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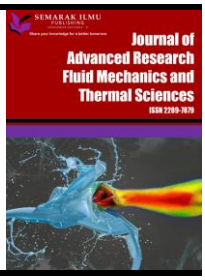

Biogenic Synthesis and Characterization of AgNPs Using CEPS: Cytotoxicity and Antibacterial Activites

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ABSTRACT

This study was amid to biogenic synthesis of Silver Nanoparticles (AgNPs) and evaluated its chemical-physical and biomedical properties. The currently used reducing agent is Polyalthia sclerophylla (CEPS). As a reducer, a crude extract of Polyalthia sclerophylla leaves (CEPS) was used, while silver nitrate (AgNO3) was used as an initiator. Two samples were prepared and named AgNPs-a and AgNPs-b, respectively. The prepared samples were carried out to characterize their biological, physical, and chemical properties. Energy Dispersive X-Ray Analysis (EDX) and ultraviolet-visible spectroscopy were the first techniques utilised to emphasise the formulation of AgNPs (Uv-vis). The morphology and size of the particles are determined using scanning transmission electron microscopy (STEM) and scanning electron microscopy (SEM). AgNPs were tested for cytotoxicity against Mg-63 human cells (a type of osteosarcoma cell and its osteoblast-like cells) using the Alamar blue assay, and their antibacterial properties were investigated against Gram-positive Staphylococcus aureus (S. aureus) and Gram-negative Escherichia coli (E. coli). The wavelength of AgNPs-a was 436 nm, while AgNPs-b was 441 nm, according to the data. According to SEM and STEM images, the shapes of the prepared samples were spherical. The particle sizes were not the same, with AgNPs-a having a diameter size range of 48nm to 68nm and AgNPs-b having a diameter size range of 59nm to 77nm. The availability of Mg-63 cells in prepared samples was greater than 89% for all concentrations. AgNPs-a inhibited bacteria growth more effectively against both bacteria, with results against S. Aureus and E. coli at 80M/ml of 24mm and 22mm, respectively, compared to AgNPs-b at the same concentration of 20mm and 18mm. According to our results obtained from chemical-physical techniques, the shapes of both AgNPs-a and AgNPs-b were similar, while the sizes of the particles were different. The antibacterial effect affects the difference as smaller sizes more inhibition for bacterial growth. The current study has shown that non-toxic produced samples can be utilised as an antibacterial agent, with nano-sizes that can be employed safely in the medical and biological areas.

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1. Introduction

Synthesis and characterization of nanoparticles are very important and exciting for researchers because the nanoparticles can be applied widely in chemical, physical, biological, and medicinal areas. Silver nanoparticles (AgNPs) are gaining attention from researchers because of their unique and desired qualities, which include antibacterial effects, high surface-to-volume ratios and catalytic properties.

Nanomaterials have recently encouraged researchers, due to their unique properties to find easy, fast and economical ways to synthesise them [1]. Many chemical elements have been converted, from normal size to nanoscales, such as gold (Au), silver (Ag), lead (Pb), and others [2-4]. Ag metal is considered one of the most nano-synthesized compared with other metals due to its unique features, making it applicable for various applications such as medical, industrial and biological [5-8].

Synthesis of AgNPs was done previously using many techniques. Through the previous studies, researchers faced many challenges during its implementation, such as time of preparation, cost of the materials used, and not being eco-friendly for the environment. The physical and chemical methods of the nanoparticles of silver are needed requirements, and one of the important ones is the cost of the preparation is very high. However, the green chemistry of AgNPs, compared with the chemical and physical methods, has many advantages [9, 10].

In previous studies, the researchers developed the methods used to synthesize AgNPs, in the green chemistry way, according to three steps: stability, stenosis, and reduction. Green chemistry methods use extracts from natural sources from many biological materials, including amino acids, vitamins, plants, and enzymes [11,12]. The advantages of green chemistry include being eco-friendly, cheaper, and appropriate to the synthesis of large-scale from scaled-up. In the present study, the AgNPs were synthesized via green chemistry way with non-toxic and environmentally friendly biological materials [13,14].

Polyalthia sclerophylla is a group of species distributed in the tropics and subtropics. The species were discovered from the sources in the Species 2000 & ITIS Catalogue of Life: April 2013, which belongs to the Annonaceae family for its medicinal properties. Also, 17 species have been identified in this Polyalthia species. Polyalthia has therapeutic qualities such as antibacterial, anti-inflammatory, anticancer, antihyperglycemic, antifungal, antioxidant, hepatoprotective, and anti-HIV-1 action from the leaves and twigs [15-18]. Because of the antibacterial capabilities of the Polyalthia genus, Polyalthia sclerophylla was chosen as a reducing agent to manufacture AgNPs to boost the ability of bacteria growth suppression. Moreover, according to previous studies, there was no study reported to synthesise AgNPs from CEPS. Thus, the present study is reported to be the first study. The present study conducted to synthesize and characterize the AgNPs using green synthesis method and evaluate their biomedical properties for biomedical applications.

2. Methodology

2.1 Materials

The *Polyalthia sclerophylla* leaves (LPs) have been collected locally from Perak, Malaysia. Silver nitrate (AgNO3) and ampicillin were purchased from the Bendon brand (Malaysia).

2.2 Methods

2.2.1 LPS crude extract preparation

The LPs were cleaned with water a few times to eliminate all dust and fungus from the leaves. Then, the LPs were dried using sun-dry for seven days. The dried LPS were crushed and ground completely to obtain the powder of LPs. The dried LPs were extracted using the hot-extraction method using a soxhlet extractor, the distilled water (DW) as a solvent. The crude extract of LPS was prepared, and approximately 10 g of LPS powder was placed in the round bottom flask of the soxhlet with 100 ml DW. The extraction period was between 2-5 h using reflux heating. Then, the extraction reached all possible leaves, and the round bottom flask was cooled at room temperature. The mixture was filtered, and a rotary evaporator was used to dry it and store it at -4 ^{o}C for future work.

2.2.2 Synthesis method of AgNPs

Two concentrations of $AgNO_3$ ($1*10^{-3}$M and $1*10^{-4}$M) were used to synthesize two samples named AgNPs-a and AgNPs-b. Briefly, 100 ml of DW was used to dissolve $1*10^{-}3$M of $AgNO_3$ in a conical flask. The conical flask was placed on the hot plate at 80 ^{0}C for 30-60 minutes. Then, 10 ml of CEPS was added gently to the solution, and the solution colour changed from colourless to yellow-brown, which indicated the conversion between the Ag ion to Ag0. The AgNPs-a solution was centrifuged at 14000 rpm for 15 mins, and the bottom part of the microtube was collocated, represented by AgNPs-a, in which the upper part was dismissed. A similar procedure was repeated to prepare $1*10^{-4}$M as an AgNPs-b. Figure 1 shows the process used to synthesize AgNPs.

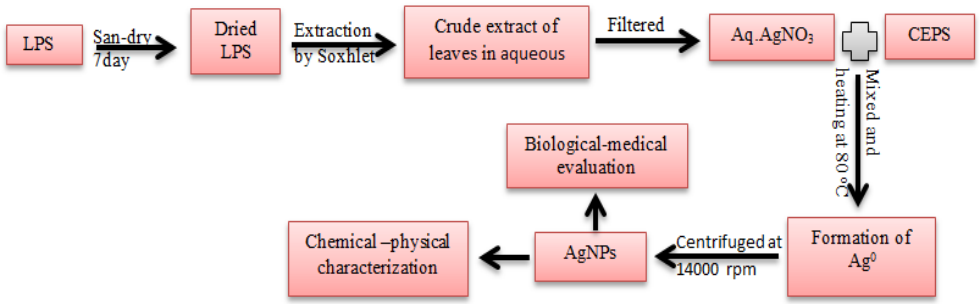


Fig. 1. The preparation Scheme of silver nanoparticles

2.2.3 Cytotoxicity of AgNPs

Alamar blue assay was used to investigate the cytotoxicity of the prepared samples against the MG-63 human cells line. The prepared samples have been downhiller for 24 h in the medium 200 mg/ mL to check the viability of the cells. The present study did not include the materials which represented the negative control. A 0.2 µm of syringe was used for sterilization. The ratios of W/V of 25, 50, 75,100, and 200 mg/mL were dissolved in the extracts in the medium. MG-63 cells have been implanted for 24 h in 24-multiwell plates $1×10^5$ cells/mL were combined with diluted extracts. The incubator of CO_2 has used to incubate the mixture all day at 37 °C. After completing the incubation process, the next step was determining the cell viability using Alamar blue assay. Cultures were stained and incubated in the CO_2 incubator at 37 °C for 24 hours.

2.2.4 AgNPs antibacterial activity

The antibacterial properties of produced AgNPs against E. coli and S. aureus were investigated using a paper disc diffusion test. This approach was employed in this investigation since it is simple and readily available in our facility. Furthermore, it is not expensive. Bacteria were grown on nutrient agar media. Saturated in 1 ml of distilled water, 10 g of AgNPs-s and AgNPs-b were tested against the bacteria. CEPS was also investigated. Following that, AgNPs-a and AgNPs-b concentrations of 10, 20, 40, and 80 M/ml was dissolved in distilled water and passed through a 6 mm disc filter. After 24 hours of incubation, inhibited zones were assessed, as well as the extent of antibacterial effects on *S. aureus* and *E. coli.*

3. Results and Discussion

The current study used CEPS to perform biogenic green synthesis and characterise AgNPs. The first indication that Ag ion was being converted to Ag0 was the change in colour of the solution from colourless to yellow-brown, which was caused by surface plasmon resonance (SPR).

3.1 Uv-Vis Spectroscopy

Uv-vis spectroscopy was utilised to confirm AgNP fabrication, with SPR of the AgNPs attributed to optical characteristics [19,20]. SPR band generation is caused by incoming light on the surface of particles, which results in wave absorption in visible light [21,22]. AgNPs were shown to absorb and have a peak in the 390-460 nm region [23].

Figure 2 shows absorbance peaks of $AgNO_3$, CEPS, and AgNPs a and b. Extract of the leaves and $AgNO_3$ did not show any absorbance peaks in the wavelength range of 200-600 nm. The outcome of different concentrations of $AgNO_3$ was observed. The absorbance peaks were shifted from 436 nm in AgNPs-a to 441 nm in AgNPs, along with the increased concentrations of the $AgNO_3$. This shafting could be attributed to a variety of factors, including particle size and shape [24,25].

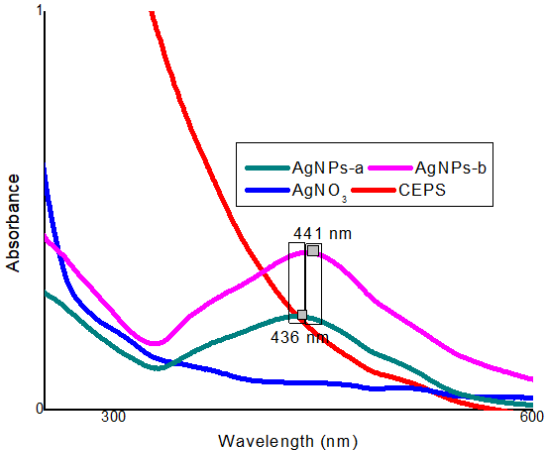
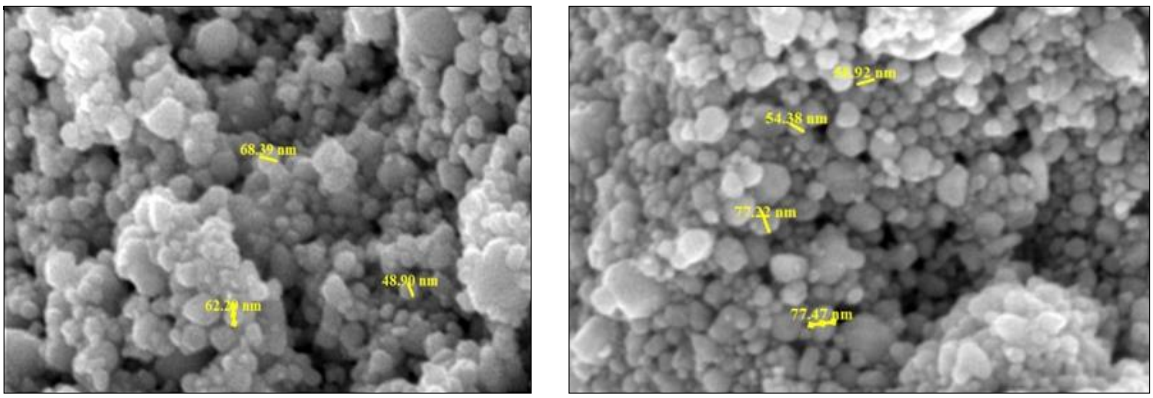


Fig. 2. Uv-vis spectrum of the a) AgNPs-a, b) AgNPs-b, c) $AgNO_3$ and CEPS

A similar impact for varying concentrations of $AgNO_3$ was observed in the work of Karimi *et al.,* [26], where the wavelength peaks switched from red (higher wavelength) to blue (lower wavelength) as the concentration of $AgNO_3$ increased. The association between the size of the Ag particles and the absorbance peak has already been proven. The red shift indicates a larger size when compared to the blue shift [27]. Our study has affirmed the reason that the shaft was due to the size of the particles according to SEM and STEM analysis.

3.2 SEM

Figure 3 shows the SEM images of AgNPs-a, and AgNPs-b, both of the samples have shown the same surface morphology and the shapes of the particles for the samples were spherical. The sizes of the particles decreased with the increase in the concentrations of $AgNO_3$. The SEM photos also reveal that the Ag-a particle range was from 48nm to 68nm, whereas the Ag-b particle range was from 59nm to 77nm.



(a) (b)

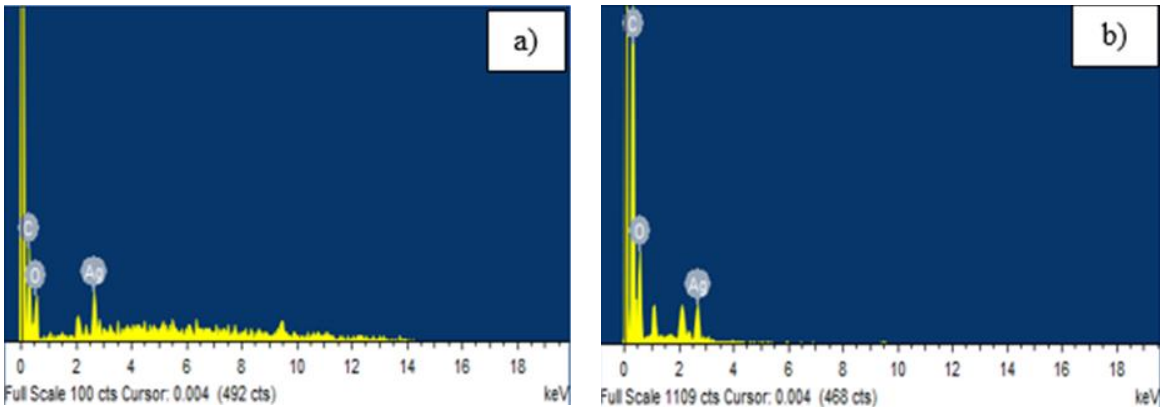
Fig. 3. SEM images of a) AgNPs-a and b) AgNPs-b

Htwe *et al.,* [24] conducted a study to determine the influence of $AgNO_3$ concentrations on the morphology of AgNPs under SEM imaging. The SEM data demonstrate that the AgNPs have a spherical form with a homogeneous surface. Meanwhile, the diameters of the Ag particles differed. The particle size decreased as the concentration of $AgNO_3$ rose. This study's findings back up our findings.

3.3 EDX

EDX technique has been used to emphasize the peaks of the AgNPs, which the strong peak for the Ag has been obtained at 3 keV. Previous studies documented the peak of silver nanoparticles in 3 keV for silver nanoparticles from 5-250 nm [28].

The EDX spectrums of AgNPs-a and AgNPs-b are shown in Figure 4(a) and 3(b), respectively. The presence of Ag elements, along with oxygen and carbon, is observed in both spectra. The spectrum AgNPs-b has shown carbon with a higher percentage than the silver element, while for spectrum AgNPs-a has shown carbon with a lower percentage than carbon. Due to the presence of oxygen and carbon in the CEPS, oxygen and carbon peaks were identified. These findings imply that AgNPs are bordered by carbon and oxygen [29]. The EDX profile displays a spectrum signal in the Ag region of about 3 keV, which corresponds to metallic Ag absorption due to surface plasmon resonance (SPR) of AgNPs with sizes ranging from 20 to 350 nm [30,31].

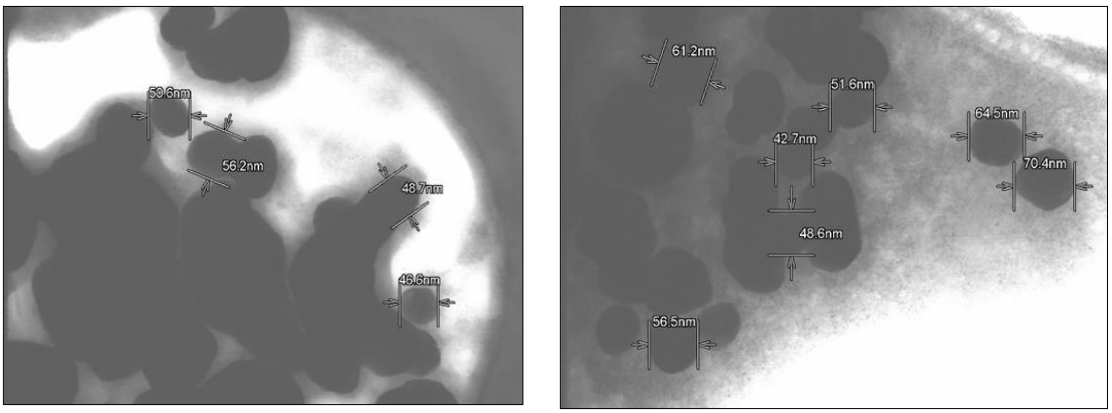


(a) (b)

Fig. 4. EDX spectrum of a) AgNPs-a and b) AgNPs-b

3.4 STEM

STEM was utilised to check the form and morphology of the silver nanoparticles that had been synthesised. Figure 5 depicts the nano-graphs of AgNPs-a and AgNPs-b samples. The shape of the produced samples was spherical, which is identical to the obtained results that have been mentioned in the SEM analysis. Figure 5 depicts the particle size distribution. AgNPs-a has the largest particles having a diameter of 46-56 nm, and AgNPs-b has a particle size ranging from 42 nm to 70 nm. These results are aligned with the SEM-obtained results. In AgNPs-b, there were some particles with more than 100 nm, which might be due to them clumping together while being thawed and put on a copper grid before the examination.



(a) (b)

Fig. 5. STEM image of (a) AgNPs-a and (b) AgNPs-b

3.4 Antibacterial Activities of AgNPs-a and AgNPs-b

Table 1 shows the two parts; the first part explains the positive (ampicillin) and negative (DW) controls along with the CEPS, while part two represents the ability of AgNPs-a and AgNPs-b to inhibition of bacteria growth against *E. coli* and *S. aureus*. The first part shows the ability of CEPS to be used as an antibacterial agent, showing significant activity against both bacteria reaching 8.8 mm and 7 mm at higher concentrations. This ability was increased in the AgNPs-a and AgNPs-b. The results of the current study revealed a strong effect on bacterial growth, with the inhibitory growth of AgNPs-a reaching 24 mm and 22 mm against E. coli and S. aureus, respectively. Meanwhile, the AgNPs-b showed 20 mm and 18 mm against both pathogens, respectively.

Table 1
Antibacterial activities of AgNPs-a, AgNPs-b and CEPS, with controls

No	Conic	CEPS		Ampicillin		DW	
		E. coli	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
1	10 µM/ml	6 mm	5 mm	21 mm	18 mm	0	0
2	20 µM/ml	7 mm	5.4 mm	24 mm	19 mm	0	0
3	40 µM/ml	8.4 mm	6.2 mm	28 mm	24 mm	0	0
4	80 µM/ml	8.8 mm	7 mm	33 mm	29 mm	0	0
No	Conic	AgNPs-a		AgNPs-b			
		E. coli	*S. aureus*	*E. coli*	*S. aureus*		
1	10 µM/ml	14 mm	15 mm	13 mm	12 mm		
2	20 µM/ml	16 mm	16 mm	16 mm	15 mm		
3	40 µM/ml	20 mm	20 mm	18 mm	16 mm		
4	80 µM/ml	24 mm	22 mm	20 mm	18 mm		

Results showed that AgNPs-a had more effect than AgNPs-b due to distinct particle size. As mentioned previously, the smaller size of the nanoparticles can show a higher effect against bacteria [23]. The reason for that can be explained by two or three mechanisms. The most acceptable one is that the small particle size has more surface area, which has more advantage to these particles entering the bacteria cell wall than leading to accumulating particles then damaging the ROS, which makes it dead[1]. In the present study, CEPS was used to enhance the ability of AgNPs against bacteria.

3.5 Alamar Blue Assay

The Alamar blue assay was used to investigate the cytotoxicity of AgNPs in Mg-63 human cells. According to Figure 6, the cell viability was greater than 89% when treated with AgNPs-a and AgNPs-b at all concentrations. The cell viability of Mg-63 in the presence of AgNPs was determined using the Alamar blue test. Figure 6 depicts the cell vitality of Mg-63 after the treatment of prepared samples. AgNPs-2 have a cell viability of more than 89% at all doses. The outcomes of the present study showed there was no toxicity for the prepared samples, even at the higher concentrations that demonstrated to use of them safely and can be applied in the medical and biological field.

Table2:
The cell viability of Mg-63 attained after exposure to the AgNPs-a and AgNPs-b for 24 h

Concentrations	Control	AgNPs-a	AgNPs-b	Cell viability	
25 mg/ml	0.253	0.21	0.223	83	88.14
50 mg/ml	0.277	0.259	0.241	93.5	87
75 mg/ml	0.304	0.277	0.267	91.11	87.82
100 mg/ml	0.238	0.213	0.212	89.49	89.07
200 mg/ml	0.305	0.267	0.346	87.54	113.44
AVERAGE				89.03	93.6

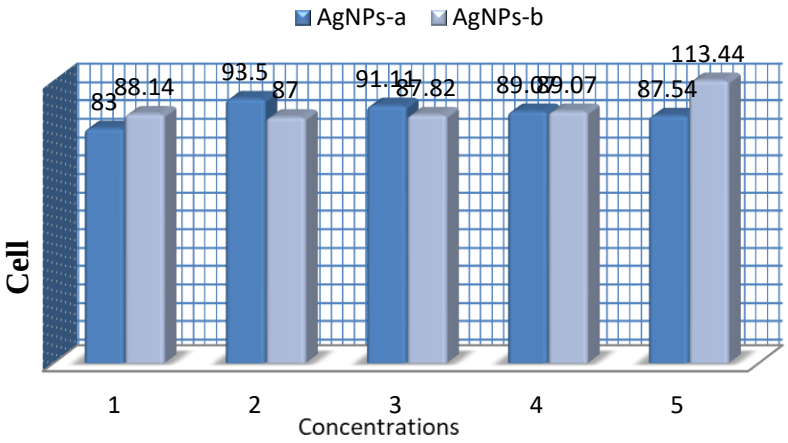


Fig. 6. The cell viability of MG-63 percentages at several concentrations of a) AgNPs-a and b) AgNPs-b

The findings were consistent with those reported by Albers *et al.,* [32]. According to the literature, AgNPs may be hazardous at greater concentrations, implying that AgNPs might be used in the human body with a controller dose to benefit from the unique qualities of AgNPs while avoiding the danger of side effects by utilising a few ratios of it. The investigations have shown that there is a spectrum of non-toxic amounts. 10 g/mL [33], 25 g/mL [34], 40 g/mL [35], 50 g/mL [36], 100 g/mL [37], 160 g/mL [38], and 300 g/m. Many researchers have observed that AgNPs toxicity can be indicated below when it is made using natural materials to lessen the possible cytotoxicity of silver nanoparticles [39].

4. Conclusions

The current study reported the successfully synthesized of AgNPs by the green method. The size obtained for the two different samples has a similar shape. Synthesized silver nanoparticles were characterized by using several characterisation techniques. AgNPs-a showed a smaller size than AgNPs-a in STEM and SEM. The shape was spherical. In the antibacterial part, AgNPs-a were more effective than AgNPs-b. The current work has revealed non-toxic nano-sized produced samples can be utilized safely in medical and biological sectors.

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