Novel silver nanocluster based on vancomycin antibiotic with peroxides like activity for colorimetric detection of S. aureus bacteria in milk samples

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Research Article

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Abstract

A fast and colorimetric new strategy based on dual detection elements in one nanostructure as aptamer-silver nanocluster (apt-AgNC) is described for the specific and sensitive detection of *Staphylococcus aureus* (SA) based on silver nanocluster (AgNC) conjugated to oligonucleotide aptamer. AgNC is the first peroxidase activity research of NC based on vancomycin. This probe can specifically bind to the surface of SA and thus decrease the peroxidase activity of the apt-AgNC. Finally, in the presence of TMB as substrate and H_2O_2, the blue color of the solution in the infected milk samples was decreased. The detection limit for SA was 80 CFU mL^{-1}, and the processing time could occur during 45 min. This suggested SA detection technique has a number of appealing features, including high sensitivity, ease of use, quick testing time, and low cost.

1. Introduction

Staphylococcus aureus (SA) is one gram-positive bacteria found in various locations throughout the body, such as the conjunctiva, nose, and skin. It is responsible for several diseases, including pneumonia, endocarditis, arthritis, and bacteremia Pebdeni et al. 2022). Pathogenicity is caused by pathogenic agents and extracellular protein toxins by SA. Despite the fact that SA does not produce spores, it can contaminate food during preparation, processing, and handling. Ingestion of contaminated food containing enterotoxins generated by SA causes food poisoning; staphylococcal food poisoning generally occurs between 30 minutes to 8 hours. The consumer's health determines the severity of the ailment and the amount of poison consumed. As a result, early identification of SA is critical (Pebdeni et al. 2020). SA produces some virulence factors which cause mastitis. Bovine mastitis is the most common and costly illness in the milk and dairy busines. Vancomycin, a last-resort antibiotic, works against bacteria by decreasing cell wall formation, modifying cell wall permeability, and potentially leading to cell wall eradication (Zhu et al. 2021). Because of the optical features of metal nanoclusters (NC), including absorption and fluorescence properties, it received increased interest. Nanoclusters have sub-nanoscale dimensions (Nemati et al. 2022; Shokri et al. 2020). According to publications in the literature, the optical features of these NCs are attributed to the quantum confinement effect caused by the size of the clusters (Zhu et al. 2021). The ultra-small size, strong photoluminescence, biocompatibility, and photostability characteristics of metal NCs make them highly appealing. These intriguing features are ideal for biosensing applications. Silver nanocluster (AgNC) is one of the most researched metal NC due to its simple manufacturing procedure. However, because silver is more reactive and quicker to oxidize in its zero-valent state than gold, it is more challenging to synthesize AgNCs and explore their characteristics than gold equivalents, which have been investigated extensively. As a result, both basic and practical science depends on the availability of high-quality AgNC with well-defined size, structure, and surface (Yun-Peng et al. 2020). AgNCs have been effectively prepared using the direct reduction approach in organic and aqueous mediums. In a reducing agent, this synthetic process involves fast reductive development of intermediate AgNC and gradual size focusing on monodisperse Ag NC. The reducing agent, such as ascorbic acid and $NaBH_4$, is often used to make AgNC with a range of ligands,

including thiolates, DNAs, peptides, proteins, and polymers (Kermani et al. 2018; Joshi et al. 2015). Colorimetric biosensing is a technology based on a color change that can accurately detect various targets and biomolecules. It has gained popularity because of the visible color change, ease of operation, and quick read-out (Tarokh et al. 2021). Because of features like low cost, simplicity, speed, and the lack of need for expensive apparatus, peroxidase-like activity has gotten a lot of interest for detecting harmful microorganisms. The catalytic oxidation of 3, 3', 5, 5'-tetramethylbenzidine (TMB) by H_2O_2 produces a blue color that can be seen with the naked eye. As a result, developing a new colorimetric approach for sensitive and selective detection of harmful bacteria is critical (Pebdeni et al. 2021; Pebdeni, Hosseini 2020; Pebdeni et al. 2022). In the selection of DNA aptamer for SA, some components of the SA membrane, such as protein A and clumping factor A, are utilized as targets in the search for DNA aptamer, which have provided effective SA-specific aptamer (Roupioz 2017; Cao et al. 2009; Moon et al. 2015; Chang et al. 2013). Aptamer-based approaches have lately been the focus of study for diagnostic purposes because to their simplicity, cheap cost, and selectivity (Borghei et al. 2020). Here, for the first time, the AgNC based on vancomycin with excellent peroxidase activity was synthesized. We successfully combined AgNC based on vancomycin as a template and specific aptamer to prepare a biosensor for colorimetric detection of SA by using dual recognition elements in one nanoprobe. Specifically, Ag NCs were synthesized by peptide template conjugated to amine-functionalized aptamer in the presence of EDC/NHS that have suitable peroxidase activity in TMB and H_2O_2 (Scheme1). Simultaneously, using two recognition elements in one nanostructure is useful. Because in other sensors which reported dual recognition methods for the detection of SA, they used two distinct recognition receptors. The method of concurrently employing a dual receptor of vancomycin and aptamer in a unique biosensor with peroxidase-like activity was devised in this research, which has never been done before. Two nanostructures are required in other dual strategies to detect bacterial targets, such as one for the immobilization of aptamer or cDNA and another for the immobilization of vancomycin or other recognition elements (Yu et al. 2017; You et al. 2019; Cheng et al. 2016; Zhong et al. 2015; Meng et al. 2017). Still, in our work, one nanostructure was created with two recognition components. It may be used to identify a specific target and generate a signal that is easy and quick to make, as well as the target detection procedure, which improved selectivity and sensitivity and assured that NCs had a high affinity for complete SA whole cells.

2. Experimental

2.1. Materials

Vancomycin hydrochloride, silver nitrate ($AgNo_3$), N-(3-(diethylamino)-propyl)-N'-ethyl carbodiimide hydrochloride (EDC), ascorbic acid, N-hydroxysuccinimide (NHS), TMB, hydrogen peroxide (H_2O_2), and sodium hydroxide provided from Sigma-Aldrich. Tris-EDTA and HCl were purchased from Merck. Amino modified aptamer for detecting the SA (5'-NH2- GCG CCC TCT CAC GTG GCA CTC AGA GTG CCG GAA GTT CTG CGT TAT-3') obtained from Pishgam Company (Tehran, Iran). The bacterial strain and its culture media were provided from Baharafshan Institute of Research and Development (Tehran, Iran). Other

compounds were analytical reagent grade and were utilized without additional purification as received. Raw milk was purchased at a nearby market (Tehran, Iran). Throughout the process, sterile deionized water was used.

2.2. Apparatus

A Perkin-Elmer lambda 25 UV-Vis spectrometer was used to measure UV-Vis absorption spectra in the range of 200–800 nm. A transmission electron microscope (Zeiss, EM10C, 80 KV, Germany) was used to examine the size and structure of the AgNC. A Zetasizer Nano-ZS90 Malvern was used to perform dynamic light scattering (DLS) measurements at room temperature. The map analysis was done by FE-SEM (MIRA II, TESCAN).

2.3. Synthesis of the AgNC based vancomycin and apt-NC

First, we mixing the vancomycin hydrochloride (1 wt%) with a 5 mL $AgNo_3$ solution (5 mM) and stirring it for 10 min at room temperature. A mixture generated due to the interactions between silver ions, amine and hydroxyl functional groups of vancomycin was obtained. After the additional of the 1 M NaOH solution, a purple color is seen while adjusting the pH to 9–11. Following that, ascorbic acid (10 mM) was gently added and mixed at room temperature for 3 hours until the color of the solution turned light brown, indicating that the AgNC had been prepared. Filters were used to eliminate bigger precipitates from the synthesis AgNC. Then, 2 mg EDC and 1 mg NHS were added to 2 mL of the synthesized AgNC and agitated for 1 hour to activate the carboxyl groups of NCs. Then, 10 µL amino-modified aptamers (100 µM) were incorporated into the mixture overnight at room temperature with constant stirring in a dark place. The aptamers were conjugated to the NCs through the interaction of the –C = O groups of AgNC with the -NH groups of the aptamers leading to the formation of amide bonds, resulting in the apt-AgNCs conjugates.

2.4. Bacterial culture

Available gram-positive and negative strains of bacteria such as SA (ATCC 29213), *E. coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 10145), *Salmonella typhimurium* (ATCC 14028), and *Bacillus subtilis* (ATCC 168) were all cultivated in sterile Luria- Bertani broth (LB broth) and incubated overnight at 37°C. The bacteria were then centrifuged and transferred to nutrient agar mediums, where they were incubated for 1 day at 37°C. Several bacterial colonies of various strains were diluted in PBS and their absorbance was measured using UV–Vis spectroscopy at a wavelength of 600 nm (OD600). The number of bacterial cells was estimated employing the gold standard method of plate counting. The conventional bactericidal halo test was used to compare the antibacterial activity of vancomycin and AgNC.

2.5. Detection of SA For bacterial assays

After optimizing the experimental parameters, a series of concentrations ($10–10^8$ CFU mL^{-1}) of SA solution were incubated with the apt-AgNCs and at room temperature, the suspension was gently shook. After the incubation time, the peroxidase activity of NCs was measured through a UV-Vis spectrophotometer in the presence of TMB and H_2O_2.

2.6. Real Sample Analysis

Milk is among the foods that prone to be infected by SA during its preparation. To study the analytical reliability of this NC, the proposed colorimetric method was employed to detect SA in milk. Milk samples were obtained from a local supermarket and diluted ten times with deionized water. Known concentrations of SA (10^7, 10^5, 10^3 CFU mL^{-1}) were spiked to milk. The blank sample (negative control) was sterilized milk by autoclave. Finally, the spiked real samples were incubated to apt-AgNC at optimal conditions. Thus, the peroxidase-like activity of the apt-AgNC was measured after the addition of TMB and H_2O_2.

3. Results And Discussion

3.1. Characterization

The dispersity and the size of the NC has been investigated through TEM. According to TEM analysis, the particles are spherical in shape, well-dispersed, and uniform, with an average diameter of 3 nm (Fig. 1a). An elemental mapping reveals the presence of Ag atoms that showed in Fig. S1a. The formation of stable NCs was achieved by reducing Ag + to Ag in the presence of ascorbic acid as the reducing agent. As a result, AgNC was synthesized in the current investigation using a one-pot process in aqueous medium. The products were described both before and after aptamer bonding. The chemical, physical, and optical properties of AgNCs resulted from vancomycin based on AgNCs. Because of their small size, AgNCs have molecule-like properties, such as a high UV–Vis absorption band. Employing UV–Vis spectroscopy of the AgNCs, and apt-AgNCs were analyzed in the region of 200–800 nm (Fig. 1b).

The absorption peak seen at 414 nm is due to metal silver nanoclusters' well-known Surface Plasmon Resonance (SPR) absorption, which is a key property of metal nanocluster doped thin films utilized to construct SPR-based sensors and nonlinear photonic devices (Moon et al. 2015; Ferraris et al. 2010; Esmaeillou et al. 2017). As a result, the vancomycin absorption peak was seen in AgNC absorption. Interband electronic transitions of the NCs from distinct energy levels generate AgNC's multi-band optical absorption. The absorption peaks of AgNC, and apt-AgNC were tested to corroborate the immobilization of aptamers on AgNC. The variations in AgNCs absorbance before and after aptamer immobilization are shown in Fig. 1b. In comparison to AgNCs, the absorption spectra of apt-AgNCs showed an absorption peak at around 260 nm, indicating that the aptamers were successfully conjugated to the AgNCs. Vancomycin shows a strong peak at 280 nm. The unique absorption of vancomycin was visible in AgNC, indicating that the NC had been successfully synthesized. Antimicrobials having broad-spectrum activity, such as Ag nanostructure, have been studied extensively in nanotechnology. After accessing the permeability of the bacterial cell membrane, the Ag nanostructure alters sulfur-containing amino acids and phosphorus (DNA) after accessing the cell, preventing replication. Previous studies using sophisticated electron microscopy revealed that positively charged AgNPs promote cell lysis by causing wall weakening, pore development, and leaking of cell content. Once within the cell, the silver

nanoparticles disintegrate, releasing highly reactive silver species with antibacterial properties (Lara et al. 2019). Vancomycin as template of the AgNC can also enhance antibacterial activates against gram-positive bacteria. Also, in this research, the enhancing the antibacterial activity of AgNC compared of vancomycin alone is very interested and using the AgNC based on vancomycin as an antibacterial agent is excellent (Fig. S1b).

3.2. NCs Peroxidase-like activity

The peroxidase-like activity of AgNC was analyzed using TMB and H_2O_2. The strategy for synthesizing the AgNC relied on reducing the Ag by ascorbic acid as the reducing agent in the formation of NCs. To study the peroxidase-like activity of the AgNC, TMB was chosen as the substrate, which can be oxidized by hydrogen peroxide to form a bluish product during the peroxidase-like activity. Figure 2 was shown the peroxides activity of apt-AgNC, which decreased in the presence of SA as a specific target.

Oxidation of the chromogenic TMB in the presence of the AgNCs was investigated, resulting in a visible blue color. The presence of silver ions in the NC structure is responsible for their absorption and peroxidase-like capabilities. The absorbance intensity at 652 nm, which is the typical peak of oxidized TMB, increased significantly following the addition of AgNC, as seen in Fig. S2. The optimal concentration of TMB and H_2O_2 that led to the highest absorption of peroxides activity of AgNC during the optimum duration of 6 minutes was chosen. When the H_2O_2 concentration was set to 10 mM, different concentrations of TMB were utilized as the substrate. As shown in Fig. S2, the 13 mM of TMB show the highest peroxides activity of NC during 6 min. When the concentration of TMB was fixed at 13 mM, the different concentration of H_2O_2 (3–20 mM) was tested. The 10 mM of H_2O_2 shows the highest peroxides activity of NC.

Optimization of the incubation time required for the binding of the apt-AgNC by dual recognition factor to the surface protein of target bacteria is shown in Fig. S2. The absorbance is related to the high concentration of bacteria with different time durations in the presence of TMB (13 mM), and H_2O_2 (10 mM) was measured. Fig. S3 shows the optimization of the required time for the apt-AgNCs to attach to the whole cell of specific bacteria. The absorbance of samples containing high quantities of bacteria was monitored during the presence of TMB and H_2O_2. The optimal period to incubate apt-AgNCs with SA bacteria was 45 min.

3.3. Principles of SA detection

A specific concentration of SA was added to the prepared apt-AgNC, and the alterations in the peroxidase-like activity were monitored after the addition of TMB and H_2O_2. According to Fig. 3, following the addition of a specific target to the NC solution, the peroxidase-like activity of the NC was decreased. At the same time, the color of the control is blue. This process demonstrated that both bio elements, including aptamer and vancomycin, had joined SA specifically. The calibration curve, as illustrated in Fig. 3, was achieved in values ranging from 10^2 to 10^8 CFU mL^{-1} using the linear regression equation $y=-0.0657 + 0.914$ ($R^2 = 0.984$). The sensitivity of NC was investigated, and the LOD of the nanosensor

was estimated to be 80 CFU mL^{-1} (S/N = 3). A comparison of the proposed colorimetric aptasensor with various previously published SA detection approaches is shown in Table 1. The results were compared to those of other instances described, demonstrating that this approach provides an extended dynamic range and suitable LOD. Our nanosensor system offers a number of unique characteristics. It's easy for synthesis and one step process of detection.

Table 1
Comparison of the analytical performances between the method and the reported methods for the detection of SA.

Method	Nanostructure	bioreceptor	Analytical range (CFU mL^{-1})	LOD (CFU mL^{-1})	Ref.
Colorimetric	dsDNA-SYBR Green complex	aptamer	-	81	(Yu et al. 2020)
LSPR	Au nanodisk	aptamer	$10–10^4$	10^3	(Khateb et al. 2020)
Chemiluminescence	alkaline phosphatase-conjugated phe11-protonectin	IgG	10^3-10^7	$2.9×10^2$	(Fan et al. 2019)
Colorimetric Fluorescent	Magnetic NP, cDNA- upconversion NP	aptamer	$56–5.6 × 10^6$	22	(Ouyang et al. 2021)
Microscopic Detection	SiO_2-AuNP Core–Shell	Bacteriophage	-	$8 × 10^4$	(Imai et al. 2019)
Lateral flow assay	AuNP	IgG Vancomycin	10^3-10^7	10^3	(Zhao et al. 2021)
Fluorescent	Carbon dot	Vancomycin	$3.18×10^5–1.59×10^8$	$9.40×10^4$	(Zhong et al. 2015)
Colorimetric	Apt-AgNC	Aptamer Vancomycin	10^2-10^8	80	This work

3.4. Evaluation of selectivity

The selectivity test of NC's for SA was tested in the same settings with other pathogens such as *E. coli, Salmonella typhimurium, Bacillus subtilis*, and *Pseudomonas aeruginosa*. The findings revealed that non-

specific strains couldn't be attached to their whole cell of them. Thus, their absorbance is higher than in the presence of SA (Fig. 4). The specificity of the applied aptamer and vancomycin toward the surface structures of the target is referred to as NC selectivity. Also, AgNC can attach to gram-positive bacteria; however, NC's selectivity and specificity against SA bacteria increased with the aptamer. It has a propensity for not attaching to other bacteria.

3.5. Analytical application in real sample

The diagnostic ability of the aptasensor was tested using milk as a real sample. As a result, the aptasensor was used to assess the presence of SA in the spiked sample of milk. The results from the new apt-AgNC approach and the standard technique were remarkably similar. As shown in Table 2, the sensor shows excellent recovery confirming the method's suitability for detecting SA in milk or other food samples. Moreover, contaminated milk samples were examined simultaneously employing the developed method and the classic plate counting. The recoveries are between 91 and 102%, and the relative standard deviations (RSD) are between 1.8 and 3.2%, indicating that the developed colorimetric sensing platform has a great potential for the quantitative measurement of target in real samples.

Table 2
Determination of SA in spiked real samples using the peroxidase activity of sensing platform

Real sample	Spiked concentration (CFU mL^{-1})	Measured concentration (CFU mL^{-1})	Recovery (%)	RSD (n = 3, %)
Milk 1	1×10^{7}	102×10^{7}	102	3.2
Milk 2	1×10^{5}	95.5×10^{5}	95	2.6
Milk 3	1×10^{3}	9.1×10^{3}	91	1.8

4. Conclusion

Conclusively, to detect pathogenic SA bacteria, a unique and sensitive dual recognition elements biosensor platform was developed using the apt-AgNC based on vancomycin. Because of the strong affinity of the aptamer and vancomycin for the whole cell of SA, the peroxidase activity of apt-AgNC was reduced when SA was added. Among other pathogenic bacteria studied, this sensing platform exhibited great sensitivity for SA and had an excellent detection limit of 80 CFU mL^{-1} in the linear range of 10^{2} to 10^{8} CFU mL^{-1}. The assay's ability to detect the target SA was confirmed in spiked milk as real samples with high percent recoveries. Furthermore, by modifying the aptamers, the sensing platform can be employed to detect various gram-positive bacteria, which will widen the scope of the discovered method's use.

Declarations

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Compliance with Ethical Standards

Funding Financial support was provided by the Faculty of new sciences and technologies, Tehran University, Tehran (Iran).

Conflict of Interest Azam Bagheri pebdeni· Morteza Hosseini, declare no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Data Availability Data will be made available on reasonable request.

Informed Consent Not applicable.

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Figures

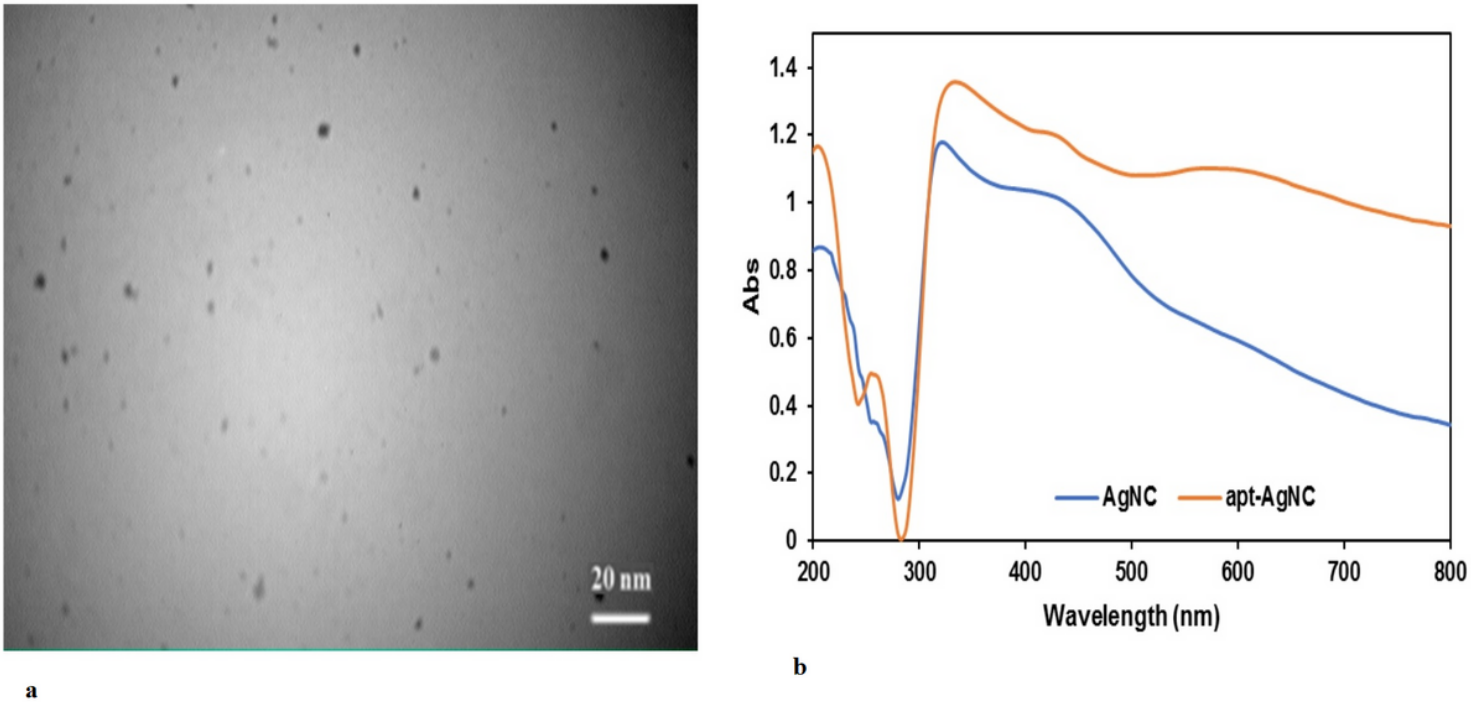

Figure 1

(a) TEM image of AgNC, (b) UV-Vis spectroscopy of vancomycin, AgNC, and apt-AgNC.

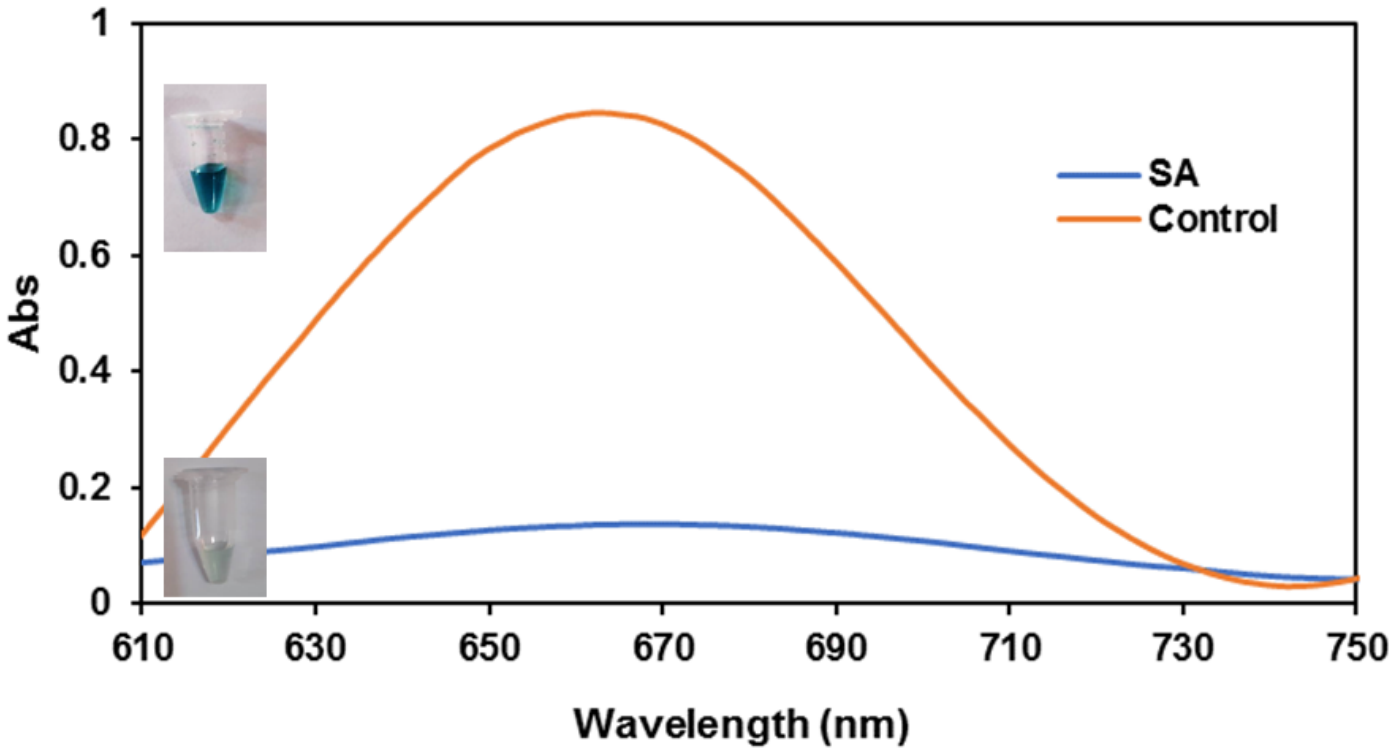

Figure 2

Absorbance spectra of AgNC before and after the addition of *S*A in the presence of TMB- H_2O_2. (Inset) images related to the color change of the proposed NCs.

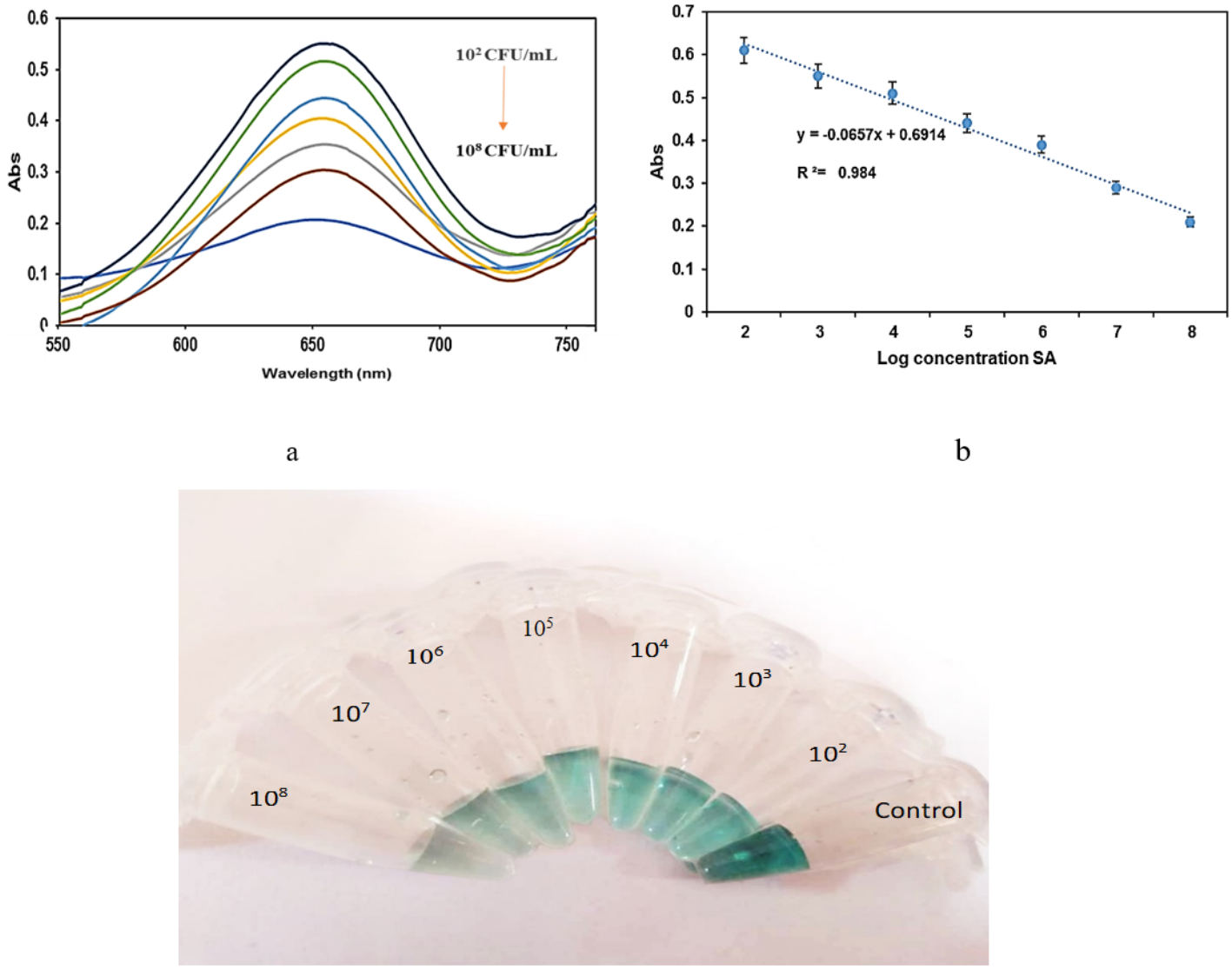

Figure 3

(a) The calibration curve with the logarithm of different concentrations of SA in the range of 10^2-10^8 CFU mL^{-1}, (b) The linear rang of SA concentrations at 652 nm wavelength, (c) color change observed with the naked eye for NCs with increasing concentration of SA.

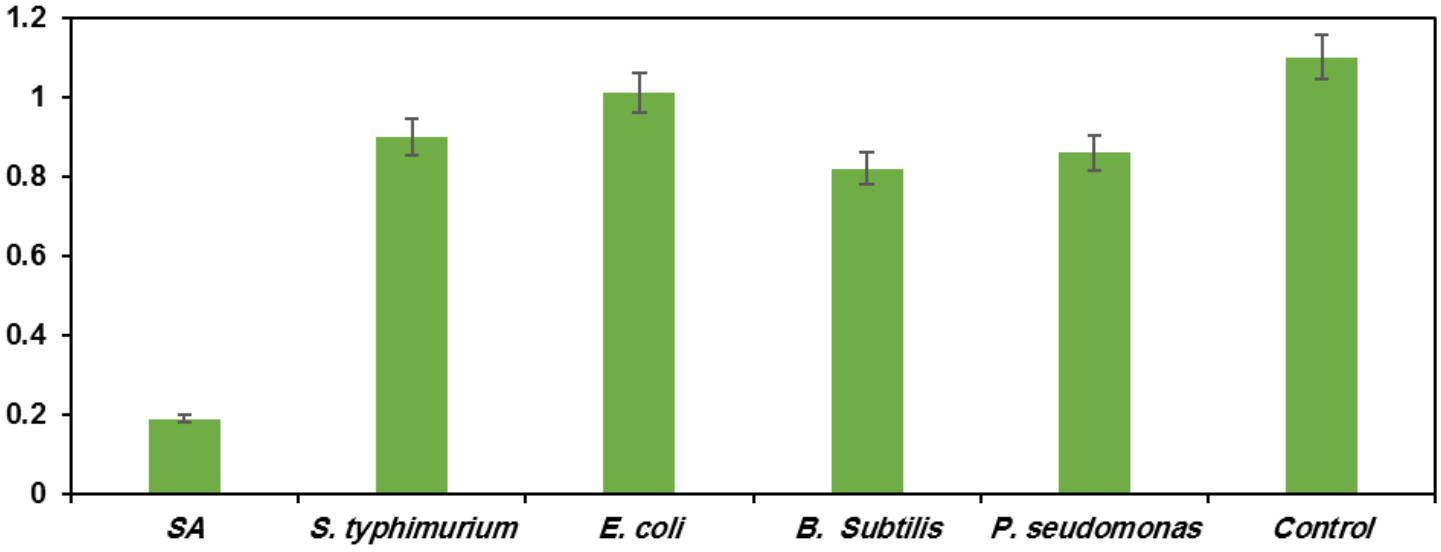

Figure 4

The selectivity of the apt-NC by the same concentration of different bacteria.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Scheme1.png
- Supplementarydata.docx