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1 Singlet Oxygen Generation Efficiency and
2 Antimicrobial Ability in Glutathione Protected
3 Ag₃₁ 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 Ag₃₁SG₁₉ 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, Ag₃₁SG₁₉ was found to have
7 inhibitory activity against *Escherichia coli* and *Staphylococcus aureus* with a diameter
8 of 15.3 ± 0.58 nm and 16.7 ± 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 Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ 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⁺ ions from the nanoclusters. These multimechanistic
14 antimicrobial effects make the synthesized Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ nanoclusters was revealed.
 - 23 • Ag₃₁SG₁₉ nanoclusters showed a higher efficiency for ¹O₂ production
24 compared with new methylene blue under 473 nm irradiation.
 - 25 • Enhanced antibacterial activity of Ag₃₁SG₁₉ 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 Ag^+ -rich nanoclusters, it has been proposed to have both efficient

1 ROS generation and abundant Ag⁺ 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⁺ and thereby enhanced antibacterial performance. In a previous
6 study, glutathione (GSH)-protected silver nanoclusters which have been produced
7 mainly as mixture (Ag_n(SG)_m) [19, 25] were reported to have strong activity against
8 *Pseudomonas aeruginosa* due to the easily oxidized and released Ag⁺ on the surface
9 of AgNCs. It has been reported by Yuan et al. that the ultrasmall Ag⁺-rich
10 nanoclusters (GSH-capped Ag⁺-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 (Ag₃₁SG₁₉) 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 (AgNO₃), (±)-α-lipoic acid, tetramethylammonium borohydride
23 ((CH₃)₄N(BH₄)), sodium borohydride (NaBH₄), trisodium citrate (TSC), 1,3-
24 diphenylisobenzofuran (DPBF), sodium hydrate (NaOH), and 2,7-
25 dichlorodihydrofluorescein diacetate (H₂DCF-DA) were purchased from Sigma
26 Aldrich. L-glutathione (GSH) reduced were procured from Carl Roth. Ammonia
27 (NH₄OH), 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 Ag₃₁SG₁₉ synthesis, the procedures of cyclic reduction under oxidative conditions were the same as previous synthesis[17]. Here, in a dark environment, 1 mL of AgNO₃ solution (55 mg/mL) was dropwise added to an solution of L-glutathione (200 mg in 35 mL of H₂O) 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 NaBH₄ 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 AgNO₃ 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 NaBH₄ (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 H₂O 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 Ag₂₉DHLA₁₂ 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% NH₄OH 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 NaBH₄ 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 AgNO₃ 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 ¹O₂ generation measurements*

26 The singlet oxygen generation efficiency was measured by an indirect method
27 with the ¹O₂ 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 1.37×10^{-6} M and 6.15×10^{-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. Δt 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 ~ 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 μM . Freshly prepared H_2DCF solution was used for further.

29 For oxidants generation indication, 2 mL mixture aqueous solution containing
30 prepared H_2DCF (6.15×10^{-5} M) and $Ag_{31}SG_{19}$ (1.37×10^{-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 Ag₃₁SG₁₉ and plasmonic AgNPs*

5 The antibacterial activity of Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉
14 was prepared. Here, the bacterial inoculum was prepared by adjusting the cell
15 concentration of respective cultures to 1.5×10^8 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 μ l of Ag₃₁SG₁₉ 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.

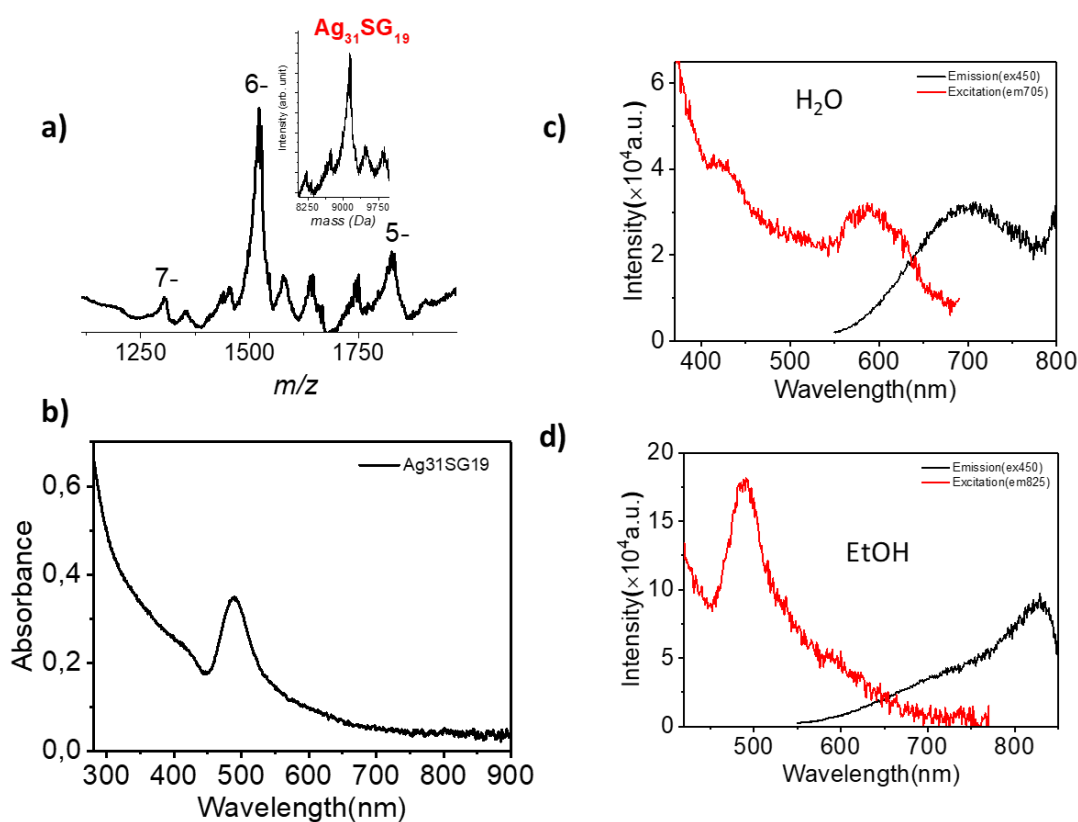
22 *2.6.2 The Minimum Inhibitory Concentration analysis*

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 Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ (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 μ 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 μ l) was added to each of the well followed

1 by incubation in dark for 30-45 mins. MIC value of Ag₃₁SG₁₉ 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

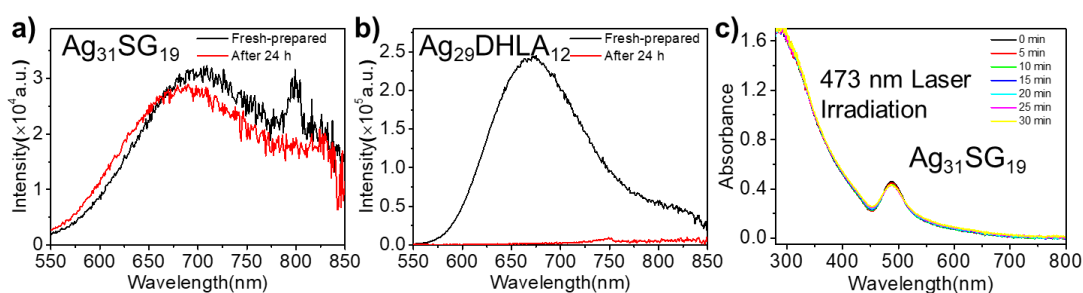
6 3.1 Characterization of atomically precise Ag₃₁SG₁₉ silver nanoclusters and their 7 (photo)stability.



8
 9 *Figure 1 Mass spectrometry (a) and optical (b-d) characterization of Ag₃₁SG₁₉. (a) ESI-MS spectrum*
 10 *recorded in the negative mode of ionization. Inset : Deconvoluted ESI-MS spectra. (b) Absorption*
 11 *spectrum of Ag₃₁SG₁₉ in water. Emission (at excitation 450 nm) and Excitation spectra (at emission*
 12 *705 nm (c) and 825 nm (d) of Ag₃₁SG₁₉ (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]⁵⁻ through [M-7H]⁷⁻. 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 Ag₃₁SG₁₉ (9164 Da) (see Figure S1). Experimental and simulated isotope pattern of

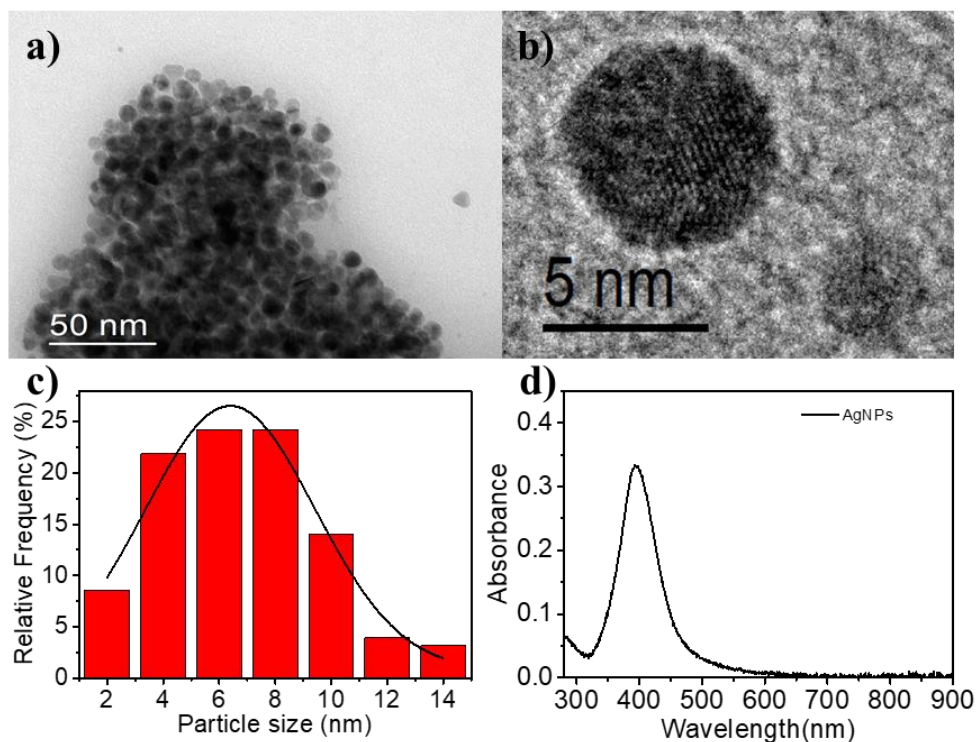
1 [M-6H]⁶⁻ were shown in Figure S2. The pattern shape and mass position match well,
 2 though the resolution of our mass spectrometer is too low to resolve isotope pattern.
 3 The absorption spectra (Figure 1b) showed the presence of an intense visible band
 4 centred at ~490 nm, in agreement with previous findings for which a structure for
 5 Ag₃₁ was proposed (see Fig. S3).[17] Excitation and emission spectra for Ag₃₁SG₁₉
 6 nanoclusters dispersed in water and ethanol were also analyzed (Figure 1c and 1d). As
 7 already observed for Ag₂₉ nanoclusters[28], the solvent-dependent emission spectra
 8 were also evidenced for Ag₃₁SG₁₉ 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 H₂O and ethanol. Stability and
 11 photostability are generally important issues, that limit the applications of thiolate
 12 protected silver nanoclusters. As the, Ag₂₉DHLA₁₂ 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 Ag₂₉ in less than 24
 16 hours (Figure 2b). On the other hand, the Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ and Ag₂₉DHLA₁₂for fresh and 24 h old solutions under normal*
 23 *conditions ((a) and (b) and (c) absorption spectra of Ag₃₁SG₁₉ 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.



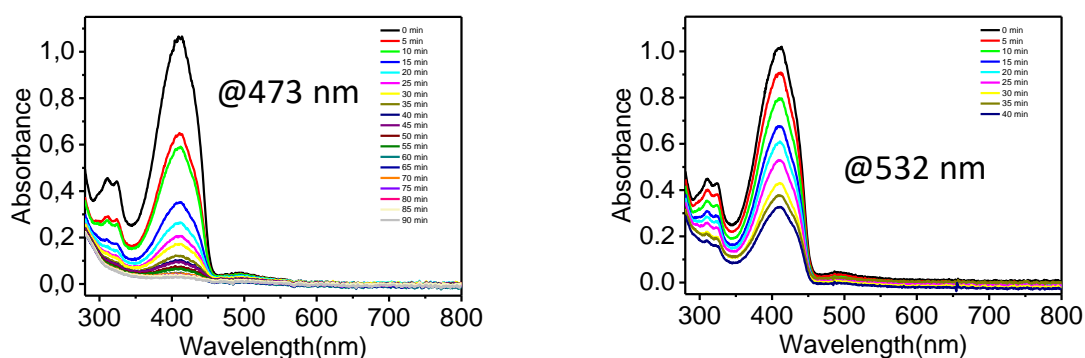
3

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 Ag₃₁SG₁₉ 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 ¹O₂ 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 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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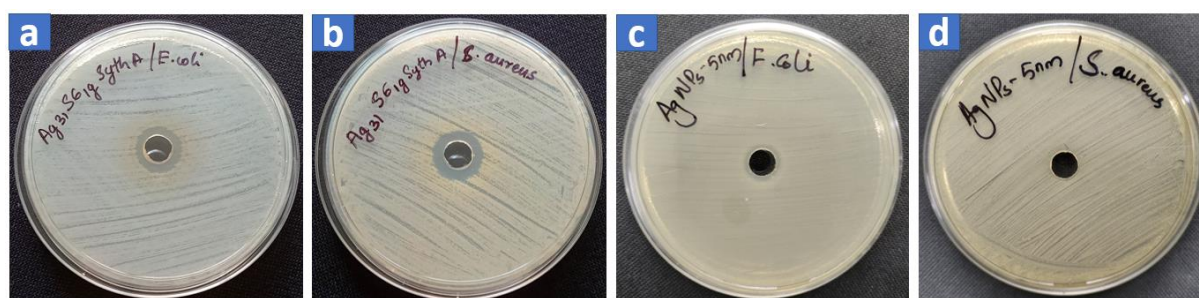
Ag ₃₁ SG ₁₉	705 nm	870.4±19.7 (7.2%)	3.505
NMB	652 nm	0.70 (MeOH)[42]	2.587
AgNPs			0.612

1

2 3.3 Antibacterial activity analysis of Ag₃₁SG₁₉

3 3.3.1 Agar well diffusion analysis

4 The antibacterial activity analysis of Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ 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) Ag₃₁SG₁₉ against *E. coli*, (b) Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ against *S. aureus* and *E. coli* was determined using the
16 standard microtiter plate assay. Here, the MIC value of Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ was evaluated against *E. coli*
20 and *S. aureus* as test organisms. From the agar well diffusion analysis, Ag₃₁SG₁₉ (20
21 mg/ml) was found to have significant antibacterial activity against both bacteria with

1 a zone of inhibition measuring 15.3 ± 0.58 and 16.7 ± 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 Ag₃₁SG₁₉. However, the zone of inhibition for plasmonic
4 AgNPs was observed to be 12.3 ± 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 Ag₃₁SG₁₉ required to inhibit the growth of *E. coli* and *S. aureus*.
7 Here, the MIC of Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ when compared with *E. coli*.

12 In the study, Ag₃₁SG₁₉ 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 Ag₂₉ nanoclusters protected by DHLA ligands.
17 Here, Ag₃₁SG₁₉ 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 (¹O₂ 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 Ag₃₁SG₁₉.
25 However, we cannot exclude that the generation of oxidizing species (H₂O₂, radicals)
26 upon irradiation may play a role on the antimicrobial activity of Ag₃₁SG₁₉. 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 Ag₃₁SG₁₉ is prone to produce such
31 oxidizing agents in addition to ¹O₂ 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 Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ 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 ¹O₂ generation for Ag₃₁SG₁₉ 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 Ag₃₁SG₁₉ 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 ₃₁ SG ₁₉	312.5	156.2	Our work
Glutathione-protected Ag ⁺ -Rich NCs	450.0	358.0	Ref.[24]
Ag _n (GSHR) _m	1687.5	1687.5	Ref.[25]

19

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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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