Supplementary Information

Water-Soluble N-Heterocyclic Carbene Stabilized Gold Nanoparticles by Top-Down Synthesis: Performance in Catalysis and Photoacoustic Imaging

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Experimental

General

Solvents and reagents (reagent grade) were commercially available and used without further purification. The DDS@AuNPs and imidazolium salts **NHC1** and **NHC2** were prepared following established procedures as described previously.[1–3] UV-vis absorption spectra were recorded in toluene, MilliQ water, or PBS 1x (pH 7.4) on an Agilent Instruments Cary 60 spectrometer. TEM images were acquired on a JEOL 100 CX. Before measurement, the samples dissolved in water were deposited on a copper/carbon grid with 200 mesh pores. Measurements were taken after the grids had been dried for 12 hours. The micrographs were analyzed using Image J software.[4] ^{1}H NMR spectra were recorded in D_2O on a Bruker AV400 UltraShield (400 MHz). Thermogravimetric analysis was performed on TGA Q5000 from TA INSTRUMENTS under argon atmosphere (gas flow = 25 mL/min; ramp = 10 °C/min to 800 °C). Dynamic light scattering measurements and zeta potentials were measured on a Zetasizer Nano ZS (Malvern Instruments) in MilliQ water. XPS spectra were recorded with an Axis Supra system (Kratos, UK) at a base pressure of 1×10^{-8} Torr, using a monochromatic Al Kα source (1486.6 eV) at 15 mA emission current. A pass energy of 40 eV, a step size of 0.1 eV, and a dwell time of 1000 ms was chosen in hybrid lens mode and with a slot collimation mode, resulting in an estimated spot size of 2 x 1 mm. Binding energies were corrected based on the Au(0) 4f signal at 84 eV. The Au 4f region (100 – 70 eV) was recorded one time after collecting a fast survey spectrum, and before collecting the remaining O 1s, C 1s, Au 4f, N 1s and S 1s regions with the same settings. This procedure was repeated one time to check for beam-induced spectral changes, while the Au 4f region was recorded twice in the second run for high-resolution spectra. The measurement procedure ended with a fast survey spectrum. The built-in charge neutralizer was turned on during all measurements with a 0.45 A current, 1 V bias and 5 V balance. Data quantification was performed using CasaXPS software Version 2.3.22 and a Shirley-type background.[5] Au(0) 4f line shapes were determined based on an internal Au metal reference and used for spectra fitting. Since only ratios within the Au 4f region are reported, area estimations using relative sensitivity factors were not necessary. Fluorescence measurements and kinetics were performed on an Infinite 200 Pro Plate reader with i-control software (Tecan) using a Thermo Fisher Scientific Nunclon 96 Flat Bottom Transparent Polystyrene plate.

Preparation of the NHC@AuNPs via top-down (TD) approach

The corresponding imidazolium salts (0.06 mmol, 1.0 eq.) and KO^{t}Bu (**NHC1** = 0.06 mmol, 1.0 eq., for **NHC2** = 0.12 mmol, 2.0 eq.) were dissolved in dry DMF (3 mL) and stirred for 30 min under argon atmosphere to generate the corresponding carbene NHC species.[6] The freshly synthesized thioether stabilized nanoparticles (DDS@AuNPs) (10 mg) were dissolved in dry hexane (3 mL) and added to the NHC reaction mixture. After stirring overnight, the resulting precipitated NPs were washed with DMF (3 x 2 mL) and toluene (3 x 2 mL) and centrifugated after each washing (2000 rpm, 10 min). The resulting solid NHC@AuNPs (ca. 2 mg) were dissolved in MilliQ water (ca. 10 mL) and purified by dialysis for 48 h, using treated cellulose dialysis membrane (Sigma-Aldrich, D9652) with a molecular weight cut-off of 14 kDa. The MilliQ water for dialysis was changed four times over 48 h. The NPs were then lyophilized to obtain powders.

AuNP1-TD: ^{1}H NMR (400 MHz, D_2O): δ 7.33 (H_b), 4.41 (H_c), 4.29 (H_f), 3.67 (H_e), 3.00 (H_c), 2.95 (H_f), 2.39 (H_d), 2.05 (H_g), 1.93 (H_d), 1.36 (H_h), 0.95 (H_i).

AuNP2-TD: ^{1}H NMR (400 MHz, D_2O): δ 7.54 (H_b), 7.40 (H_c), 7.00 (H_d), 6.18 (H_d), 4.35 (H_e), 2.88 (H_g), 2.30 (H_f).

ICP-MS analysis

The gold content in the NP samples was determined using ICP-MS. First, the NP samples were digested using 1 mL of HNO_3:HCl (1:1, ROTIPURAN®supra 69%, Carl Roth and ROTIPURAN®supra 37%, Carl Roth, respectively). Afterwards, 5 mL of ultrapure water were then added before digesting for 40 min using a microwave digestion system (Multiwave 5000, Anton Paar) at 190 ºC (Rotor 24 HVT50). Samples were then diluted to 25 mL with ultrapure water and measured by ICP-MS (Nexion 5000, PerkinElmer) with autosampler (4DXFast Dxi NexION SC4 DX). SmartTune standard solution (NexiON Setup Solution, Pure Plus, PerkinElmer) containing 200 ng/L of Be, Ce, Fe, In, Li, Mg, Pb, U was used alongside the internal standard solution of Re 10 µg/L (PerkinElmer) in 2% HNO_3 and 2% HCl. Calibration curve of gold metal was performed in 2% HNO_3 and 2% HCl (100 µg/mL gold in 2% HCl, TruQms, PerkinElmer). All measurements were repeated three times.

Cytotoxicity Evaluation

Cell viability assays were carried out using gastric parietal cells (HGT-1, Laboratory of Pathological Anatomy, Nantes, France). The cells were grown in DMEM Glutamax (Gibco), supplemented with 10% Fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (BioReagent) under standard conditions at 37 ºC and 5% CO_2 in a humidified incubator. For cell maintenance, cells were passaged every 3-4 days using trypsin/EDTA (BioReagent) to detach them. To perform the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma), 50,000 cells per well in 200 µl of DMEM were seeded in a transparent 96-well plate. After incubating for 24 h until the wells reached confluence, cells were treated for 24 h with **AuNP1-TD** and **AuNP2-TD** at a range of concentrations (100, 60, 30, 6, 0.6 µg/ml) in DMEM (n=3). After 24 h, the medium was removed, and 100 µl of MTT solution was added to each well. After 10 min of incubation, the supernatant was removed and 150 µL of DMSO was added. The plates were read using an Infinite 200 Pro Plate Reader (550 nm absorbance and 690 nm reference wavelength). The data was processed using GraphPad Prism 6.

Catalytic reduction of 4-nitrophenol

The catalytic reduction of 4-nitrophenol was performed as previously reported with 0.2 mg (Au = 10.9 µg/mL) of **AuNP1-TD** and 0.3 mg (Au = 57.45 µg/mL) of **AuNP2-TD**.[7] In detail, the reduction of 4-nitrophenol was monitored by *in situ* UV-vis absorption spectroscopy with a spectrum measured every 30 seconds between 300–820 nm with an average time of 0.1 s, interval of 1 nm, and scan rate of 600 nm/min, until a plateau was reached. Firstly, 100 µL of a 4-nitrophenol aqueous solution (3 mM) was added to the cuvette containing the TD NHC@AuNPs and 1.9 mL of MilliQ water. This was then followed by the quick addition of a freshly prepared $NaBH_4$ aqueous solution (1 mL, 30 mM) to the cuvette while starting the analysis. The final concentration of $NaBH_4$ (15 mM) in the cuvette was used in excess with respect to the concentration of 4-nitrophenol (0.15 mM) to ensure a *pseudo*-first order reaction.

Catalytic reduction of resazurin

Stock solutions of all four NPs (TD and BU) were prepared (0.6 mg/mL), along with an aqueous stock solution of resazurin (1.5 mM). The solutions were diluted further to obtain a NP concentration of 2 µg/mL and 5 µM of resazurin per well (with a total volume of 90 µL) within a 96-well plate. Prior to each

measurement, 10 µL of NH_2OH (3 mM) was added to give a final volume of 100 µL and concentration of 0.3 mM per well (final concentrations of NPs = 1.8 µg/mL and resazurin = 4.5 µM). The final content of Au in each well was as follows: **AuNP1-TD** = 0.196 µg/mL, **AuNP2-TD** = 0.689 µg/mL, **AuNP1-BU** = 0.227 µg/mL, and **AuNP2-BU** = 0.522 µg/mL. The reactions were carried out at room temperature and followed by fluorescence spectroscopy with an excitation wavelength of 532 nm (excitation bandwidth 9 nm) and an emission wavelength of 584 nm (emission wavelength 20 nm). With 50 flashes and an integration time of 20 µs, the emission spectra were recorded over 6 h with 3 repeats for each NP. The calibration curve of the product (resorufin) was performed between 0 and 4.5 µM concentration.

Catalytic oxidation of 3,3,5,5-tetramethylbenzidine (*TMB)*

The procedure for the oxidation of TMB in the presence of H_2O_2 was adapted from the literature[8] and monitored *in situ* by UV-vis absorption spectroscopy between 800-300 nm with a scan rate of 600 nm/min. The experiments were performed at 37 °C with stirring at 120 rpm for 3 h with a scan taken every 15 min. A baseline scan containing the NHC@AuNPs, H_2O_2 and acetate buffer was performed before each measurement. For the experiments, 15 µL (**AuNP1-TD**), 4.3 µL (**AuNP2-TD**) or 30 µL (BU AuNPs) of an aqueous solution of AuNPs (0.6 mg/mL) was first added to the cuvette along with 200 µL of H_2O_2 (20 mM) and acetate buffer (20 mM, pH 4.0) to reach an overall volume of 1830 µL. A freshly prepared solution of TMB in DMSO (100 µL, 2.5 mM) was quickly added to initiate the measurement. The final concentration of TMB and H_2O_2 in the cuvette was 0.13 mM and 2 mM, respectively. The final gold content in the cuvette for each NP was as follows: 0.05 µg/mL for **AuNP1-TD** and **AuNP2-TD**, 0.12 µg/mL for **AuNP1-BU** and **AuNP2-BU**. The same experiments were repeated with **AuNHC1** (14.8 µL) and **AuNHC2** (13.8 µL) to achieve an overall gold content of 0.12 µg/mL in each cuvette. Uncatalyzed reactions were followed as control in the absence of gold sources.

The reaction was followed using the absorption of the product at 652 nm, the concentration at each time point was calculated using the reported extinction coefficient (3.9×10^{-4} $M^{-1}cm^{-1}$). The linear interval of the reaction progression (0-3.5 min) was considered to determine the initial reaction rate. This was corrected by extracting the uncatalyzed reaction rate and used to calculate the TOF considering the amount of Au in the catalytic system.

Optoacoustic Tomography

Tissue phantom

Several testing samples were prepared containing gold nanoparticles (AuNPs) at concentrations of 0.3, 0.7, and 1.1 mg/mL in MilliQ H_2O. A tissue-mimicking phantom was fabricated to measure the photoacoustic response of each sample. This phantom consisted of an agar cylinder (representing the skin) with a diameter of 19 mm, housing two straws (representing vessels) embedded within it. The agar phantoms were prepared by heating a solution of 1.5% w/v agar (05039, Fluka) in water, and then adding 2.1% v/v Intralipid (I141, Sigma-Aldrich). The two straws were vertically positioned in the center of an open 20 mL syringe (with the injection end removed) using a support. The Agar solution is then poured into the assembly and left to solidify. The phantom had a scattering coefficient of ~5 cm^{-1} due to the intralipid and the absorption coefficient was assumed to be negligible. For each measurement, one of the straws was filled with the nanoparticle solution, while the other was filled with MilliQ H_2O as reference. These straws were placed symmetrically with respect to the center of the measuring chamber, which minimizes differences in laser fluence between the two. This setup allows normalization of the AuNP signal relative to the water reference. The acoustic coupling is identical for both straws due to the uniform phantom material, and the quantity of sample in each straw is the same. Additionally, any wavelength-dependent fluence differences are corrected by the MSOT system's internal power meter, which automatically scales the data across wavelengths. The setup can be visualized in Figure S12.

Photoacoustic tomography

The measurements were conducted using a photoacoustic (PA) imaging system Multi-Spectral Optoacoustic Tomography (MSOT inVision 256-TF, iThera Medical). This system employs a tunable optical parametric oscillator pumped by an Nd:YAG laser. It generates excitation pulses of 9 ns, in the near-infrared spectrum, with a repetition rate of 10 Hz and a peak pulse energy of 80 mJ. A fiber bundle provides homogenous ring-shaped line illumination around the phantom. The photoacoustic signals are captured by an array of 256 toroidally focused piezoelectric transducers with a center frequency of 5 MHz and a bandwidth of 60%. These transducers are arranged in a concave configuration, covering an angular range

of 270° and a radius of curvature of 4 cm. The peak fluence occurs between 700–900 nm (~20 mJ/cm²), while at the extreme edges (680 nm and 980 nm), the fluence drops to ~12.8 mJ/cm².

According to the ANSI Z136.1 standard (Table 5b for single pulse skin exposure) and the IEC 60825-1 standard (Table 8 and correction factor C4, Figure 6), for pulse durations $t < 10^{-7}$ s, the single-pulse MPE for the skin is given in terms of radiant exposure (H) by the following formula:

$H = 20 \cdot C_A$ mJ/cm^2

Where:

- H is the Radiant Exposure MPE (J/cm^2).
- C_A is the wavelength-dependent correction factor.
    - For wavelengths λ between 400 – 700nm: $C_A = 1.0$
    - For wavelengths λ between 700 – 1050nm: $C_A = 10^{0.002 \cdot (\lambda - 700)}$

MPE Calculation for MSOT

1. For 660 nm:
    - $C_A = 1.0$
    - MPE $= 20 \cdot 1.0$ mJ/cm^2 $= 20$ mJ/cm^2
2. For 980 nm:
    - $C_A = 10^{0.002 \cdot (980 - 700)} = 10^{0.002 \cdot 280} = 10^{0.56}$
    - $C_A \approx 3.63$
    - MPE ≈ 20 mJ/cm$^2 \cdot 3.63 \approx 72.6$ mJ/cm^2

For each sample, five specific positions were selected, spaced 1 millimeter apart along the long axis, thus covering a total length of 4 mm. For each position, the sample was illuminated with 65 different wavelengths in a range from 660 to 980 nm, 5 nm spaced. The PA signals were acquired with 16 averages to increase the SNR.

Figures

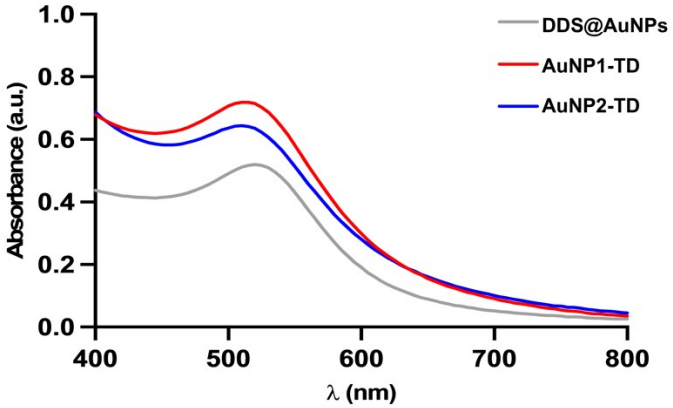

Figure S1. *UV-vis absorption spectra overlay of DDS@AuNPs (grey) in toluene, and* ***AuNP1-TD*** *(red) and* ***AuNP2-TD*** *(blue) in MilliQ water.*

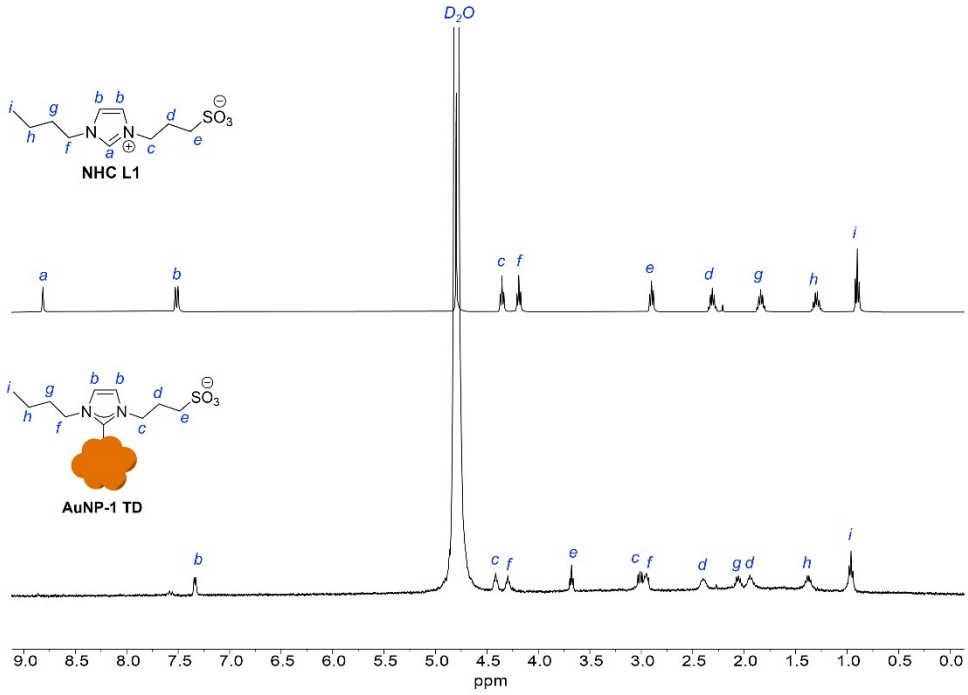

Figure S2. *^{1}H NMR spectra in D_2O of the imidazolium salt (****NHC1****, top) and* ***AuNP1-TD*** *(bottom). The peaks are assigned to the protons in both structures.*

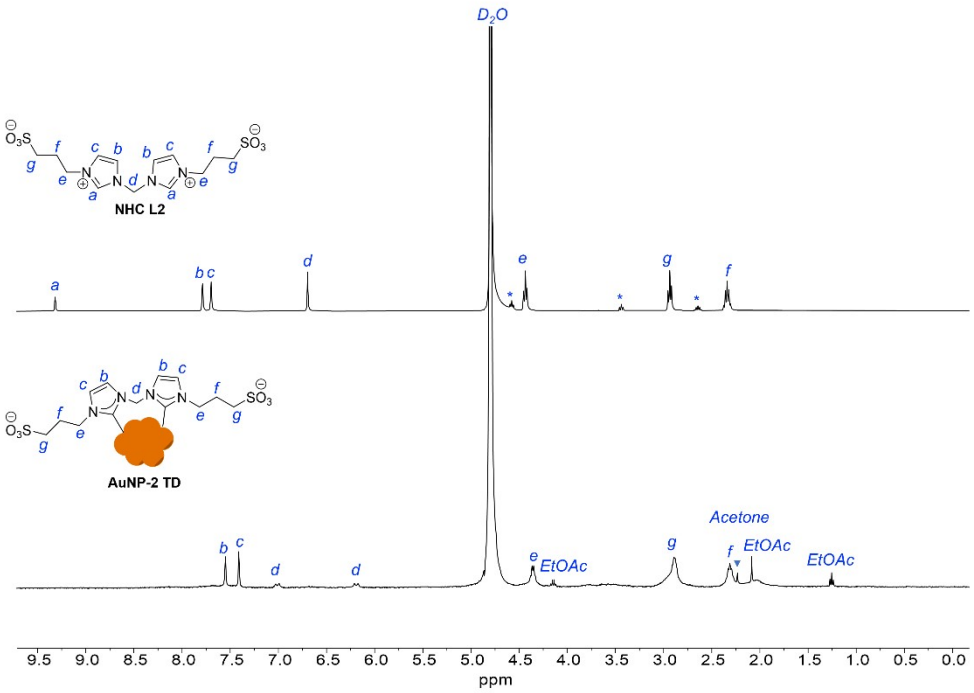

Figure S3. *^{1}H NMR spectra in D_2O of the imidazolium salt (**NHC2**, top) and **AuNP2-TD** (bottom). The peaks are assigned to the protons in both structures. *Residual 1,3-propane sultone.*

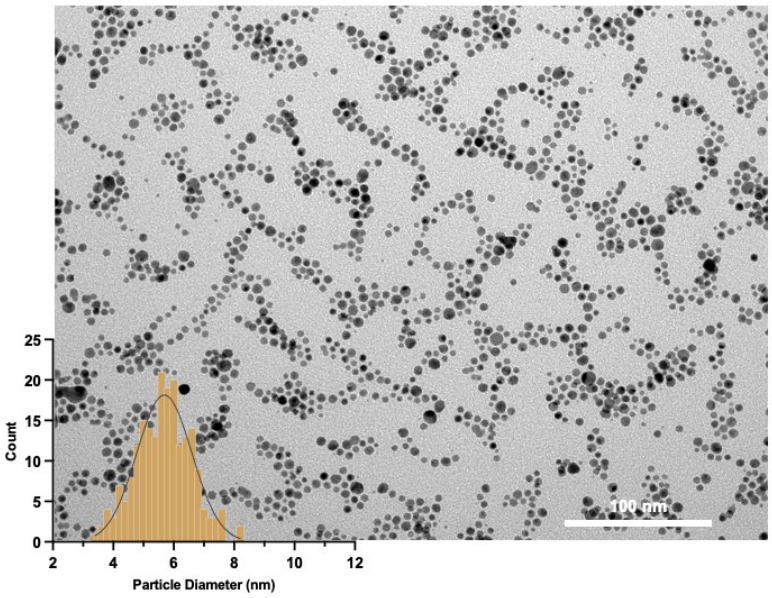

Figure S4. *TEM representative image of the DDS@AuNPs with size histogram. Average particle size measured = 5.7 ± 0.9 nm.*

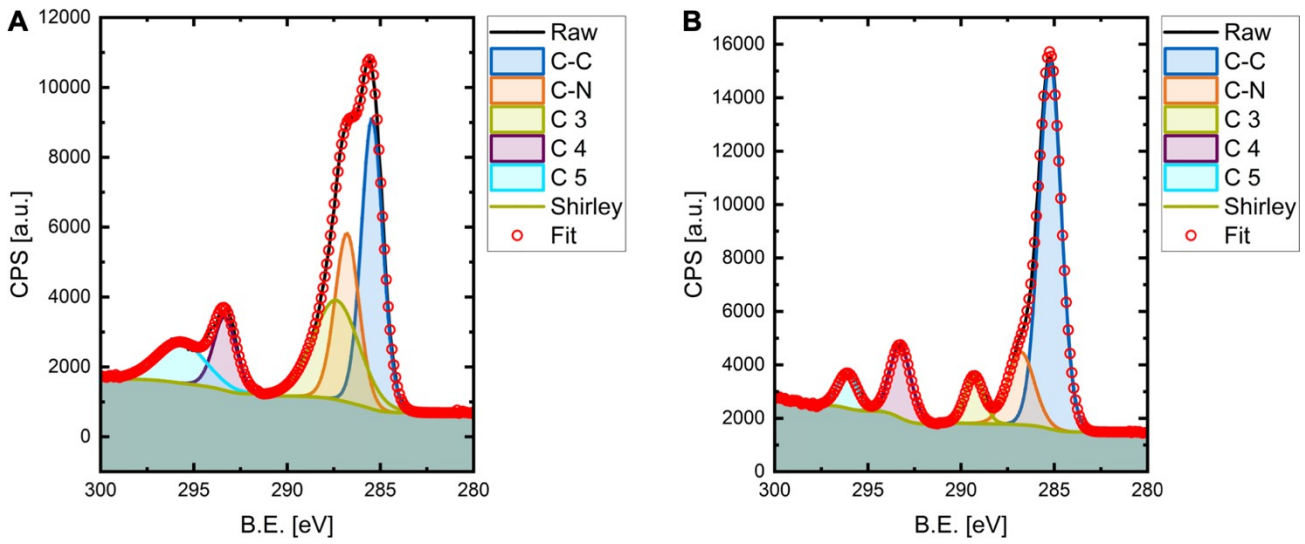

Figure S5. *XPS experimental spectra and fitting of C 1s for* ***A. AuNP1-TD*** *and* ***B. AuNP2-TD*** *with deconvolution of the peaks.*

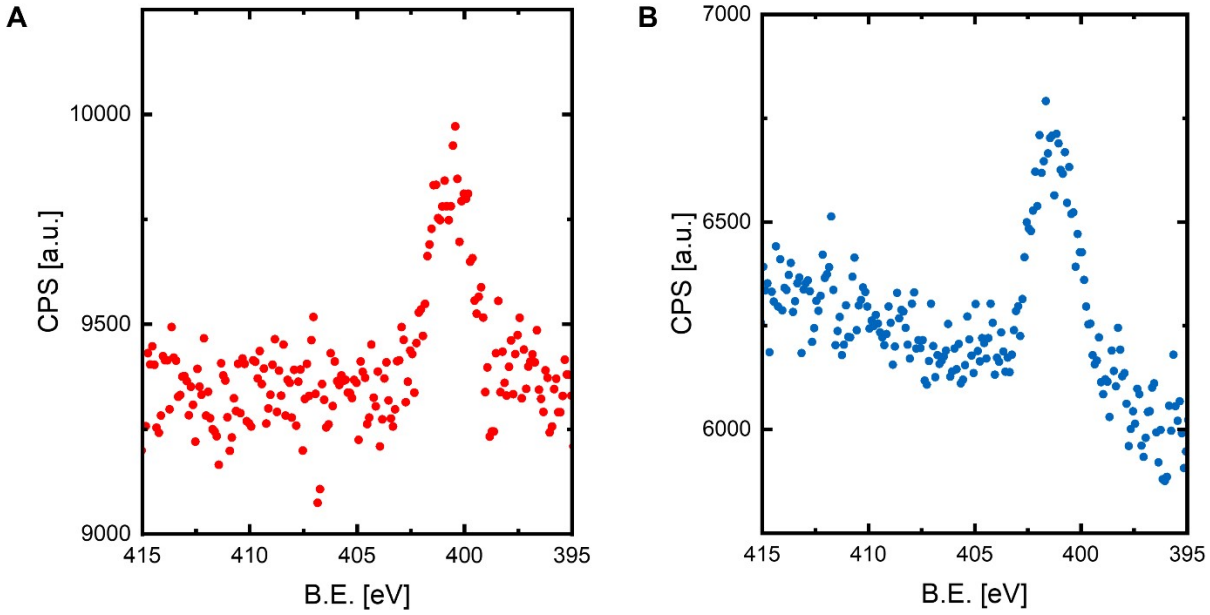

Figure S6. *XPS experimental spectra of N 1s for* ***A. AuNP1-TD*** *and* ***B. AuNP2-TD****.*

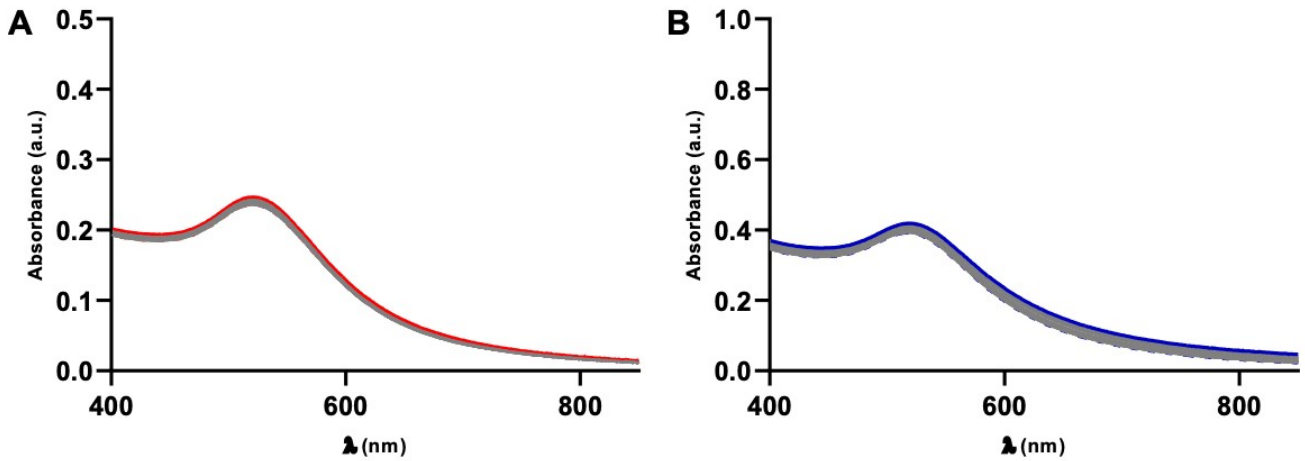

Figure S7. *UV-vis absorption spectra of* ***A***. ***AuNP1-TD*** *and* ***B***. ***AuNP2-TD*** *in MilliQ water over time (16 h) at 37 ºC.*

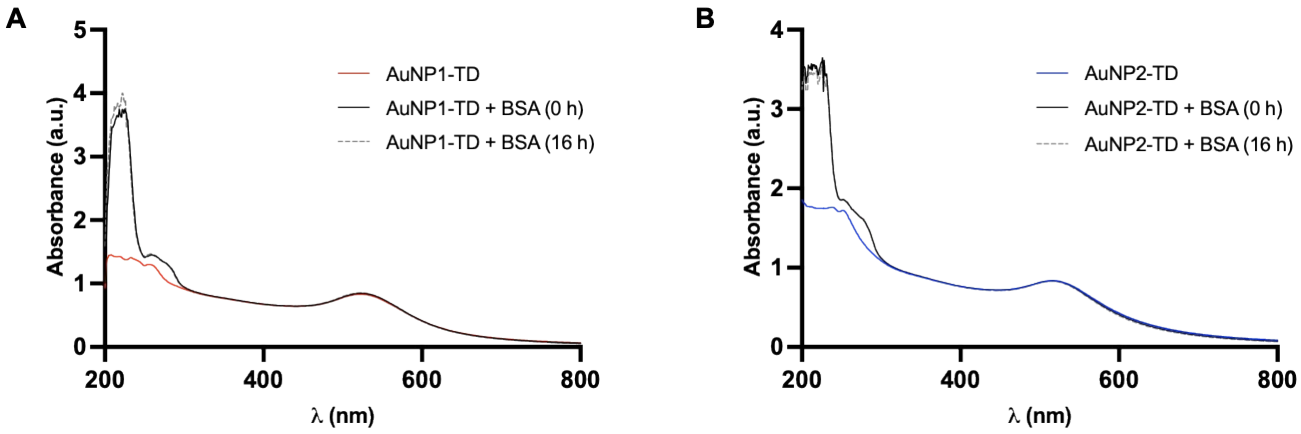

Figure S8. *UV-vis absorption spectra of* ***A***. ***AuNP1-TD*** *and* ***B***. ***AuNP2-TD*** *in PBS 1x (pH 7.4) in the presence of BSA (17 mM) at time 0 h and after 16 h at 37 ºC.*

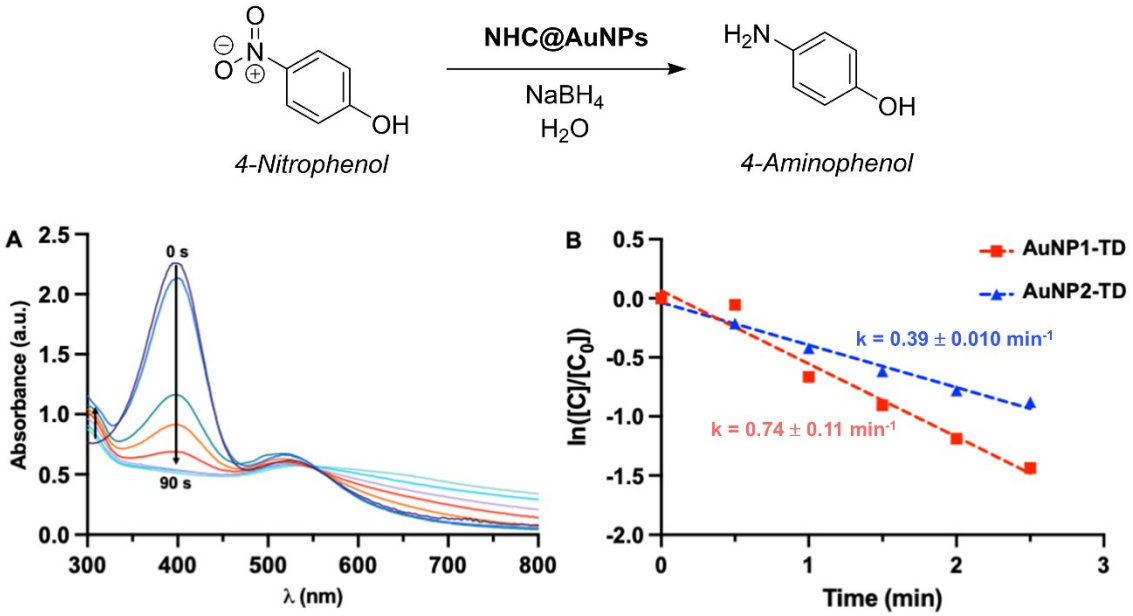

Figure S9. A*. UV-vis absorption kinetic studies for the reduction of 4-nitrophenol (4NP) catalyzed by NHC@AuNPs in water at r.t.* ***B****. Plot of ln([C]/[C$_0$]) vs. time for* ***AuNP1-TD*** *(red) and* ***AuNP2-TD*** *(blue) showing first-order kinetics and calculated rate constants. *Obtained as a mean value from three independent experiments with the corresponding standard deviation.*

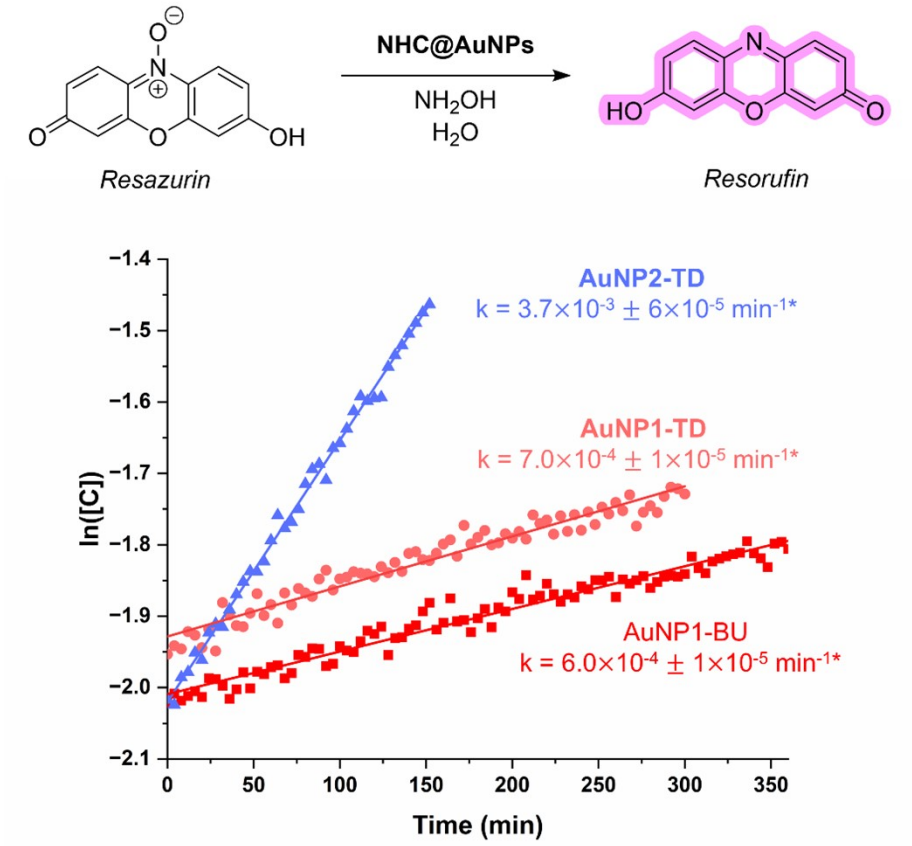

Figure S10. *Fluorescence emission studies for the reduction of resazurin to resorufin catalyzed by NHC@AuNPs with NH_2OH in aqueous solution at room temperature. Plots of ln([C]) vs. time with* ***AuNP1-TD***, ***AuNP2-TD***, *and* ***AuNP1-BU*** *with calculated rate constants. *Obtained as a mean value from three independent experiments with the corresponding standard deviation.*

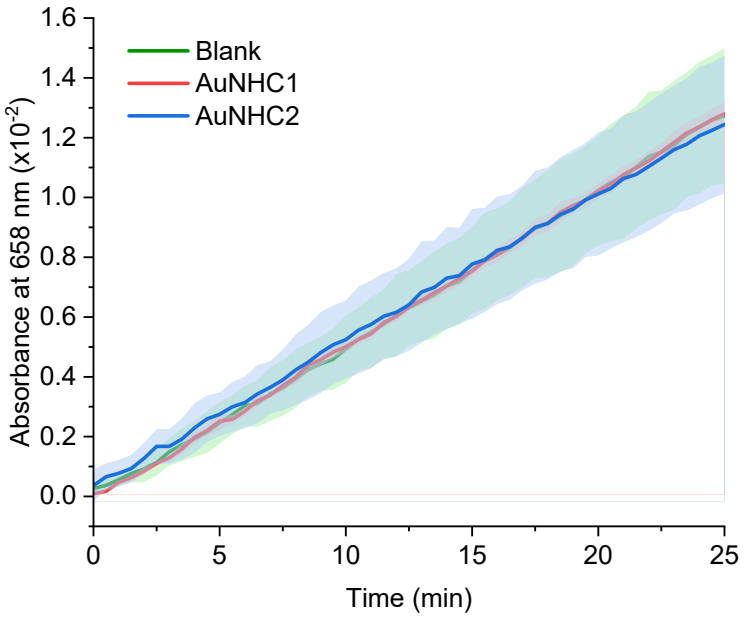

***Figure S11**. UV-vis absorption kinetics at 658 nm for the oxidation of TMB in the presence of H_2O_2 with **AuNHC1** (red line, Au = 0.12 µg/mL), **AuNHC2** (blue line, Au = 0.12 µg/mL) and no catalyst (green line, blank) over 25 min at 37 ℃ in NaOAc buffer (pH 4.0). Lines corresponding to an average of two experiments with standard deviation represented by splice.*

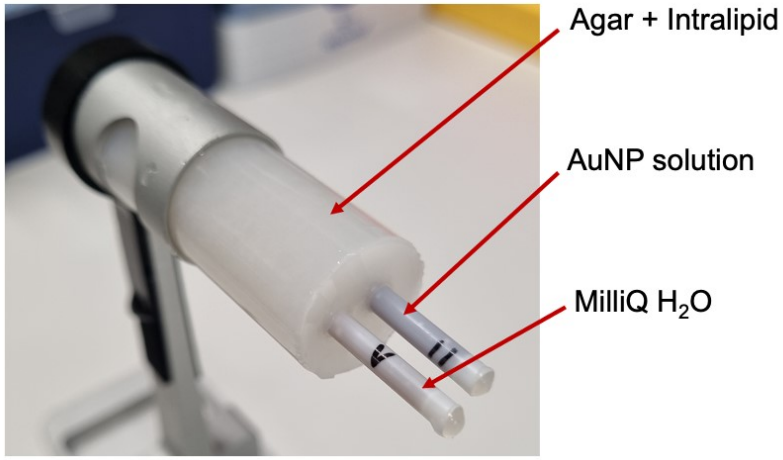

***Figure S12.** Image of the tissue-mimicking phantom setup mounted on the measurement holder.*

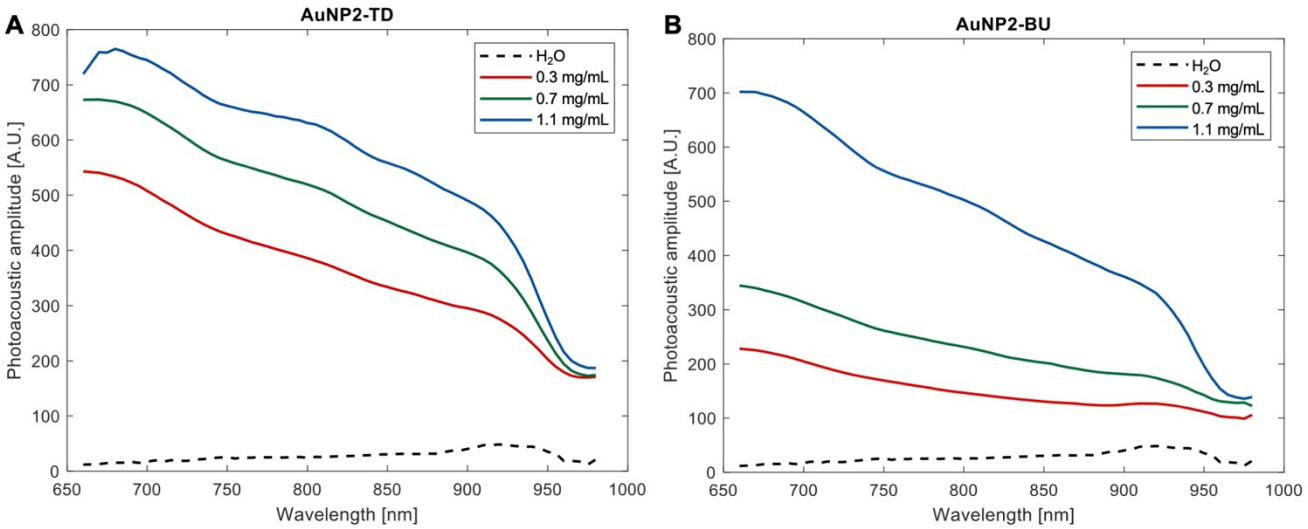

Figure S13. *Average photoacoustic spectra of* ***A***. ***AuNP2-TD*** *and* ***B***. ***AuNP2-BU*** *sample cross-section at various NP concentrations from 660-980 nm.*

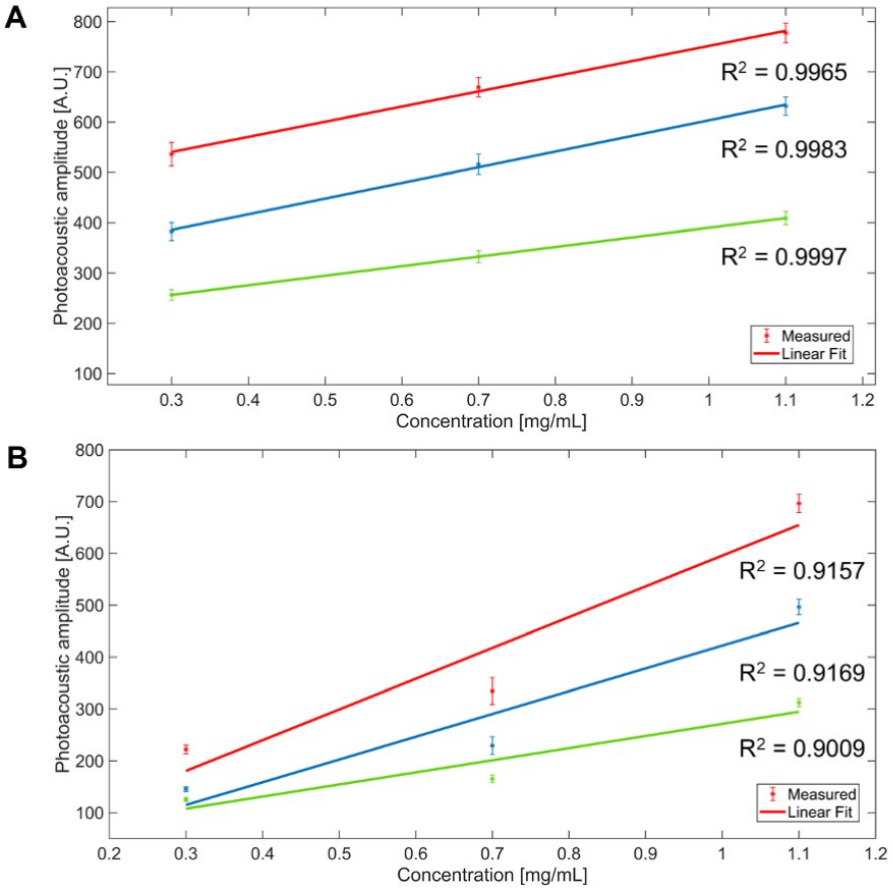

***Figure S14**. Photoacoustic response of the sample cross sections at varying AuNP concentration **A**. **AuNP2-TD** and **B**. **AuNP2-BU** at three different wavelengths: 675 nm (red), 805 nm (blue) and 930 nm (green). The straight lines represent the linear fit of the measured values with R^2 values for each line displayed.*

Tables

Table S1. XPS data (BE and composition) for **AuNP1-TD** and **AuNP2-TD.**

	AuNP1-TD			AuNP2-TD		
Peak	**BE (eV)**	**%At Conc.**	**Area (a.u.)**	**BE (eV)**	**%At Conc.**	**Area (a.u.)**
Au 4f 7/2 (Au(0))	84.00	97.88	69694.73	84.00	96.12	7820.09
Au4f 7/2 (Au(I))	85.54	2.12	1510.35	85.8	3.88	314.9
C 1s (C-C)	285.44	33.5		285.22	61.61	
C 1s (C-N)	286.79	21.89		286.78	14.63	
C 3	287.36	24.6		289.29	6.73	
C 4	293.33	9.12		293.25	11.66	
C 5	295.67	10.89		296.08	5.36	
S 2p	168.69		2040.43	168.45		2019.4

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