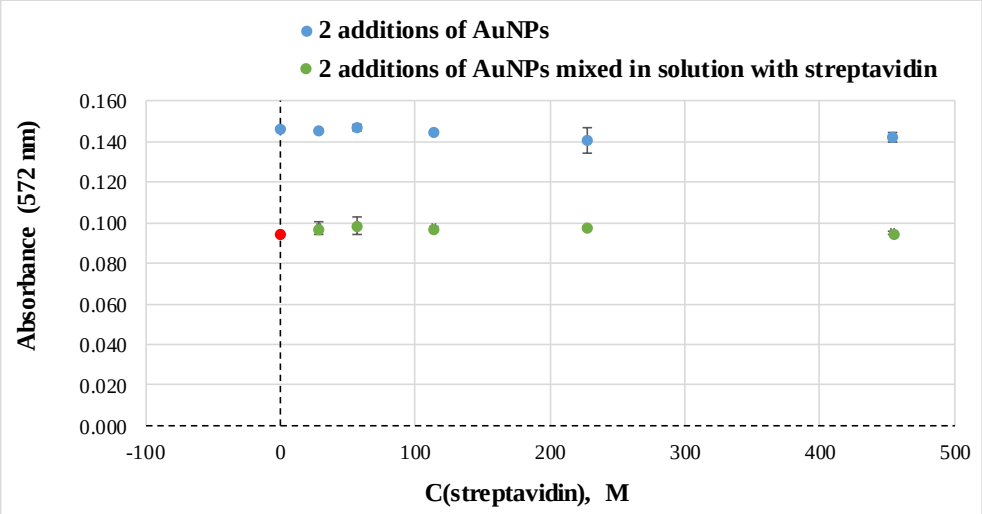


Figure 14. Results of the experiment with decreased concentration of detergent (0.01%). (a) 2 additions of biotin-AuNPs (45X dilution), (b) 2 additions of biotin-AuNPs (45X dilution) (mixed in solution and incubated together with streptavidin before adding). Red data point indicates absence of 2nd addition of biotin-AuNPs in control well.

In both cases, the difference in the signals between the control wells and test wells and signal increase were not observed. It was assumed that the streptavidin was getting engulfed by the biotin-AuNPs (see discussion in Section 4.1.8).

Similar experiment was done (Experiment 6), but incubation time for the blocking step and pre-mixing the reagents (streptavidin with biotin-AuNPs) was prolonged. In the first case, biotin was added to the wells for blocking and incubated overnight. In the second case, streptavidin was mixed with biotin-AuNPs in solution in microcentrifuge tubes and incubated overnight. The following day reagents were added sequentially. The prolongation of the incubation time did not alter the outcome. The results of the experiment are presented in ANNEX 1.

4.1.7. Biotin-FITC experiment

Fluorescence quenching measurements were made with biotin-FITC to test if the streptavidin used in this study is active (*i.e.*, capable of binding biotin). FITC is a fluorescent dye with an excitation peak at approximately 495 nm and an emission peak at 519 nm. All fluorescein derivatives are

prone to contact quenching; depending on pH and hydrophobicity of the surrounding environment, the fluorescence quantum yield of FITC can be altered substantially [37]. This can be utilized for measurement of biological interactions; for instance, in case of biotin-FITC, the quenching effect does predominantly occur when 2 or more biotin-FITC molecules are bound to the streptavidin due to the spatial proximity of the fluorophores. Results of the biotin-FITC experiment are presented in Figure 15.

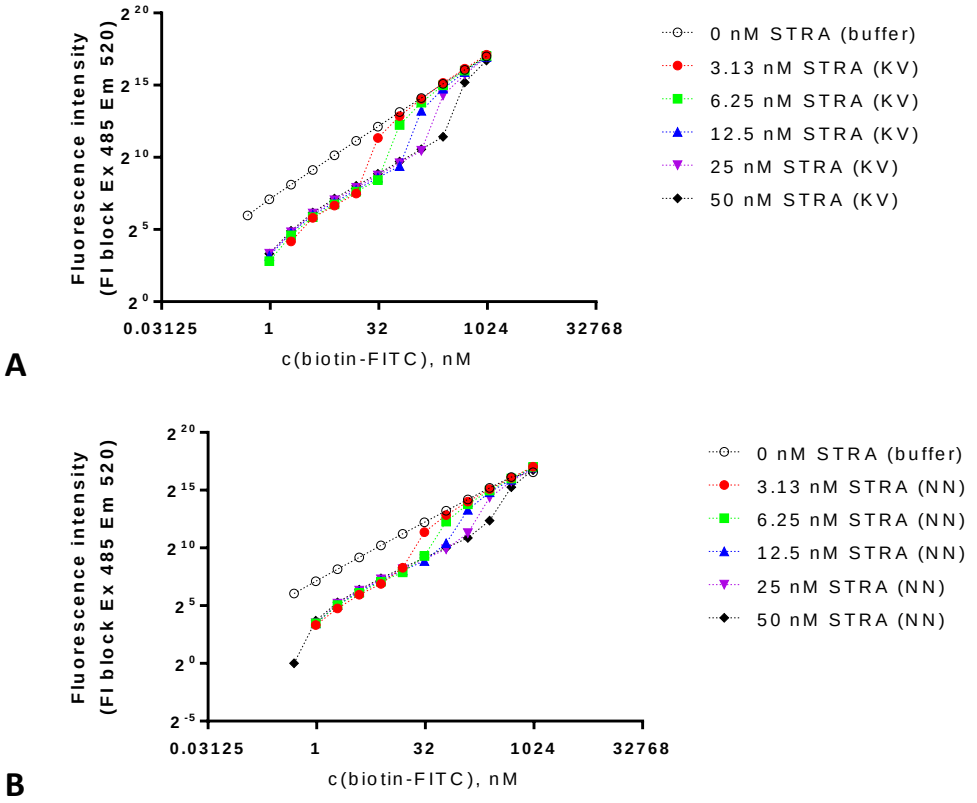


Figure 15. Results of biotin-FITC experiment. (A) KV – control streptavidin, (B) NN – streptavidin used in the given studies. For better visual representation, both axes are in logarithmic scale. Nominal concentrations of streptavidin are shown in the graph legend. Results of one representative experiment are shown.

If streptavidin is not present in the solution, the increase of biotin-FITC signal is linearly dependent on the concentration of the molecule, whereas, upon addition of streptavidin, there is a "kink" (an increase in the value of the slope) in the graph that separates the upper plateau (non-quenched FITC) from the bottom plateau (quenched FITC). The location of the "kink" in the graph depends on the concentration of the binding protein: the more streptavidin is added, the more biotin is

required to saturate all the binding sites, and hence the "kink" is shifted to the right at increasing concentrations of streptavidin. In this way, the concentration of the active streptavidin can be assessed: *e.g.*, in case of titration with 12.5 nM nominal concentration of streptavidin (both the control and the tested solutions), the data point corresponding to 62.5 nM biotin-FITC is on the bottom plateau (quenched FITC), whereas the data point corresponding to 125 nM biotin-FITC is on the top plateau (non-quenched FITC). Given that a maximum of 4 biotin moieties can bind to streptavidin, the concentration of the active streptavidin should be ca 15.6 nM, which is even slightly higher than the nominal concentration (the difference could probably be attributed to the fact that vendors usually provide a slightly higher amount of protein than ordered to compensate for the putative loss of activity during transportation and aliquoting). From the results of the experiment (Figure 15), it was concluded that the streptavidin used in the given study is active. Thus, the absence of upscaling is not caused by the inactive streptavidin.

4.1.8. Conclusion from streptavidin-biotin approach

Overall, several hypotheses were tested in studies with biotin-AuNPs and streptavidin, and different parameters (concentrations, incubation times, washing times, washing solutions, and blocking regimes) were varied to achieve signal amplification. However, satisfactory results were not obtained, possibly due to the engulfment of streptavidin by the biotin-AuNPs.

The following hypothesis is proposed for explaining the results of the streptavidin-biotin experiments: all 4 biotin-binding sites of streptavidin are bound by the biotin molecules from the same biotin-AuNP. This is supported by the fact that dimensions of streptavidin (4.2 nm × 4.2 nm × 5.6 nm) [38] are smaller than the length of the polyethylene glycol linker length between biotin and AuNP (5000 Da polyethylene glycol length is approximately 30 nm) [39].

To resolve this issue, the use of commercially available AuNPs functionalized with streptavidin is proposed. Another solution is to use click chemistry reactions for binding sequentially added reagents. Due to the formation of a covalent bond between the AuNPs, there is also no risk regarding the slow dissociation of the binding partners or competition of surface-bound reaction centers with those present in solution (which was possible in the case of experiments with biotin-AuNPs and streptavidin).

4.2. Click chemistry approach

Streptavidin well plates were also used for the click chemistry approach. Biotin, derivatized with an azide group, was used as a linker between streptavidin and the gold nanoparticles: it binds efficiently to streptavidin at the bottom of the plate, and the azide group can react with the alkyne on DBCO-AuNP. Similar to the streptavidin-biotin method, DBCO-AuNPs and azide-AuNPs are, therefore, added in sequential order to amplify the signal.

4.2.1. Experiment 1

AuNPs were diluted 65-fold in PBS. The well plate was incubated with the AuNP solutions for 2 h at 30 °C after each addition. Mean values and standard deviations of 2 replicates are presented in the graph (Figure 16).

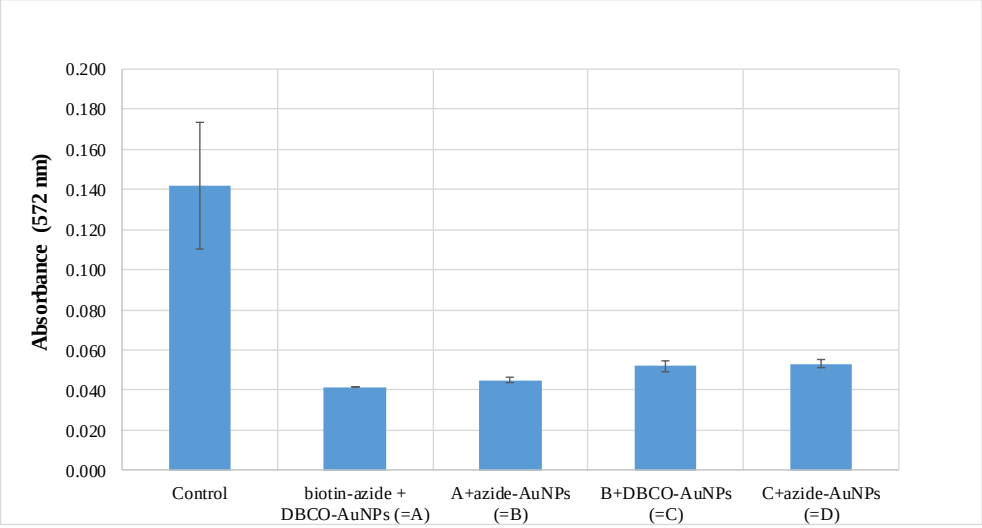


Figure 16. Result of the click chemistry Experiment 1. Control – no biotin-azide added (only 1 addition of DBCO-AuNPs and 1 addition of azide-AuNPs (65X dilution)).

It was observed that signals in control wells (no biotin-azide) were higher compared to the wells with biotin-azide. Furthermore, no substantial increase of absorbance values could be seen after sequential addition of azide-derivatized and DBCO-derivatized AuNPs. Next, for characterization of surface homogeneity, microscope images of the wells were taken (Figure 17).

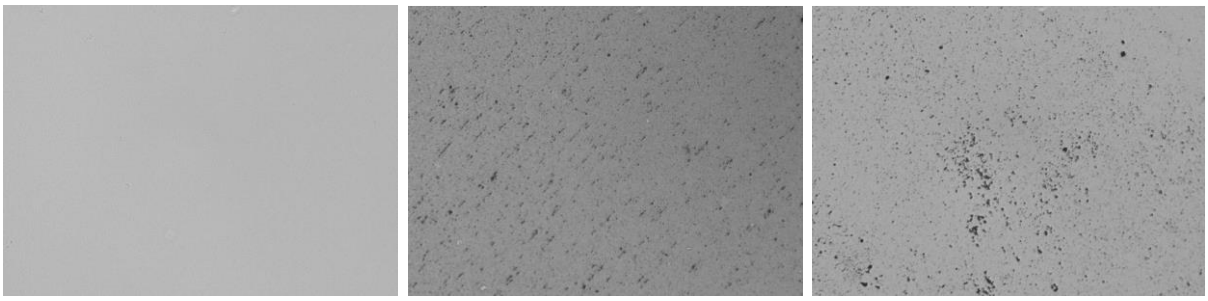


Figure 17. Microscope images of the wells (20× objective). (a) empty well, (b) well A1 on the plate (control) (DBCO-AuNPs + azide-AuNPs), (c) well A5 on the plate (2 additions of DBCO-AuNPs + 2 additions of azide-AuNPs).

From the absorbance values and the images, it was observed that AuNPs had formed aggregates (*i.e.*, large "clumps" of AuNPs) and were non-specifically bound to the bottom of the well. It is important to note that there were aggregates present even in control wells where biotin-azide was not included (Figure 17 (b)). The absorbance in the non-control wells (containing biotin-azide) is suspected to be lower because biotin-azide blocks the surface, and the AuNPs cannot adsorb onto the surface to such extent as in the control wells. Further experiments were made to study this phenomenon.

4.2.2. Experiment 2: consecutive addition of single type of AuNPs

From the previous experiment it was not clear if the aggregates had formed due to the DBCO-azide reaction or due to non-specific binding of the AuNPs. For this reason, an experiment was done where azide-AuNPs and DBCO-AuNPs were not added to the same well (*i.e.*, only DBCO-AuNP solutions were added to the wells in row A, and only azide-AuNP solutions were added to the wells in row B). Otherwise, the experimental conditions were kept constant. The results can be seen in Figure 18.

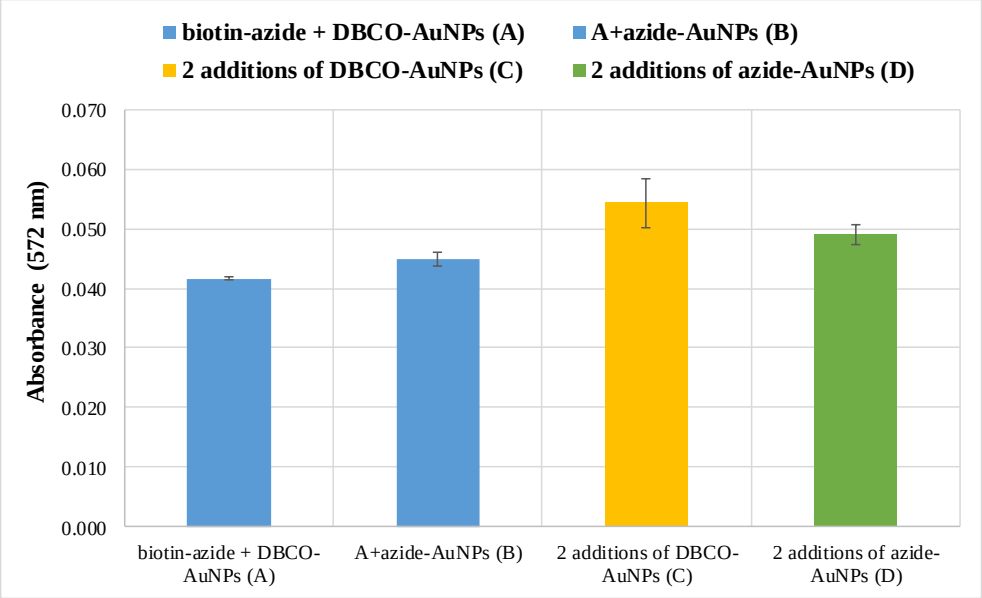


Figure 18. Results of Experiment 2 with 2 additions of DBCO-AuNPs and 2 additions of azide-AuNPs. Absorbances of wells where biotin-azide, DBCO-AuNPs and azide-AuNPs are sequentially added are shown for comparison.

Microscopy images were also taken to see if aggregation still took place (Figure 19).

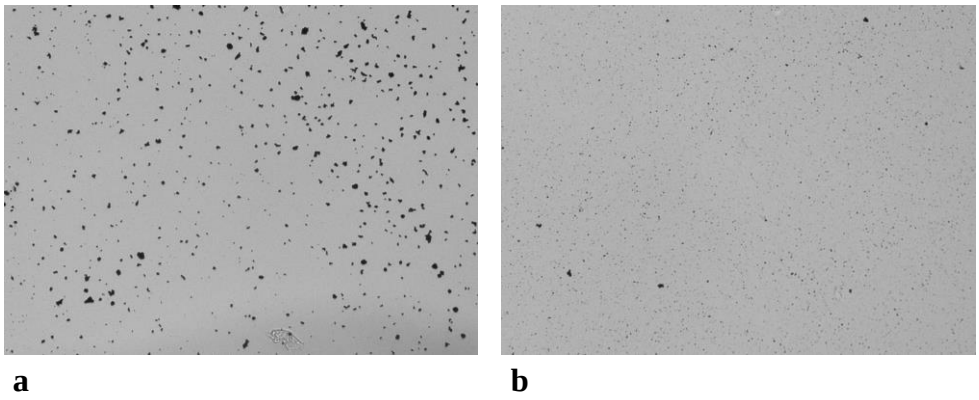


Figure 19. Microscope images of the wells (20× objective). (a) well A1 (double addition of DBCO-AuNPs), (b) well A2 (double addition of azide-AuNPs).

From Figure 18 and Figure 19, it can be seen that absorbances are higher for wells where different surface modified AuNPs were not mixed and that aggregates still formed at the bottom of the well. Therefore, it was concluded that non-specific binding (not the DBCO-azide reaction) of the AuNPs to the well was the reason why the aggregates could be seen at the bottom of the well. Moreover, the DBCO-AuNPs were more prone to aggregation (possibly, due to the hydrophobic nature of DBCO). The following experiments were done to eliminate the non-specific binding.

4.2.3. Experiments 3 and 4: effect of BSA and DMSO on non-specific binding of AuNPs

1% BSA and 0.2% DMSO were tested as additives in the incubation solution. It was hypothesized that BSA would block the non-specific binding sites in the well and decrease formation of aggregates at the bottom of the well. As it was speculated that non-polar ends of the AuNPs surface modification led to the non-specific binding, DMSO was hypothesized to alleviate this problem, leading to better suspension stability. Other parameters of the experiment were kept constant. The results of the experiment are shown in Figure 20.

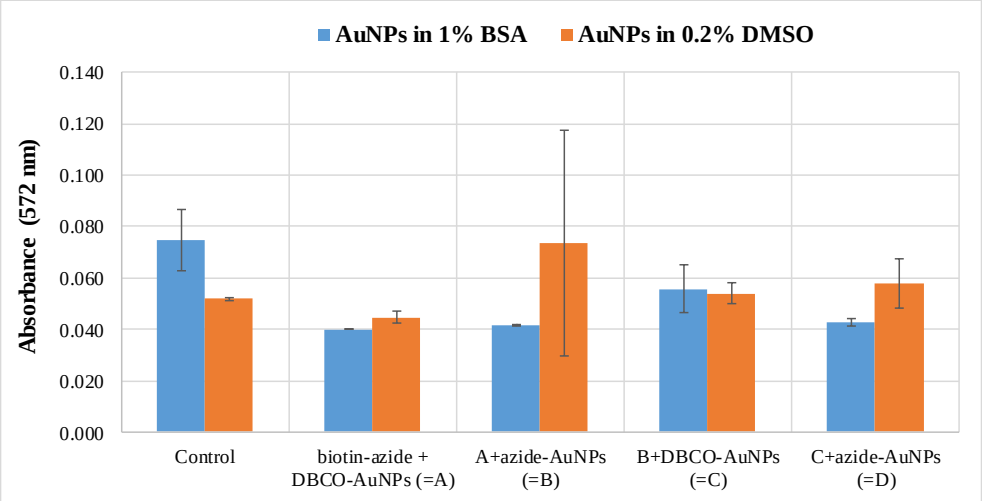


Figure 20. Results of the experiment where 1% BSA and 0.2% DMSO (65X dilution of AuNPs) used to decrease the non-specific binding and aggregation at the bottom of the well.

It was observed that non-specific binding in control wells as compared to the well blocked by the addition of biotin-azide was slightly reduced in both cases. This is concluded because the difference between the control well and wells indicated in column A in Figure 20 is smaller than in previous experiments (*e.g.*, Figure 16). It was noted during these experiments that aggregates were forming already in the AuNP stock solution. It was speculated that this leads to precipitation of the large aggregates in the wells. Moreover, the formation of aggregates would lead to a decrease in the apparent concentration of the AuNPs in the solution and, therefore, a slower DBCO-azide reaction. Therefore, in the subsequent experiments, different approaches were tested to eliminate the aggregation in the solutions before applying them to the wells.

4.2.4. Experiments 5, 6 and 7: effect of vortexing, sonication, and increased concentration of detergent on aggregation of AuNPs

To get rid of aggregation the following steps were taken. Stocks of AuNPs were diluted in 0.01% Triton-X in PBS solution, and the washing steps were done using 0.1% Triton-X-PBS solution. In vortexing experiment (Experiment 5), stocks of AuNPs and all dilutions were vortexed, and in sonication experiment (Experiment 6), the stocks and dilutions were sonicated for 5 min before each addition. In Experiment 7, 0.1% Triton-X in PBS solution was used both as a solvent and washing solution. Other parameters of the experiments were kept constant. Results of 3 experiments are presented in Figure 21.

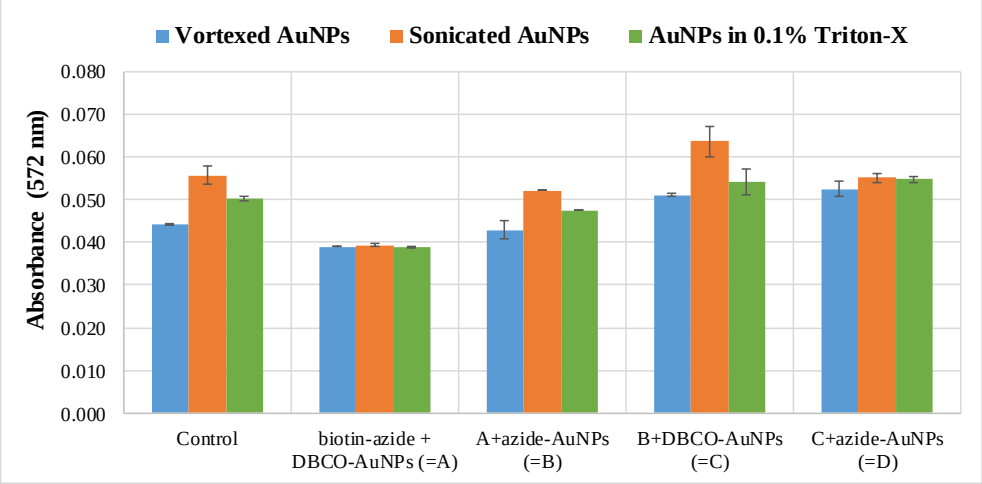


Figure 21. Results of the experiments 5,6 and 7. AuNPs; 65X dilution.

From the results, it can be seen that vortexing, sonicating, and increasing the concentration of the detergent helped to decrease the non-specific binding to a slight extent compared to previously conducted experiments (*i.e.*, absorbance in control wells and wells in column A are similar compared to Figure 16, and the absorbance in the control well is lower than in Figure 16). Nevertheless, the non-specific binding still occurred in the control wells in all 3 experiments (ANNEX 2). In case of experiments with vortexed AuNPs as well as AuNPs dissolved in 0.1% Triton X, it was observed that absorbance values increased after each addition of AuNPs, but the difference between values was low (no practical signal amplification could be achieved).

4.2.5 Conclusion from click chemistry approach

Based on the experimental results with AuNPs that have DBCO and azide functional groups, it was concluded that the amplification of the signal could not be achieved due to aggregation of the AuNPs. As the AuNPs aggregate, the concentration of particles in the solution decreases leading to lower reaction rates, and the aggregates can sediment and adsorb non-specifically onto the well plate. It is suspected that the AuNPs had aggregated in stock solution already upon arrival due to PBS that was used as solvent for the nanoparticles. The AuNPs can aggregate because the negative charge on the surface of AuNPs is shielded by the ions in PBS [40]. Thus, new AuNPs must be ordered in another solvent to conduct further experiments.

5. SUMMARY

In the present study, the aim was to develop a novel signal amplification method where sequential addition of reagents (AuNPs) is used for achieving enhanced signal. Two approaches were investigated to achieve signal upscaling:

- streptavidin-biotin approach, based on non-covalent interaction of biotin (immobilized onto AuNPs) and streptavidin;
- click chemistry approach, based on the strain-promoted azide-alkyne cycloaddition reaction (the reactive groups were immobilized onto AuNPs).

In total, 6 experiments for the streptavidin-biotin approach and 7 experiments for the click chemistry approach were done. In the streptavidin-biotin approach, modified parameters were the following: concentration of AuNPs, blocking of the plate surface with biotin solution, incubation time, and detergent concentration. In the click chemistry approach, varied parameters were: different solvents, vortexing and sonication of AuNPs, the concentration of a detergent solution.

Two types of measurements were made in these approaches: absorbance at 572 nm (corresponding to AuNP absorption maximum) and brightfield microscopy (for assessment of homogeneity of the formed surface). It is speculated that biotin-streptavidin approach did not give upscaling of the signal due to engulfment of the streptavidin into the polyethylene glycol layer of a single AuNP. Several issues were observed for click chemistry approach, such as aggregation of AuNPs, and non-specific binding of AuNPs to the bottom of the plate. Overall, although signal upscaling could not be achieved during this thesis, the knowledge gained from these experiments will be used as the basis for further research. In case of streptavidin-biotin approach, the AuNPs with streptavidin surface modification could be used, and in the case of click chemistry approach, AuNPs in a more suitable solvent could be tested. Moreover, one of the prospective methods that can be applied in the future is enzymatic amplification where the signal upscaling is achieved via repeated catalytic conversion of the substrate.

6. ACKNOWLEDGEMENT

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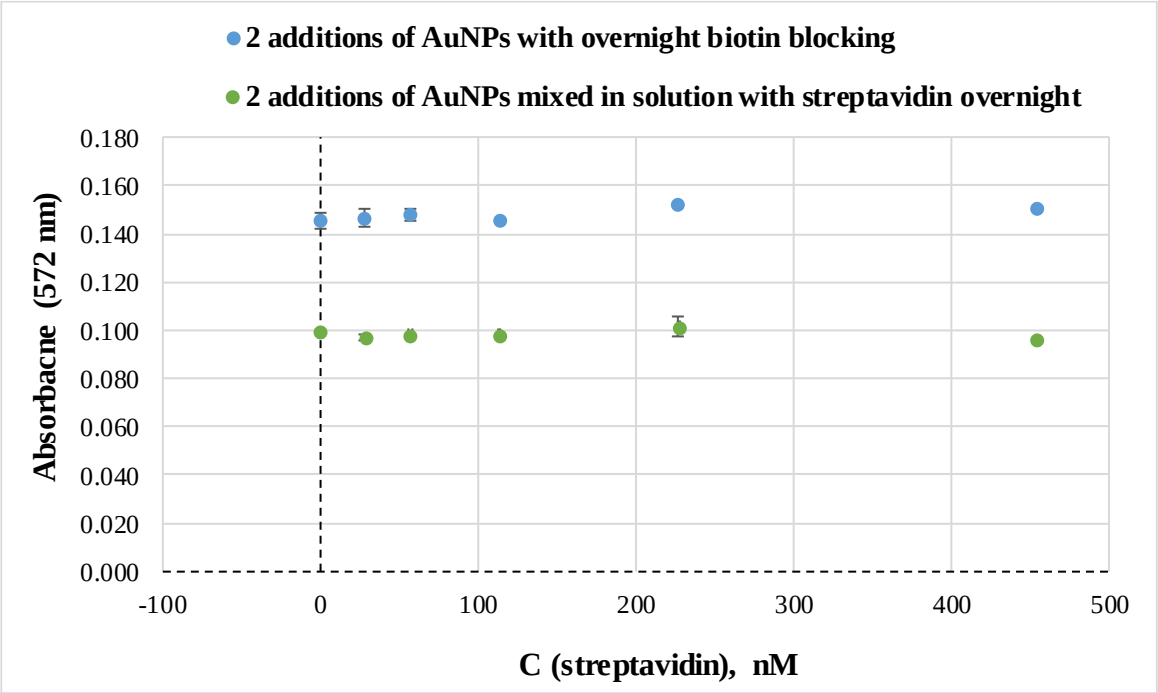
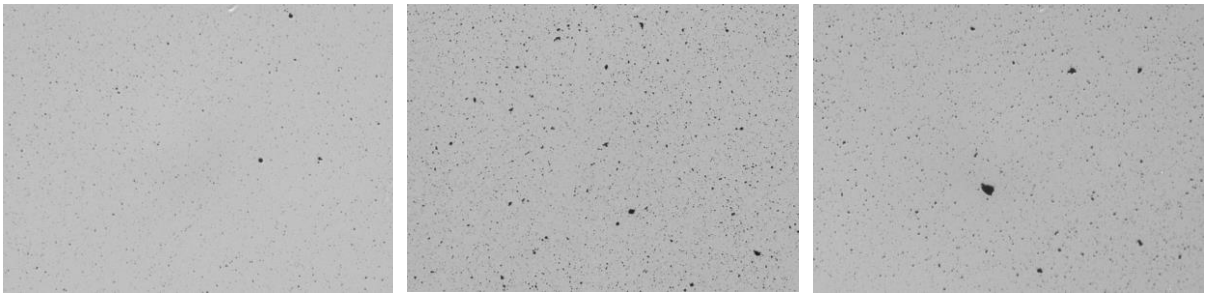
ANNEXES

ANNEX 1. Experiment 6 in streptavidin-biotin approach



Results of the experiment with overnight (24 h) incubation (a) 2 additions of biotin-AuNPs (45X dilution) with overnight biotin blocking, (b) 2 additions of biotin-AuNPs (45X dilution) (mixed in solution and incubated together with streptavidin overnight).

ANNEX 2. Microscopy images from the experiments 5, 6 and 7



a **b** **c**

Microscope images of the wells (20× objective). (a) well A1 (control well from the Experiment 5), (b) well A1 (control well from the Experiment 6), (c) well A1 (control well from the Experiment 7).

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Naila Nasirova

25/05/2021

INFORMATION SHEET

Signal upscaling with the sequential addition of reagents for enhancement of detection sensitivity

In the present study, a simplified model system for signal upscaling using sequential addition of reagents was aimed to be developed using gold nanoparticles (AuNPs). For achieving this goal, two approaches were investigated: streptavidin-biotin and click chemistry. In the streptavidin-biotin approach, modified parameters were the following: concentration of biotin-AuNPs, blocking of the plate surface with biotin solution, incubation time, and detergent concentration. In the click chemistry approach, varied parameters were: different additives in PBS, vortexing and sonication of AuNPs (derivatized with azide and alkyne), the concentration of a detergent solution. Two types of measurements were made in these approaches: absorbance at 572 nm (corresponding to AuNP absorption maximum) and brightfield microscopy (for assessment of homogeneity of the formed surface). Although signal upscaling could not be achieved during this thesis, the knowledge gained from this experience will be used as the basis for further research.

Keywords: sensitivity, gold nanoparticles, signal upscaling, streptavidin, biotin, click chemistry

CERCS: P300 analytical chemistry

INFOLEHT

Signaali võimendamine detekteerimise tundlikkuse suurendamiseks reagentide järjestikuse lisamise teel

Käesoleva uurimustöö eesmärk oli välja töötada signaali suurendamise süsteem, kasutades kulla nanoosakeste (AuNP) järjestikust lisamist. Selleks uuriti kahte metoodikat: streptavidiin-biotiin ja *Click*-keemia. Streptavidiin-biotiini metoodika puhul muudeti järgmisi parameetreid: AuNP-de kontsentratsioon, plaadi pinna blokeerimine biotiiniga, inkubatsiooniaeg ja pindaktiivse aine kontsentratsioon. *Click*-keemia metoodika puhul muudeti järgmisi parameetreid: lahuse komponendid, AuNP-de intensiivne segamine ja ultraheliga töötlemine, pindaktiivsete ainete kontsentratsioon. Katsete käigus kasutati kahte tüüpi mõõtmisi: neeldumine lainepikkusel 572 nm (vastab AuNP neeldumismaksimumile) ja heleda välja mikroskoopia (pinna homogeensuse hindamiseks). Ehkki selle lõputöö käigus ei õnnestunud signaali võimendumist saavutada, kasutatakse nendest eksperimentidest saadud teadmisi edasiste uuringute jaoks.

Märksõnad: tundlikkus, kulla nanoosakesed, signaali võimendamine, streptavidiin, biotiin, *click*-keemia

CERCS: P300 analüütiline keemia