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1 Singlet Oxygen Generation Efficiency and  
2 Antimicrobial Ability in Glutathione Protected  
3 Ag<sub>31</sub> Nanoclusters

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1 **Abstract:**

2 Ligand protected silver nanoclusters (Ag NCs) have recently been reported to  
3 have significant applications as antimicrobial agents. In the current study, glutathione  
4 (SG) protected silver nanocluster was synthesized leading to Ag<sub>31</sub>SG<sub>19</sub> by cyclic  
5 reduction and oxidative condition and was evaluated for its singlet oxygen generation  
6 and antibacterial activity. From the results of the study, Ag<sub>31</sub>SG<sub>19</sub> was found to have  
7 inhibitory activity against *Escherichia coli* and *Staphylococcus aureus* with a diameter  
8 of  $15.3 \pm 0.58$  nm and  $16.7 \pm 0.58$  nm respectively. The MIC was also calculated to  
9 have 0.3125 mg/ml and 0.1562 mg/ml respectively. In addition, the Ag<sub>31</sub>SG<sub>19</sub> was  
10 found to have high efficiency for the generation of singlet oxygen. Based on the  
11 results of the study, the antimicrobial mechanisms of Ag<sub>31</sub>SG<sub>19</sub> could be predicted to  
12 be mainly because of the generation of singlet oxygen species in addition to the  
13 activity due to the release of Ag<sup>+</sup> ions from the nanoclusters. These multimechanistic  
14 antimicrobial effects make the synthesized Ag<sub>31</sub>SG<sub>19</sub> to be a promising candidate for a  
15 broad range of antimicrobial applications.

16 **Keywords:**

17 silver nanoclusters; antimicrobial agent; quantum clusters; reactive oxygen species

18

19

20 **Highlights:**

- 21
- 22 • Good photostability of Ag<sub>31</sub>SG<sub>19</sub> nanoclusters was revealed.
  - 23 • Ag<sub>31</sub>SG<sub>19</sub> nanoclusters showed a higher efficiency for <sup>1</sup>O<sub>2</sub> production  
24 compared with new methylene blue under 473 nm irradiation.
  - 25 • Enhanced antibacterial activity of Ag<sub>31</sub>SG<sub>19</sub> nanoclusters compared to  
26 previous works was presented.

## 11 Introduction

2 Bacterial infections have caused serious threat to human health with massive  
3 global economic loss.[1] Even though antibiotics are being considered as the most  
4 effective antibacterial medicine, their widespread use has already resulted in the  
5 development of antimicrobial resistance (AMR) among microbial pathogens.[2] Here  
6 is the relevance of exploring nanotechnological advances for the discovery of new  
7 nanoscale materials with high antimicrobial efficiency and safety.[3, 4] Nanomaterials  
8 generally cause the generation of reactive oxygen species (ROS) and hence exhibit  
9 potential antibacterial activity. The targeted killing action of nanomaterials against  
10 pathogenic bacteria hence could be due to the ROS which act as strong oxidants  
11 leading to oxidative damage of cellular constituents.[5, 6]

12 Among the various nanomaterials, ligand protected noble metal nanoclusters  
13 (NCs) are novel entities having a well-defined number of metal atoms and ligands.[7-  
14 9] When protected by the biomolecular templates, the nanoclusters can exhibit strong  
15 luminescence with good biocompatibility, which make them highly appealing as  
16 theranostic probes for biomedical applications.[10-14] More importantly, NCs can  
17 exhibit their long triplet-state lifetime and the singlet oxygen ( $^1\text{O}_2$ ), the most  
18 damaging ROS species against microorganisms, has been reported to be generated by  
19 the energy transfer from NCs to molecular oxygen upon light irradiation.[15, 16]  
20 Indeed, as illustrated in Scheme S1 (in supporting materials), an efficient  
21 photosensitizer for  $^1\text{O}_2$  production requires a high triplet-state yield with a triplet-state  
22 energy larger than the energy of  $^1\text{O}_2$  (0.98 eV) for efficient energy transfer to  $^3\text{O}_2$ .  
23 From this viewpoint, the optical gap of thiolate protected silver nanoclusters is usually  
24 higher than 1 eV and since presenting long-lifetime, the triplet-state efficiency is  
25 high.[17] Consequently the nanoclusters have also been observed to display  
26 antibacterial capability after being metabolized by the bacterial cells.[18-20] In  
27 addition to their high capability to generate ROS, the antimicrobial mechanisms of  
28 metal NCs may also include the cell wall and membrane disruption, damage to  
29 intracellular components. In the case of silver-based NCs, due to their moderate  
30 stability the antimicrobial mechanisms also involve release of metal ions.[21-23] Thus  
31 for the ultrasmall  $\text{Ag}^+$ -rich nanoclusters, it has been proposed to have both efficient

1 ROS generation and abundant Ag<sup>+</sup> ions on its surface resulting in enhanced  
2 antibacterial activity.[24]

3 Compared with the AgNPs, ultrasmall AgNCs can have higher surface to volume  
4 ratio which make them to be more susceptible to the oxidative dissolution resulting in  
5 a fast release of Ag<sup>+</sup> and thereby enhanced antibacterial performance. In a previous  
6 study, glutathione (GSH)-protected silver nanoclusters which have been produced  
7 mainly as mixture (Ag<sub>n</sub>(SG)<sub>m</sub>) [19, 25] were reported to have strong activity against  
8 *Pseudomonas aeruginosa* due to the easily oxidized and released Ag<sup>+</sup> on the surface  
9 of AgNCs. It has been reported by Yuan et al. that the ultrasmall Ag<sup>+</sup>-rich  
10 nanoclusters (GSH-capped Ag<sup>+</sup>-rich Ag NCs) can have higher antibacterial activity  
11 when compared with the neutral Ag-rich counterpart.[24] Even though preliminary  
12 mass spectrometry data were reported in previous studies, insights into atomic  
13 precision for the silver cluster synthesis was not enough to explore its structure–  
14 activity relationship. Based on a facile aqueous-based synthesis,[26] the current study  
15 demonstrated the synthesis of Ag nanoclusters (Ag<sub>31</sub>SG<sub>19</sub>) using glutathione as  
16 thiolate ligand. Stability and photo-induced ROS efficiency of Ag NCs are explored  
17 and compared with ultrasmall Ag nanoparticles. Also their antimicrobial capabilities  
18 are reported.

## 29 Experimental Section

### 20 2.1 Materials

21 All chemicals were purchased and used directly without purification. Silver  
22 nitrate (AgNO<sub>3</sub>), (±)-α-lipoic acid, tetramethylammonium borohydride  
23 ((CH<sub>3</sub>)<sub>4</sub>N(BH<sub>4</sub>)), sodium borohydride (NaBH<sub>4</sub>), trisodium citrate (TSC), 1,3-  
24 diphenylisobenzofuran (DPBF), sodium hydrate (NaOH), and 2,7-  
25 dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA) were purchased from Sigma  
26 Aldrich. L-glutathione (GSH) reduced were procured from Carl Roth. Ammonia  
27 (NH<sub>4</sub>OH), diethyl ether, and ethanol were purchased from VWR Chemicals.  
28 Methanol was purchased from Honeywell. New methylene blue (NMB) was  
29 purchased from TCI. Ultrapure Milli-Q water (resistivity 18.2 MΩ) was used for for  
30 all of the experiments.

## 2.2 Instrumentation

Electrospray ionization mass spectra (ESI-MS) of silver nanoclusters were recorded on a Thermo Fisher LTQ-Velos linear ion trap mass spectrometer (mass resolution ~7500). UV-Vis absorption spectra were analysed using Avantes AvaSpec-2048 spectrophotometer with an AvaLight DH-S deuterium lamp. Transmission electron microscopy (TEM) images were obtained using a JEOL JEM 2100 microscope. Fluorescence emission spectra were studied using a Horiba FluoroMax-4 spectrophotometer and fluorescence lifetimes were measured using custom-built setup[27].

## 2.3 Synthesis of atomically precise silver nanoclusters

Two atomically precise silver nanoclusters were synthesized as our previous reports with some modification. For Ag<sub>31</sub>SG<sub>19</sub> synthesis, the procedures of cyclic reduction under oxidative conditions were the same as previous synthesis[17]. Here, in a dark environment, 1 mL of AgNO<sub>3</sub> solution (55 mg/mL) was dropwise added to an solution of L-glutathione (200 mg in 35 mL of H<sub>2</sub>O) under ice-water bath. Further to this, 800 mL of 1 M NaOH solution was added to adjust the final pH to 6~7. Then, under constant stirring, 36 mg of NaBH<sub>4</sub> in 1 mL of ice-cold water was quickly added. After 1.5 h stirring, 300 mL of hydrogen peroxide (33%) was further added to the dark brown solution. Then, 140 mL AgNO<sub>3</sub> solution (100 mg/mL) and 50 mg GSH were added after 30 min. This was followed by the addition of 500 mL of ice-cold solution of NaBH<sub>4</sub> (20 mg/mL) after 5 min. The reaction mixture was kept stirring vigorously for 1.5 h till to a brown-red solution was obtained. For purification, the solution was evaporated to ~5 mL using a rotary evaporator at 30°C. This was further precipitated with the addition of 15 mL methanol followed by centrifugation. The precipitate was further dissolved in 20 mL H<sub>2</sub>O followed by the addition of 50 ml of glacial acetic acid. This was kept still for 2 h before centrifugation at 13 000 rpm for 30 min. The precipitate was further removed and the supernatant was further concentrated by evaporator and precipitated with methanol. Then the powder was washed with methanol/diethyl ether for 3 times and dried.

The Ag<sub>29</sub>DHLA<sub>12</sub> was synthesized as per the method described previously[28] with some modification. Here, 100 mg of (±)-α-lipoic acid was dissolved in a mixture of 5 ml methanol and 10 ml water. Then, 85 mg of sodium borohydride was added and the solution was stirred for 30 min to reduce the lipoic acid. Then, excess reducer

1 was eliminated by sonication for 1 hour. Further to this, 41 mg of silver nitrate  
2 (dissolved in 15 mL of water) was added rapidly, followed by the addition of 1 ml of  
3 10% NH<sub>4</sub>OH solution. The solution was further heated at 50 °C and 85 mg of sodium  
4 borohydride was added again. After 1 hour, 42.5 mg of sodium borohydride was  
5 added further when the color became bright orange. After 1 hour, the solution was  
6 concentrated using a rotary evaporator. Then, 5 mL of ethanol and 20 mL of diethyl  
7 ether were added and was followed with the addition of 50 µL of glacial acetic acid.  
8 This was then centrifuged for 5 minutes at 9000 rpm, the precipitate was collected,  
9 washed first with ethanol/diethyl ether and then with diethyl ether. The brown powder  
10 obtained was dried by natural evaporation.

#### 11 *2.4 Synthesis of ultra-small 5-nm silver plasmonic nanoparticles.*

12 The silver plasmonic nanoparticles were synthesized as per the methods reported  
13 by Agnihotri et al.[29] Here, freshly prepared NaBH<sub>4</sub> aqueous solution (2 mM, 24  
14 mL) was added to trisodium citrate (TSC) aqueous solution (4.28 mM, 24 mL). The  
15 mixture was heated at 60 °C for 30 mins under vigorous stirring in dark. Then, 2mL  
16 of AgNO<sub>3</sub> solution (1 mM) was added drop-wise following with increase of  
17 temperature to 90 °C. When temperature was raised to 90 °C, the pH was also  
18 adjusted to 10.5 with the addition of 0.1 M NaOH. After heating for 30 minutes, the  
19 light-yellow solution was cooled down at room temperature. This silver nanoparticle  
20 solution was characterized by absorption spectroscopy, and by transmission electron  
21 microscopy (TEM). The concentration of AgNPs was verified using the absorption  
22 coefficient of  $1.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  (for 6 nm AgNPs has reported by Mulvaney et  
23 al.[30]) at plasmonic peak of ~394 nm according to Lambert-Beer law.

#### 24 *2.5 Reactive oxygen species (ROS) generation measurements*

##### 25 *2.5.1 Protocol for <sup>1</sup>O<sub>2</sub> generation measurements*

26 The singlet oxygen generation efficiency was measured by an indirect method  
27 with the <sup>1</sup>O<sub>2</sub> sensor of 1,3-diphenylisobenzofuran (DPBF).[16] Singlet oxygen  
28 generation was monitored under the excitation at 473 nm using a continuous  
29 wavelength laser (Changchun New Industries Optoelectronics Tech. Co., Ltd, China)  
30 with an output power of 250 mW and a beam diameter of 3 mm. A 532 nm  
31 continuous wavelength laser (Spectra-Physics) with an output power set to 282 mW  
32 (beam diameter: 3 mm) was also used for the comparison. A typical solution used in  
33 the experiment contained nanoclusters/nanoparticles/dyes and DPBF with a

1 concentration of  $1.37 \times 10^{-6}$  M and  $6.15 \times 10^{-5}$  M, respectively. All solutions were  
2 prepared in ethanol. For Ag NPs, the mixture solution with ethanol-water solvent  
3 (50/50 in volume ratio) was used. The samples were loaded in quartz cuvettes (1cm  
4 light path length) and the absorbance was recorded with irradiation for different  
5 time.

### 6 2.5.2 Statistical analysis for $^1O_2$ generation rate

7 The concentration of DPBF was calculated from the intensity of absorption peak  
8 at 410 nm according to Beer–Lambert law. The rate of  $^1O_2$  generation was obtained  
9 by the initial DPBF concentration change over time by a function:

$$\text{rate of } ^1O_2 \text{ generation} = \frac{c[DPBF]_{0min} - c[DPBF]_{5min}}{\Delta t} / c[NCs]$$

10

11  $c[DPBF]_{0min}$  and  $c[DPBF]_{5min}$  represent the concentration of DPBF at 0 min  
12 and 5 min under irradiation, respectively.  $\Delta t$  represents the time under irradiation (5  
13 mins), and  $c[NCs]=1.37 \times 10^{-6}$  M. The photoluminescence lifetime was calculated by  
14 two-exponential fitting in the decay curve. The uncertainty was evaluated by  
15 measuring 3 times repeatedly.

### 16 2.5.3 Protocol for oxidants generation indication

17 An excellent oxidants fluorescent indicator, 2',7' -dichlorodihydrofluorescein  
18 ( $H_2DCF$ ) was used for marking the generation of reactive oxygen species, including  
19 superoxide ( $\bullet O_2^-$ ), hydroxyl radical ( $\bullet OH$ ), and hydrogen peroxide ( $H_2O_2$ ). [31, 32]  
20  $H_2DCF$  can be oxidized to green fluorescent 2',7' -dichlorofluorescein (DCF) with  
21 an emission peak at  $\sim 525$  nm. The ROS generation can be indicated by the  
22 fluorescence change on fluorescence spectra.

23 The commercial dye 2,7-dichlorodihydrofluorescein diacetate ( $H_2DCF-DA$ ) was  
24 firstly prepared based on reported procedure [33]: 0.5 mL of 2 mM  $H_2DCF-DA$  in  
25 ethanol was added to 2.0 mL of 10 mM NaOH and stirred for 30 mins at room  
26 temperature under darkness. Then Dulbecco's phosphate-buffered saline (DPBS,  
27 pH7.2, without calcium, magnesium, Thermo Fisher Scientific) was added to a final  
28 concentration at 123  $\mu M$ . Freshly prepared  $H_2DCF$  solution was used for further.

29 For oxidants generation indication, 2 mL mixture aqueous solution containing  
30 prepared  $H_2DCF$  ( $6.15 \times 10^{-5}$  M) and  $Ag_{31}SG_{19}$  ( $1.37 \times 10^{-6}$  M) was irradiated for 10



1 mins under the excitation of 473 nm continuous wavelength laser. Before and after  
2 laser irradiation, fluorescence emission spectra were recorded with excitation  
3 wavelength at 488 nm.

#### 4 *2.6 Antibacterial activity analysis of Ag<sub>31</sub>SG<sub>19</sub> and plasmonic AgNPs*

5 The antibacterial activity of Ag<sub>31</sub>SG<sub>19</sub> and plasmonic AgNPs was studied against  
6 Gram-positive *Staphylococcus aureus* (MTCC 96) and Gram-negative *Escherichia*  
7 *coli* (ATCC 35218) by both agar well diffusion and minimum inhibitory  
8 concentration.

##### 9 *2.6.1 Agar well diffusion analysis*

10 The antibacterial activity of Ag<sub>31</sub>SG<sub>19</sub> and plasmonic AgNPs was evaluated  
11 against *E. coli* and *S. aureus* by the standard well diffusion method in Mueller Hinton  
12 Agar (MHA) plates by following the Clinical and Laboratory Standards Institute  
13 (CLSI) guidelines (CLSI M02-A13). For this, a 20 mg/ml suspension of Ag<sub>31</sub>SG<sub>19</sub>  
14 was prepared. Here, the bacterial inoculum was prepared by adjusting the cell  
15 concentration of respective cultures to  $1.5 \times 10^8$  CFU/ml (0.5 McFarland) and the  
16 organisms were inoculated onto the MHA plates as lawn culture. Then, wells were cut  
17 on the media and 60  $\mu$ l of Ag<sub>31</sub>SG<sub>19</sub> suspension was added to each well. Plates were  
18 further incubated at 37°C for 24 hours and the diameter of the zone of inhibition was  
19 measured using a zone measuring scale and the experiment was carried out in  
20 triplicates. The antimicrobial experiment was conducted under visible light exposure  
21 (400-700 nm) in Biosafety cabinet level 2.

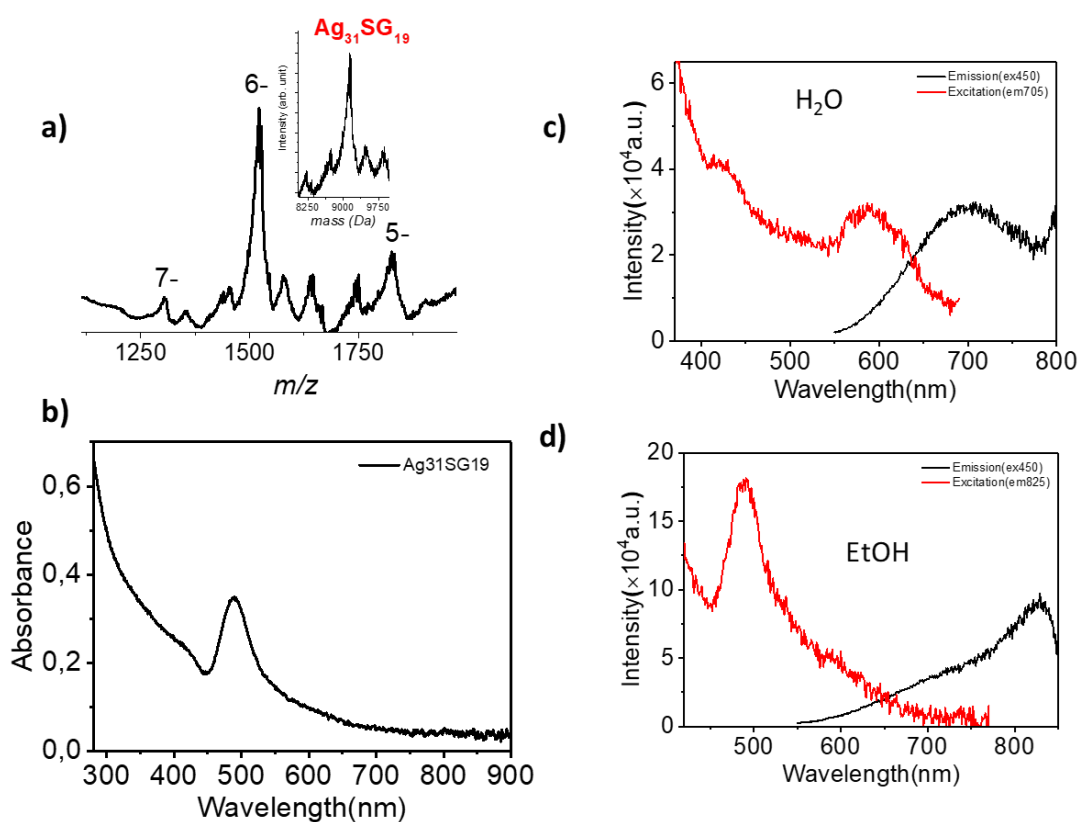
##### 22 *2.6.2 The Minimum Inhibitory Concentration analysis*

23 The Minimum Inhibitory Concentration (MIC) is defined as the lowest  
24 concentration of the antimicrobial agent that can inhibit the growth of bacteria. MIC  
25 of Ag<sub>31</sub>SG<sub>19</sub> against *S. aureus* and *E. coli* was evaluated by using the standard broth  
26 microdilution method as per the CLSI guidelines (CLSI M07-A10). For this, the test  
27 organisms were incubated overnight at 37°C in Muller Hinton Broth (MHB) and  
28 inoculum was prepared as described above. Further, serial dilution was carried out to  
29 obtain different concentrations of Ag<sub>31</sub>SG<sub>19</sub> (5, 2.5, 1.25, 0.625, 0.3125, 0.1563,  
30 0.0781, 0.0391, 0.0195, 0.0098, 0.0049, and 0.0024 mg/ml) in a 96-well microtiter  
31 plate and each well was further inoculated with 100  $\mu$ l of the selected test organisms.  
32 The plate was then incubated under shaking conditions at 100 rpm and 37°C for 24  
33 hours. After this, 0.01% Resazurin dye (30 $\mu$ l) was added to each of the well followed

1 by incubation in dark for 30-45 mins. MIC value of Ag<sub>31</sub>SG<sub>19</sub> against each organism  
 2 was identified based on the colour change observed with the addition of Resazurin.  
 3 Both sterility and growth controls were also maintained and the experiment was  
 4 carried out in triplicates.[34]

## 35 Results

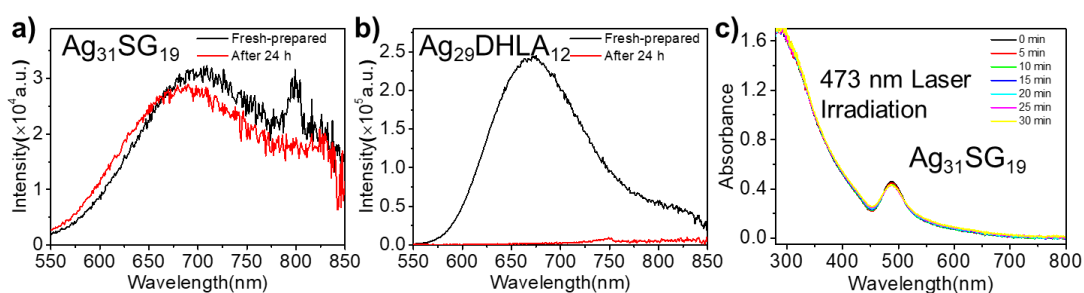
### 6 3.1 Characterization of atomically precise Ag<sub>31</sub>SG<sub>19</sub> silver nanoclusters and their 7 (photo)stability.



8  
 9 *Figure 1 Mass spectrometry (a) and optical (b-d) characterization of Ag<sub>31</sub>SG<sub>19</sub>. (a) ESI-MS spectrum*  
 10 *recorded in the negative mode of ionization. Inset : Deconvoluted ESI-MS spectra. (b) Absorption*  
 11 *spectrum of Ag<sub>31</sub>SG<sub>19</sub> in water. Emission (at excitation 450 nm) and Excitation spectra (at emission*  
 12 *705 nm (c) and 825 nm (d) of Ag<sub>31</sub>SG<sub>19</sub> (c) in water and (d) in ethanol (EtOH) (d).*

13 The purity of synthesized silver nanoclusters was examined by mass  
 14 spectrometry (Figure 1 a).[35, 36] A charge state distribution was observed from [M-  
 15 5H]<sup>5-</sup> through [M-7H]<sup>7-</sup>. Deconvolution of charge states 5 through 7 provided a mass  
 16 of 9156 Da for the intact Ag cluster and was consistent with the calculated mass of  
 17 Ag<sub>31</sub>SG<sub>19</sub> (9164 Da) (see Figure S1). Experimental and simulated isotope pattern of

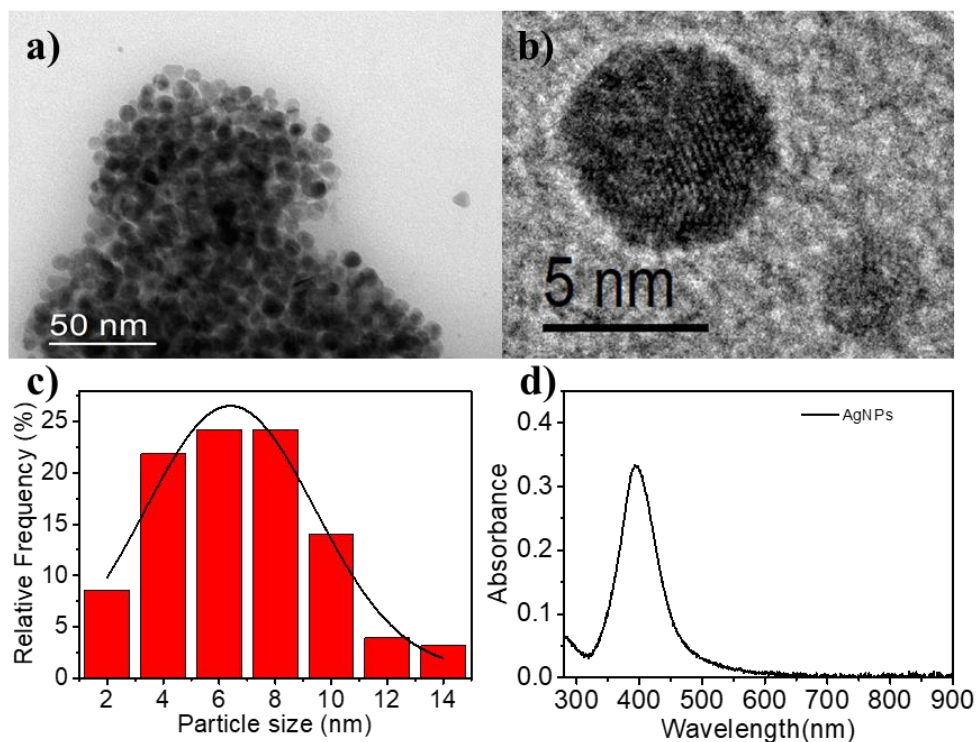
1 [M-6H]<sup>6-</sup> were shown in Figure S2. The pattern shape and mass position match well,  
 2 though the resolution of our mass spectrometer is too low to resolve isotope pattern.  
 3 The absorption spectra (Figure 1b) showed the presence of an intense visible band  
 4 centred at ~490 nm, in agreement with previous findings for which a structure for  
 5 Ag<sub>31</sub> was proposed (see Fig. S3).[17] Excitation and emission spectra for Ag<sub>31</sub>SG<sub>19</sub>  
 6 nanoclusters dispersed in water and ethanol were also analyzed (Figure 1c and 1d). As  
 7 already observed for Ag<sub>29</sub> nanoclusters[28], the solvent-dependent emission spectra  
 8 were also evidenced for Ag<sub>31</sub>SG<sub>19</sub> NCs. Such a shift might be an indication of a  
 9 stronger stabilization of the ground state than those of the excited state due to the  
 10 difference in dielectric constants between H<sub>2</sub>O and ethanol. Stability and  
 11 photostability are generally important issues, that limit the applications of thiolate  
 12 protected silver nanoclusters. As the, Ag<sub>29</sub>DHLA<sub>12</sub> nanocluster was known to have  
 13 poor stability due to degradation in few hours upon light exposure, [37, 38] it was not  
 14 possible to study its antimicrobial activity. The emission spectra for fresh and 1-day  
 15 old solutions clearly demonstrated the complete degradation of Ag<sub>29</sub> in less than 24  
 16 hours (Figure 2b). On the other hand, the Ag<sub>31</sub>SG<sub>19</sub> was found to be very stable under  
 17 normal conditions and upon constant irradiation by visible laser light as confirmed by  
 18 the emission spectra of fresh and 1-day old solution respectively (Figure 2a) and for  
 19 absorption spectra under several dozen minutes of visible laser irradiation (Figure 2c  
 20 and S4).



21  
 22 *Figure 2 :Emission spectra of Ag<sub>31</sub>SG<sub>19</sub> and Ag<sub>29</sub>DHLA<sub>12</sub>for fresh and 24 h old solutions under normal*  
 23 *conditions ( (a) and (b)) and (c) absorption spectra of Ag<sub>31</sub>SG<sub>19</sub> under 473 nm laser irradiation for 0 to*  
 24 *30 minutes).*

25 In the current study, silver nanoparticles (AgNPs) were synthesized by using a  
 26 co-reduction approach, where the nucleation and growth kinetics during the synthesis  
 27 process was precisely controlled and the AgNPs of average size ~6 nm were  
 28 synthesized with good yield and monodispersity (Figure 3). Here, the silver  
 29 nanoparticles were found to exhibit a sharp extinction peak at ~394 nm wavelength

1 which confirmed the surface plasmon behavior of such particles as opposed to the  
2 multi-band “molecular-like” behavior of silver nanoclusters.

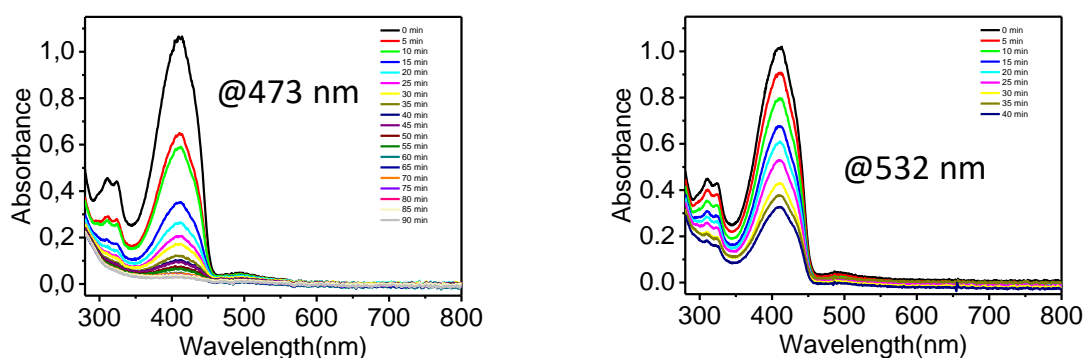


3  
4 Figure 3: Characterisation of AgNPs. (a) TEM analysis, (b) HRTEM analysis, (c) size histogram, and  
5 (d) UV-VIS spectrum of plasmonic AgNPs.

### 6 3.2 ROS efficiency of Ag<sub>31</sub>SG<sub>19</sub> in solution.

7 The indirect method was used to quantify the singlet oxygen generation by the  
8 photoexcited nanoclusters in solution. Here, 1,3-diphenylisobenzofuran (DPBF) has  
9 already been known to be highly reactive toward singlet oxygen resulting in the  
10 formation of endoperoxides (with different absorption properties in particular at 412  
11 nm) and hence can be used as an optical probe.[39, 40] Both the nanoclusters and  
12 DPBF can be excited to generate and detect singlet oxygen simultaneously. Here, the  
13 generation of singlet oxygen was triggered by the excitation of the nanoclusters with a  
14 cw laser emitting at 473 nm (and also a 532 nm) with different time of exposure. Of  
15 note, no generation of singlet oxygen species occurs under darkness. The change in  
16 the absorption of DPBF was monitored over time at 412 nm.[16] Absorption spectra  
17 and the change in the absorption of DPBF monitored over time are presented in  
18 Figure 4. The rate of <sup>1</sup>O<sub>2</sub> generation was obtained by the initial DPBF concentration

1 change over time ( $\Delta[\text{DPBF}]/\Delta t$ , at shorter time where the slope has a linear behavior)  
 2 divided by the concentration of the nanoclusters. To demonstrate the efficiency of  
 3  $\text{Ag}_{31}$  nanoclusters for  $^1\text{O}_2$  production, we compared  $^1\text{O}_2$  production by  $\text{Ag}_{31}\text{SG}_{19}$  to  
 4 that of the conventional dye photosensitizer new methylene blue (NMB). Under 473  
 5 nm irradiation, a superior  $^1\text{O}_2$  production efficiency was observed for  $\text{Ag}_{31}\text{SG}_{19}$  when  
 6 compared with NMB (Table 1). The photosensitizer ability of  $\text{Ag}_{31}\text{SG}_{19}$  is mainly due  
 7 to its high light absorption coefficients, and long lifetime to allow high intersystem  
 8 crossing efficiencies as demonstrated (Table 1). Under 532 nm irradiation (see Figure  
 9 4), a lower  $^1\text{O}_2$  production efficiency ( $^1\text{O}_2$  generation rate ( $^1\text{O}_2/\text{cluster}/\text{min}$ ) = 0.980)  
 10 was observed for  $\text{Ag}_{31}\text{SG}_{19}$  (as compared to 473 nm irradiation). Of note, the  $^1\text{O}_2$   
 11 generation rate (presented in Table 1) also depends on the absorbance of used laser  
 12 light. Quantum yield of the singlet oxygen generation allows for a normalization by  
 13 the absorbance at 473 nm of the cw laser. Using the protocol described in the  
 14 pioneering paper[41], we were able to evaluate the quantum yield of the singlet  
 15 oxygen generation (as described in supplementary materials and Figure S5). The  
 16 quantum yield of  $^1\text{O}_2$  generation was estimated to  $\sim 0.01\text{-}0.02$ .



17  
 18 Figure 4: Absorption spectra evolution of DPBF- $\text{Ag}_{31}\text{SG}_{19}$  mixture solution under cw laser (a) 473 and  
 19 (b) 532 nm irradiation.

20  
 21 Table 1 Singlet Oxygen generation rate by  $\text{Ag}_{31}\text{SG}_{19}$  nanoclusters compared to NMB dye  
 22 photosensitizer and AgNPs under cw 473 nm irradiation.

Photosensitizer	Emission	Long lifetime component (ns)	$^1\text{O}_2$ generation rate ( $^1\text{O}_2/\text{cluster}/\text{min}$ ) @473 nm
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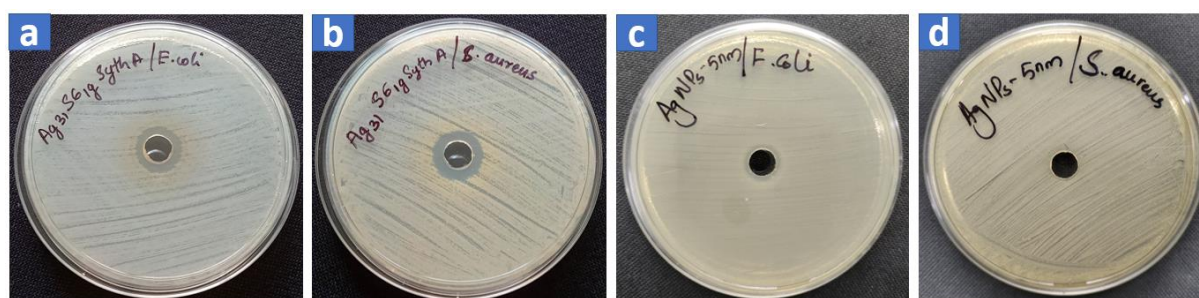
Ag <sub>31</sub> SG <sub>19</sub>	705 nm	870.4±19.7 (7.2%)	3.505
NMB	652 nm	0.70 (MeOH)[42]	2.587
AgNPs			0.612

1

### 2 3.3 Antibacterial activity analysis of Ag<sub>31</sub>SG<sub>19</sub>

#### 3 3.3.1 Agar well diffusion analysis

4 The antibacterial activity analysis of Ag<sub>31</sub>SG<sub>19</sub> was carried out against *S. aureus*  
5 and *E. coli*. Here, the zone of inhibition could be observed against both *S. aureus* and  
6 *E. coli* which indicated the Ag<sub>31</sub>SG<sub>19</sub> to have antibacterial activity. The zone of  
7 inhibition observed against *E. coli* and *S. aureus* was 15.3 ± 0.58 mm and 16.7 ± 0.58  
8 mm respectively. It was also observed that a zone of inhibition against *E. coli* was 12.3  
9 ± 0.58 mm, whereas no zone was observed against *S. aureus* (Figure 5).



10

11 Figure 5: Antibacterial activity of (a) Ag<sub>31</sub>SG<sub>19</sub> against *E. coli*, (b) Ag<sub>31</sub>SG<sub>19</sub> against *S. aureus*,  
12 (c) plasmonic AgNPs against *E. coli* and (d) plasmonic AgNPs against *S. aureus*.

13

#### 14 3.3.2 Minimum Inhibitory Concentration analysis

15 The MIC of Ag<sub>31</sub>SG<sub>19</sub> against *S. aureus* and *E. coli* was determined using the  
16 standard microtiter plate assay. Here, the MIC value of Ag<sub>31</sub>SG<sub>19</sub> against *E. coli* and  
17 *S. aureus* was found to be 0.3125 mg/ml and 0.1562 mg/ml respectively (Figure S6).

## 48 Discussion

19 In the study, the antibacterial activity of Ag<sub>31</sub>SG<sub>19</sub> was evaluated against *E. coli*  
20 and *S. aureus* as test organisms. From the agar well diffusion analysis, Ag<sub>31</sub>SG<sub>19</sub> (20  
21 mg/ml) was found to have significant antibacterial activity against both bacteria with

1 a zone of inhibition measuring  $15.3 \pm 0.58$  and  $16.7 \pm 0.58$  mm respectively against *E.*  
2 *coli* and *S. aureus*. The size of the zone of inhibition indicates the degree of  
3 antibacterial effectiveness of Ag<sub>31</sub>SG<sub>19</sub>. However, the zone of inhibition for plasmonic  
4 AgNPs was observed to be  $12.3 \pm 0.58$  mm against *E. coli* and no zone was observed  
5 against *S. aureus*. MIC analysis was carried out to find out the minimum  
6 concentration of Ag<sub>31</sub>SG<sub>19</sub> required to inhibit the growth of *E. coli* and *S. aureus*.  
7 Here, the MIC of Ag<sub>31</sub>SG<sub>19</sub> was found to be 0.1562 mg/ml against *S. aureus* and  
8 0.3125 mg/ml against *E. coli*. Overall, the results of both the experiments suggest the  
9 Ag<sub>31</sub>SG<sub>19</sub> to have significant antibacterial activity and hence it can have the potential  
10 to be used as an antimicrobial agent. Here, *S. aureus* was found to be more susceptible  
11 towards Ag<sub>31</sub>SG<sub>19</sub> when compared with *E. coli*.

12 In the study, Ag<sub>31</sub>SG<sub>19</sub> nanoclusters were produced with high purity for the  
13 quantitative measurements of ROS efficiencies upon visible laser irradiation. Here,  
14 the nanoclusters synthesized were found to be highly stable under daily conditions  
15 and also under continuous visible laser irradiation. This behavior is quite unique in  
16 particular when compared with the Ag<sub>29</sub> nanoclusters protected by DHLA ligands.  
17 Here, Ag<sub>31</sub>SG<sub>19</sub> presents high rates of singlet Oxygen generation in the visible range  
18 in particular upon 473 nm laser irradiation. Hence these Ag nanoclusters protected by  
19 glutathione were further used to investigate its antimicrobial efficacy against Gram-  
20 positive *S. aureus* and Gram-negative *E. coli*. Based on our findings on quantitative  
21 measurements of ROS efficiencies and photostability measurements in the solution,  
22 the primary mechanism (<sup>1</sup>O<sub>2</sub> generation) leading to the antimicrobial activity could be  
23 a possible mechanism. Other mechanism which arise from the degradation of  
24 nanoclusters is certainly minimal here due to the high (photo)stability of Ag<sub>31</sub>SG<sub>19</sub>.  
25 However, we cannot exclude that the generation of oxidizing species (H<sub>2</sub>O<sub>2</sub>, radicals)  
26 upon irradiation may play a role on the antimicrobial activity of Ag<sub>31</sub>SG<sub>19</sub>. To better  
27 address this point, we used a DCF-dye, as already studied by Yuan and  
28 coworkers,[43] for probing the oxidizing agents produced following photoexcitation.  
29 We adapted this method to probe type I ROS species generated following 473 nm  
30 laser irradiation, and indeed, we confirmed that Ag<sub>31</sub>SG<sub>19</sub> is prone to produce such  
31 oxidizing agents in addition to <sup>1</sup>O<sub>2</sub> species. Figure S7 displays the fluorescence  
32 spectra of DCF dye without and with 10 min laser irradiation. The strong increase in  
33 fluorescence intensity is a signature of generation of type I species.

## 1 5 Conclusion

2 In conclusion, the antibacterial performance indicated the Ag<sub>31</sub>SG<sub>19</sub> with  
3 superior stability to have enhanced antibacterial activity when compared to previous  
4 works using undefined glutathione protected silver nanoclusters (Table 2). The  
5 present study confirms that singlet oxygen generation produced by photo-excited  
6 Ag<sub>31</sub>SG<sub>19</sub> nanoclusters is the key process involved in its antibacterial mechanism,  
7 while generation of oxidizing species is also involved. Singlet oxygen generation rate  
8 as well as quantum yield for <sup>1</sup>O<sub>2</sub> generation for Ag<sub>31</sub>SG<sub>19</sub> nanoclusters are compared  
9 to NMB dye photosensitizer under cw 473 nm and 532 nm irradiations. As singlet  
10 oxygen is considered to have rapid fatal activity and the most damaging ROS species  
11 against microorganisms, the currently studied Ag<sub>31</sub>SG<sub>19</sub> nanoclusters can have  
12 significant antimicrobial therapeutic applications.

13

14 *Table 2: Minimum Inhibitory Concentration of silver nanoclusters against E. coli and*  
15 *S. aureus.*

16

Glutathione protected silver NCs	MIC (µg/mL)		Ref.
	<i>E. coli</i>	<i>S. aureus</i>	
Ag <sub>31</sub> SG <sub>19</sub>	312.5	156.2	Our work
Glutathione-protected Ag <sup>+</sup> -Rich NCs	450.0	358.0	Ref.[24]
Ag <sub>n</sub> (GSHR) <sub>m</sub>	1687.5	1687.5	Ref.[25]

19

20

21



## 1 **Declaration of Competing Interest**

2 The authors declare no conflict of interest.

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## 14 **Appendix A. Supplementary data**

15 All the data are presented in this article and the Supplementary Materials.

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