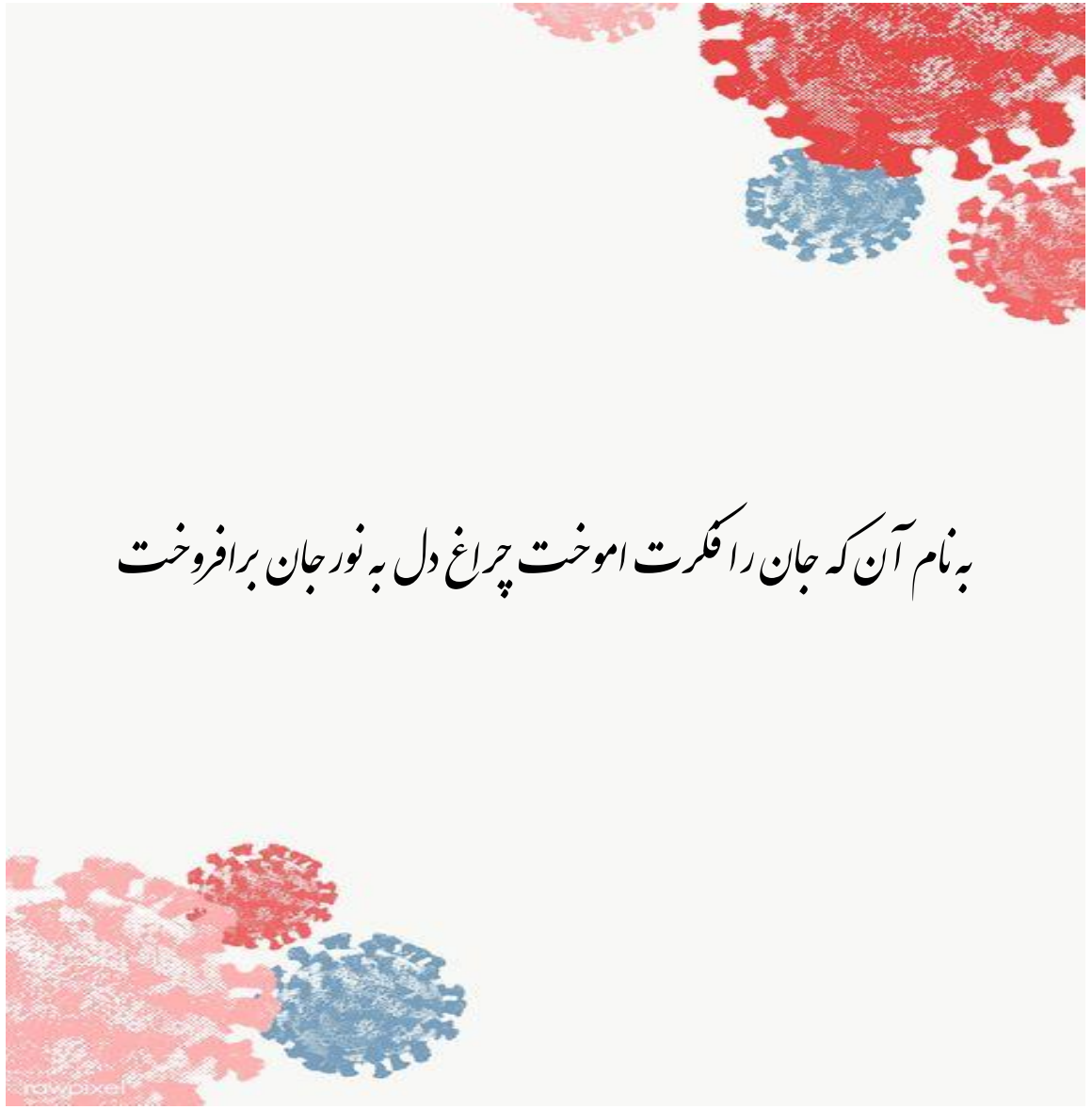

به نام آن که جان را فکرت اموخت چراغ دل به نور جان برافروخت

نام و نام خانوادگی: فاطمه نوروزی

دکترا

ورودی: مهر ۹۵

A Convenient Colorimetric Bacteria Detection Method Utilizing Chitosan-Coated Magnetic Nanoparticles

Introduction

- Bacterial contamination in myriad environments has become a serious threat to human life as it can cause various diseases.
- Among many pathogenic bacteria in water, food, or pharmaceutical products, gram-negative Escherichia coli and gram-positive Staphylococcus aureus are two major bacterial species causing disease including diarrhea, anemia, skin infections, orbital cellulitis, urinary tract infections, and eventually death
- According to the WHO, infectious diseases by food-borne pathogenic bacteria caused 240,000 deaths globally in 2010.
- Considering the seriousness of bacterial contamination, a simple, rapid, reliable, sensitive, and early-screening method to detect bacteria is critically important to conduct early-diagnosis as well as timely treatment to effectively prevent and cure the disease.

Many methods for bacteria detection

❑**The plate colony counting method:**

- As a “gold standard” because it provides a direct enumeration of bacteria through cultivation under standard conditions
- Inevitably requires specific facilities for microbial cell cultivation and skilled personnel
- A long-time analysis of up to several days for enrichment, plating and isolation, and identification

❑**Other techniques including PCR and immunological methods such as ELISA:**

- Widely used for bacterial detection based on their ability to overcome such time limitations with high detection sensitivity
- Conducted with specialized instruments, complicated sample treatment and analytic procedures
- Well-trained individuals with the appropriate expertise

*These methods cannot be conducted on-site, critically restricting their use in limited facilities or point-of-care testing (POCT) environments

Advantages and **Disadvantages**

❑To develop bacteria detection methods particularly suitable for on-site detection, colorimetric techniques have attracted increasing attention due to their easy operation and visual detection without any instrumentation/expertise.

❑ Several enzymatic assays have been reported for the colorimetric detection of bacteria, for example, T7 bacteriophages carrying the lacZ operon.

❑Glucose oxidase-mediated colorimetric detection methods were also reported for broad-spectrum bacteria carrying out glucose metabolism.

- These enzymatic methods provide simple colorimetric identification of bacteria
- Specific biological elements like phage-induced systems are often difficult to apply for broad-spectrum bacteria detection
- Enzyme instability has significantly hindered its practical utilization

Advantages and **Disadvantages**

❑To overcome these limitations, diverse nanomaterials such as:

- Nanoparticles
- Nanorods
- Nanowires
- Carbon nanostructures

❑Noble metal nanoparticles such as those composed of gold and silver :

- Their unique optical properties inducing a distinct color change by aggregation or chemical reaction on their surface. They produce a rapid and sensitive colorimetric response to target bacteria
- They generally require laborious surface modification with biomolecules such as DNA, RNA, and antibodies
- Often quite sensitive to experimental conditions causing false positives

❑ Several peroxidase-mimicking nanomaterials including:

- Gold nanoparticles
- Graphene oxide
- Iron oxide magnetic nanoparticles (MNPs)

❑They exhibit noticeable enzyme activity with extremely **high stability** and they **can be mass-produced at low cost.**

❑Like natural peroxidase, these nanomaterials can decompose H_2O_2 into an OH· radical that can oxidize peroxidase substrates such as 3,3′,5,5′-tetramethylbenzidine (TMB) or 2-2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) yielding blue and green color, respectively.

❑Pathogen-specific receptors like antibodies or DNA aptamers are conjugated on the surface of the nanomaterials and they are used to detect bacteria via sandwich-type assay procedures:

- The sandwich-type colorimetric assay is successful to detect bacterial cells, it requires tedious surface modification steps as well as many experimental procedures.

❑A label-free detection method using MNPs was also recently reported:

- DNA aptamer molecules that have a specific affinity for Salmonella typhimurium were first adsorbed onto the surface of MNPs by electrostatic interaction resulting in the inhibition of the peroxidase-like activity of the MNPs.
- Using this strategy, target S. typhimurium is detected through observing the green color from the oxidation of the ABTS substrate; however, its sensitivity is too low and the limit of detection is 7.5×10^4 colony-forming unit (CFU) mL^{-1}, presumably due to the insufficient control of the aptamer affinity between MNPs and bacterial cells.

- Chitosan, a polycationic biopolymer containing abundant amine groups in its backbone, to modify the surface of MNPs (CS-MNPs).

- **Positively charged chitosan** has been proven to show a high affinity toward broad-spectrum bacteria, majorly due to the negative surface charge as well as outer-core lipopolysaccharides of the bacterial membrane.

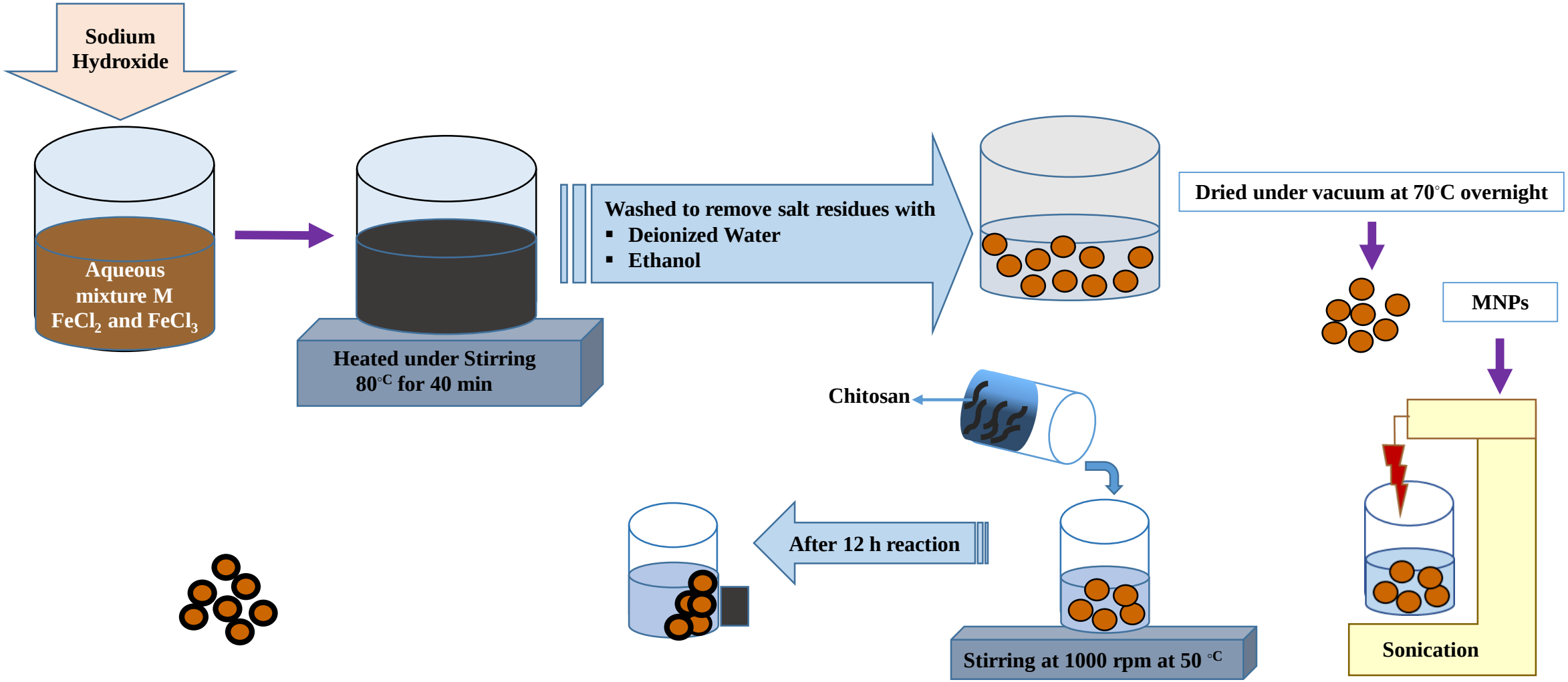
- Based on the high affinity of chitosan toward broad-spectrum bacteria, the bacterial cells would effectively bind to CS-MNPs, thereby inhibiting their peroxidase-like activity, which would produce a decreased colorimetric response.

Experimental Section

Materials

- Chitosan (CS)
- Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$)
- Iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$)
- Sodium hydroxide
- Phosphate-buffered saline (PBS)
- ABTS
- Sodium acetate
- Hydrogen peroxide (H_2O_2 30% w/v)
- Acetic acid
- Ethanol
- Glutaraldehyde (25% w/v) (Sigma-Aldrich)
- Luria-Bertani (LB) broth
- Agar powder

Synthesis of MNPs and CS-MNPs



Characterization of MNPs, CS-MNPs, and Bacteria with Nanoparticles

❑Analyzed by SEM images:

- Size
- Morphology
- Elemental composition of MNPs and CS-MNPs

*Nanoparticle samples for SEM were prepared by placing a drop of suspension on a polished wafer and dried overnight at room temperature

❑Zeta potential analysis carried out using a Zetasizer Nano-ZS

❑Using an FT-IR spectrophotometer and An X-ray diffractometer

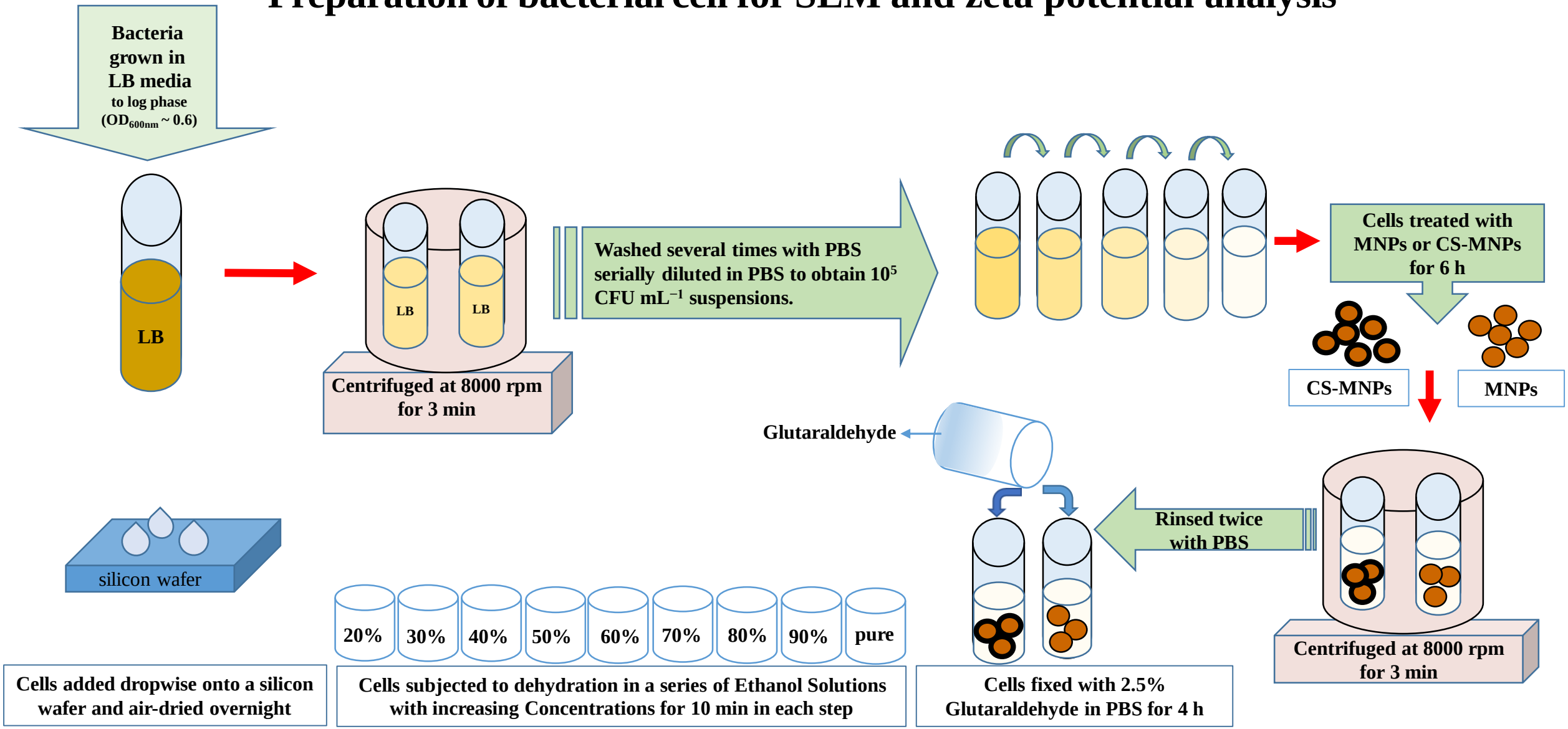
- Fourier-transform infrared (FT-IR) spectra and X-ray diffraction (XRD) patterns of MNPs, CS-MNPs, and free chitosan were obtained

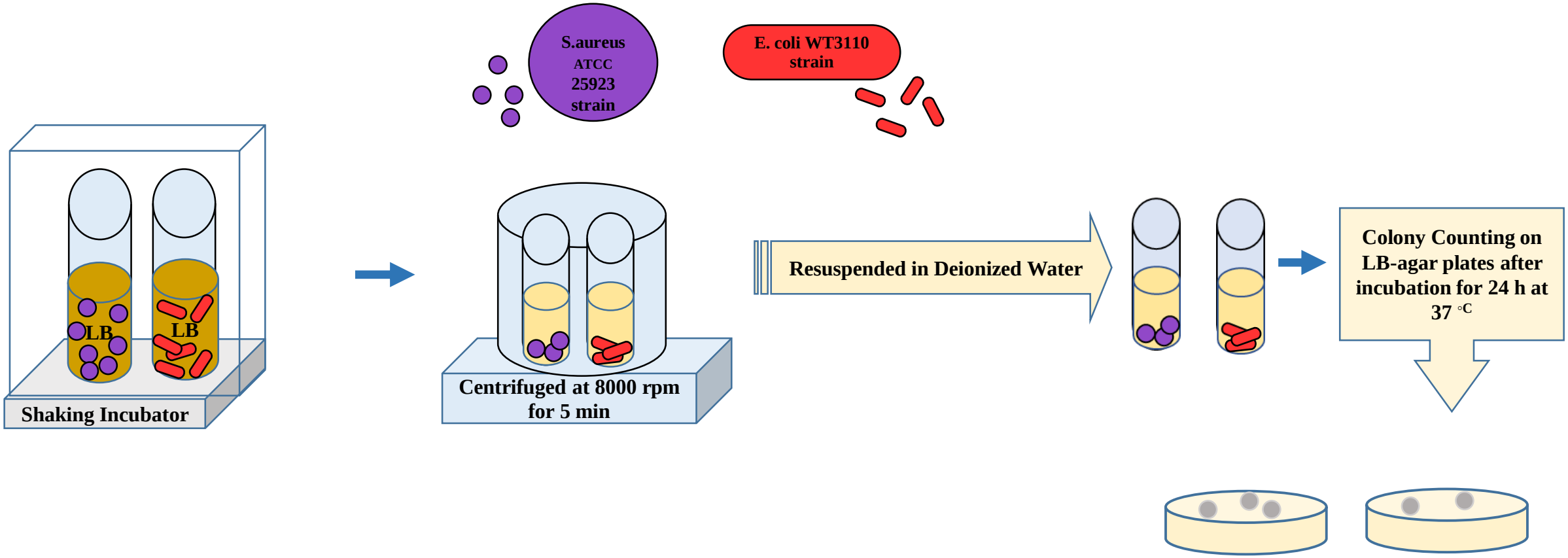
❑Analyzed by SEM and zeta potential

- Interaction of MNPs and CS-MNPs with E. coli or S. aureus

✓ **Zeta Potential analysis** is a technique for determining the surface charge of nanoparticles in solution (colloids). Nanoparticles have a surface charge that attracts a thin layer of ions of opposite charge to the nanoparticle surface.

Preparation of bacterial cell for SEM and zeta potential analysis





Selected Bacteria and Culture Conditions for the colorimetric detection experiments

Colorimetric Bacteria Detection Using CS-MNPs

❑ Colorimetric bacteria detection was carried out in transparent tubes or 96-well plates containing:

1. MNPs or CS-MNPs (0.1 mg mL−1)
2. H2O2 (2.5 mM)
3. ABTS (6 mM)
4. Bacterial cell suspension in sodium acetate buffer (50 mM, pH 4.0) at different concentrations

❑ After 10 min, nanoparticles and bacterial cells were removed by centrifugation at 12,000 rpm for 5 min.

❑ The **supernatant solution** was used to obtain images and the corresponding absorption spectra or absorption intensity at 417 nm on a microplate reader.

✓ Peroxidase substrates such as 2-20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)

Results and Discussion

Preparation and Structural Characterization of MNPs and CS-MNPs

- Because **positively-charged chitosan** has been known to present a high electrostatic **affinity toward negatively-charged bacterial cell membranes.**

- The **morphology and size** of the resulting MNPs and CS-MNPs were then **analyzed by SEM images** (Figure 1b,c).

- **Both MNPs and CS-MNPs** presented **spherical shapes** with a nearly- uniform diameter of around 30 nm.

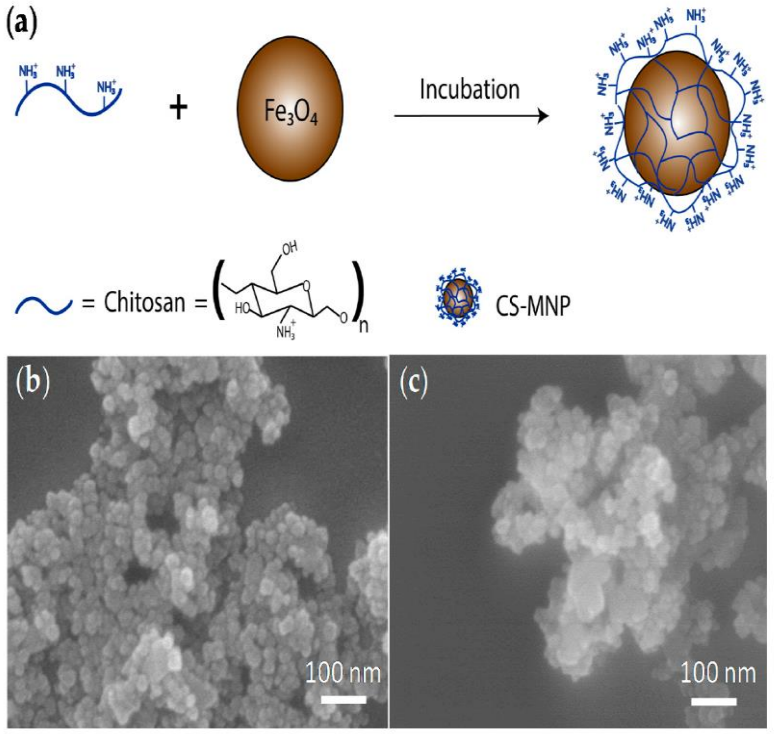


Figure 1. (**a**) Schematic illustration of chitosan coating the surface of iron oxide magnetic nanoparticles to prepare CS-MNPs. SEM image of (**b**) MNPs and (**c**) CS-MNPs.

- For the bacterial detection experiments, the surface charge of bare MNPs was +2.94 mV, and gradually increased as the concentration of chitosan also increased, as positively-charged chitosan has good adhesion to the hydroxyl group of MNPs (Figure S1).
- The SEM-EDS elemental mapping images of Fe, O, and N for CS-MNPs indicate the homogeneous distribution of each element throughout the aggregated nanoparticles, which are the main components of MNPs and chitosan (Figure 2a).
- In contrast, bare MNPs did not show any N, clearly revealing the absence of chitosan (Figure 2b).

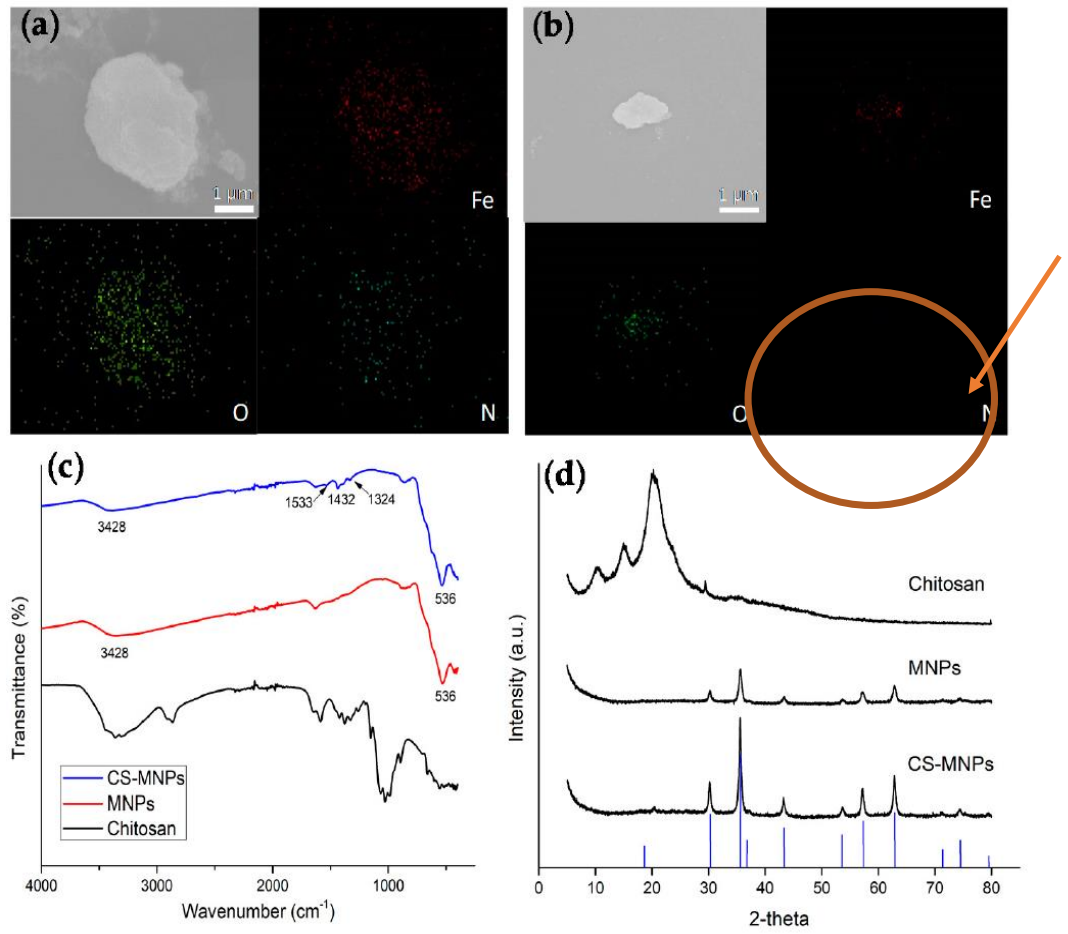


Figure 2. SEM images of (**a**) CS-MNPs and (**b**) MNPs with their corresponding EDS maps of Fe, O, and N. (**c**) FT-IR spectra and (**d**) XRD spectra for CS-MNPs, MNPs, and chitosan.

✓ Energy Dispersive X-Ray Spectroscopy (**EDS** or **EDX**) is a **chemical microanalysis technique used in conjunction with scanning electron microscopy (SEM)**. The EDS technique detects x-rays emitted from the sample during bombardment by an electron beam to characterize the elemental composition of the analyzed volume.

Rapid and Sensitive Colorimetric Bacteria Detection Using CS-MNPs

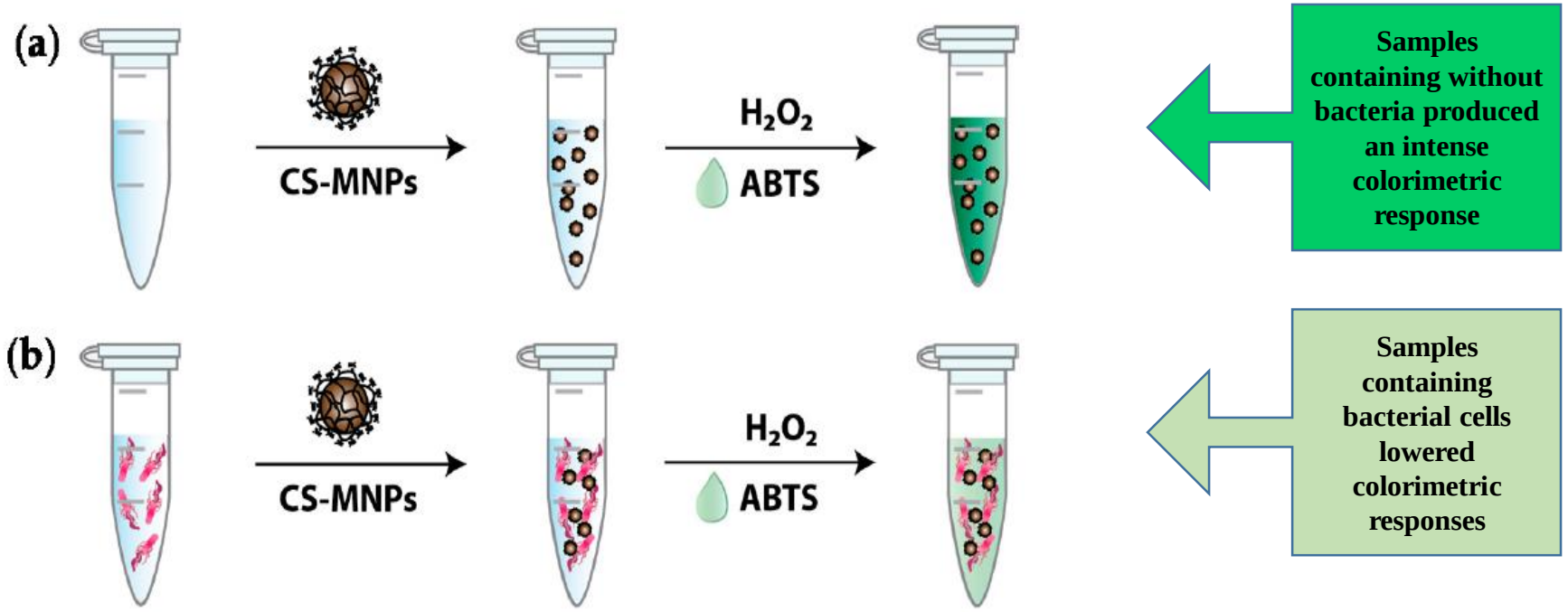
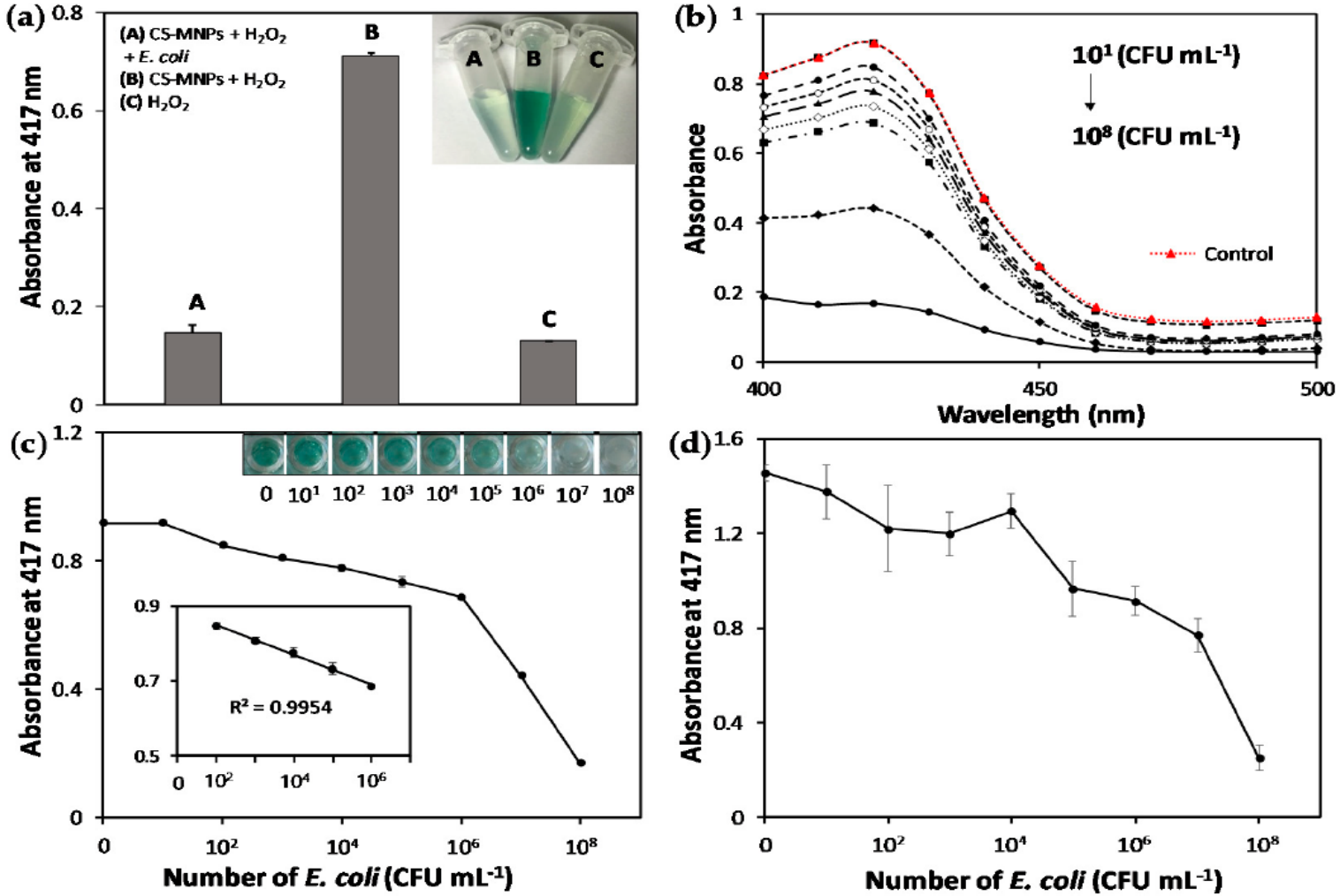


Figure 3. Schematic representation of the CS-MNPs-based colorimetric bacteria detection (**a**) in the absence or (**b**) in the presence of bacterial cells.

❑ As this color generating reaction from the ABTS oxidation takes only 10 min at room temperature.



(a)Absorption intensity of the reaction leading to ABTS oxidation using CS-MNPs in the presence or absence of E. coli (10^8 CFU mL^{-1}).
(b) Absorption spectra of the reaction tube at various *E. coli* concentrations ranging from 10^1 to 10^8 CFU mL^{-1}.
(c) Absorption intensity of solutions obtained from the CS-MNPs-based assay at various *E. coli* concentrations.
(d) Absorption intensity of solutions obtained from the bare MNPs-based assay at various E. coli concentrations.

❑Even without any detection instrument, the presence of *E. coli* could be visibly discriminated **by the naked eye at** 10^4 CFU mL^{-1}.

❑When we used bare MNPs rather than CS-MNPs for the same *E. coli*, their peroxidase activity was not efficiently inhibited by the presence of bacterial cells, probably due to:

- The inefficient binding between bacteria and MNPs, and furthermore,
- There were significant variations in the absorbance.

❑Due to their relatively higher positive charge density, CS-MNPs-based colorimetric bacteria **detection resulted in similar sensitivity for both types of bacteria.**

Demonstration of the Interaction between CS-MNPs and Bacteria

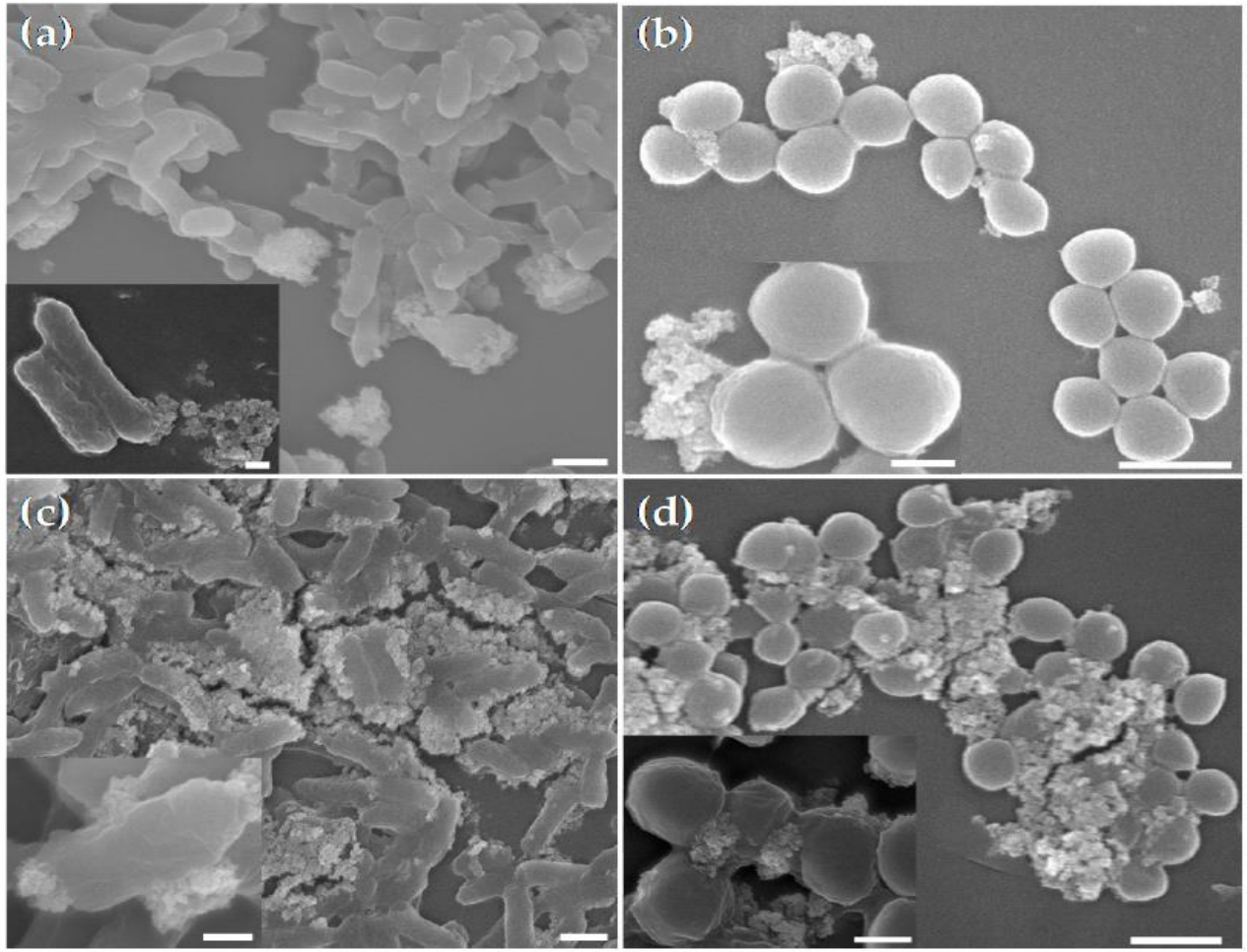


Figure 6. SEM images of (**a**) MNPs incubated with *E. coli*. (**b**) MNPs incubated with *S. aureus*. (**c**) CS-MNPs incubated with *E. coli* and (**d**) CS-MNPs incubated with *S. aureus*. Scale bars indicate 1 μm. In the insets, scale bars indicate 0.3 μm.

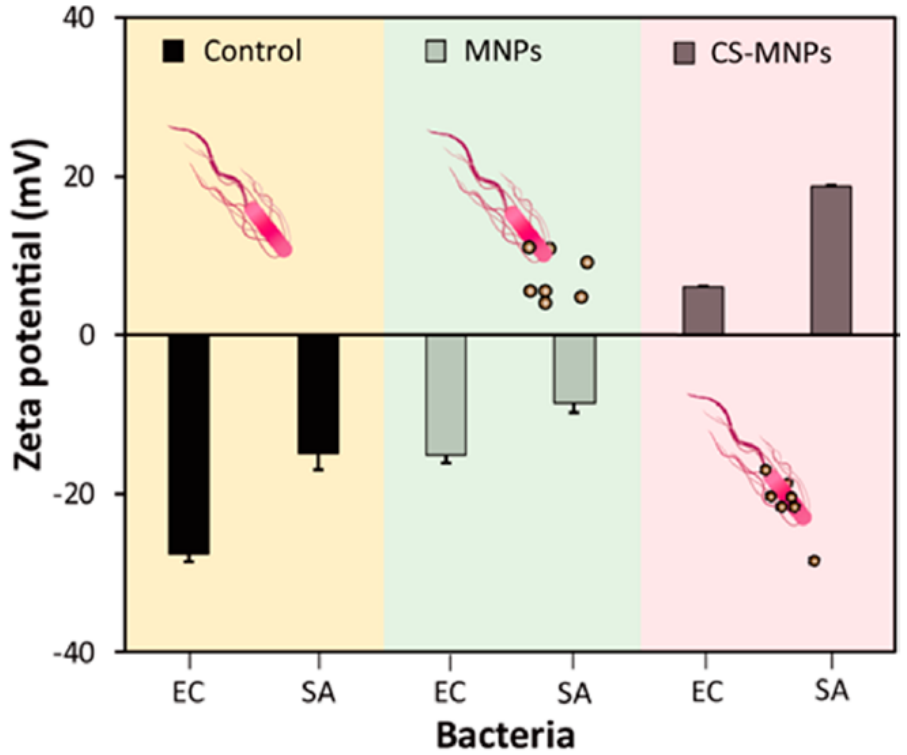


Figure 7. Zeta potential analyses of bacteria only, bacteria with bare MNPs, and bacteria with CS-MNPs. *E. coli* (EC) and *S. aureus* (SA) were used in the experiments.

Conclusions

- In summary, a simple, rapid, inexpensive, and instrument-free colorimetric bacteria detection method has been successfully developed based on the efficient electrostatic interaction between CS-MNPs and bacterial cells, as well as the concomitant inhibition of the peroxidase activity of CS-MNPs.
- This assay can be conveniently conducted and analyzed by monitoring the green color reduction within 10 min.
- Both gram-negative E. coli and gram-positive S. aureus were successfully quantified at concentrations as low as 10^2 CFU mL^{-1} spectrophotometrically and 10^4 CFU mL^{-1} by the naked eye, which is compatible with the current clinical setup.
- Based on these observations, they anticipate that this colorimetric bacteria detection assay developed using the specific affinity and catalytic activity of CS-MNPs may provide a new way to detect broad-spectrum bacteria on site.

قاصدک! هان، چه خبر آوردی؟
از کجا، وز که خبر آوردی؟
خوش خبر باشی، امّا، امّا
گرد بام و در من
بی ثمر می گردی.
انتظار خبری نیست مرا
نه ز یاری نه ز دیّاری ، باری،
برو آنجا که بود چشمی و گوشی با کس،
برو آنجا که ترا منتظرند.
قاصدک!
در دل من همه کورند و کرند.
دست بردار از این در وطن خویش غریب.
قاصدک تجربه های همه تلخ،
با دلم می گوید
که دروغی تو، دروغ
که فریبی تو، فریب.
قاصدک! هان، ولی ...
راستی آیا رفتی با باد؟
با توام، آی کجا رفتی؟ آی...!
راستی آیا جایی خبری هست هنوز؟
مانده ٔ خاکستر گرمی، جایی؟
در اجاقی- طمع شعله نمی بندم - اندک شرری هست هنوز؟
قاصدک!
ابرهای همه عالم شب و روز
در دلم می گریند ..."

— مهدی اخوانِ ثالث