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Supplementary file 1

Photothermal Therapy Using Ag/TiO_2 Core-Shell Nanoparticles Against Breast Cancer Cell Line: an I*n Vitro* and I*n Vivo* Study

Experimental Section:

Materials: In the current study, Ti plate with purity 99.9%, phosphate buffered saline (PBS), Ag nanoparticles with dimension of (20-30) nm, Polyvinyl alcohol (PVA) $(C_2H_4O)_n$ from Barcelona Espana (Didactic), Distilled water (D.W.), 96 microtiter well plate, millipore filter, 0.22, tissue culture flasks were used.

Synthesis of TiO_2:

Ti plate with purity 99.99% (2.5 x 2.5 cm^2) was used as the target to be ablated by the fiber laser. The target was placed at the bottom of 40 ml volume beaker filled once with 10 ml PBS and with D.W. once [1,2]. The solution above the target was by 1 cm. A 2 mg of PVA was solvated in the D.W. using magnetic stirrer and heating plate at temperature of 80 ºC. The specifications of the fiber laser are detailed in table S1

Table S1. The specification of the laser used to ablate the target.

Laser Type	Fiber laser (Raycus, China)
Wavelength (nm)	1060-1070
Pulse Energy (mJ)	1
Pulse duration (ns)	120
Lens focal length (mm)	254
Spot diameter (mm)	0.5
Pulse repetition frequency (kHz)	20
Used power (Watts)	10
Laser Intensity (W/cm^2)	5.10 x 10^5
Scanning speed (mm/s)	100
Ablation time (min)	15

The as-prepared solution has a faint yellow color for Ag/TiO_2 colloid (at concentration 266 µg/ml) and for the PVA/Ag/TiO_2 colloid (at concentration 400 µg/ml) (see figure S1).

Characterization:

A 3 ml of both Ag/TiO_2 and $PVA/Ag/TiO_2$ was poured inside a cuvette of a double-beam Schimadzu spectrophotometer (190-1100 nm) to perform the Uv-Visible spectrum. The topography was recorded using High Resolution Transmission Electron Microscope (HRTEM) (Zeiss Leo 912), Ab-100 KV. In which a cleaned, glow-discharged base chip is placed on top of an O-ring. Sample (1 ml) was applied to the base chip. Then, a cleaned top chip was placed on top of the base chip in which the sample was placed, and prior into the TEM for data collection it was sealed by a metal face-plate [3].

***In Vitro* study:**

Cell lines and cell maintenance. The Iraqi human breast cancer cell line AMJ13 [4] and the normal control cell line model indicated as REF (the Iraqi rat embryonic fibroblast) were used. Both cell lines were locally established, provided and authenticated at the Experimental Therapy Department/ICCMGR/ Mustansiriyah University, and were cultured at 37ºC with 5% CO_2 and maintained in growth medium RPMI-640 (U.S. Biological USA) with streptomycin (100 µg/ ml), and penicillin (100 units/ml) and with 10% Fetal Bovine Serum (FBS) (Capricorn-scientific, Germany).

Cells were harvested after trypsinized with trypsin-EDTA (T.V.) (Capricorn-Scientific, Germany) in order to be used in this study. After the cellular growth reached an adherent confluent monolayer, RPMI media was decanted off, and cells were harvested by adding trypsin-versene (as 2-3 ml) (U.S. Biological USA), the flask was racked gently. After 30 seconds trypsin-versene was poured off and added maintenance media (RPMI) then the cells were incubated at 5% CO_2 and 37 ºC until cells detached [5]. Then the cells were ready to the next experiments.

Cytotoxicity assay. In sterile conditions, Ag/TiO_2 and PVA/ Ag/TiO_2 materials were prepared as 1:1 v/v in PBS and as (1:10) v/v in D.W., and both mixtures were filtered by 0.45 sterile syringe filter (0.45 µm pore size) to be used in the cytotoxicity assay.

First of all, to conduct seeding stage, sterile 96-wells microtitration plates were used by added $1x10^4$ cells of AMJ13 or REF in 200 µl of growth media and 10% FBS to each well and sealed by self-adhesive film. Then, the plates were incubated overnight at 5% CO_2 and and 37 ºC. After that for exposure stage, the medium of each well was removed, then five dilutions of the Ag/TiO_2 colloid and control (without Ag/TiO_2) in RPMI-640 medium supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml) and without FBS as serum free media SFM were added to the wells.

Finally, and with sterile conditions the supernatants of each well were discarded, and crystal violate (C.V) stain (100 µl) were added and incubated at 5% CO_2 and 37 ºC for 20 min. After that, tap water was used to wash cells and left to dry for a while. Finally, optical density (OD) was determined at a wavelength of 492 nm with a Fluorometer (BMG LABTECH, Germany) [6 7]. Then, microscope images of both treated and control cells were captured by Leica inverted light microscope (Germany). According to the *in vitro* study, the results showed no effect of the PVA/Ag/TiO_2 on both normal and cancer cell lines which indicated the biosafety of this material, therefore only the Ag/TiO_2 was chosen for *in vivo* study.

Figure S 1. The as-prepared TiO_2 using PLAL

Characterization: The TEM image of the as-prepared Ag/TiO_2 nanoparticle core-shell and PVA/Ag/TiO_2, respectively, was studied (figure S 2). From the TEM image it was obvious that the particles were spherical but not homogeneous in its size (figure S2-a) for the Ag/TiO_2 nanoparticle core-shell. However, for the PVA/Ag/TiO_2 the TEM image has shown that the NPs were homogeneous in its shape. The presence of the PVA as a stabilizer resulted in a reduction in the size of the as-prepared NPs as depicted in figure S2-b. The dark spheres represented the Ag NPs while the bright spheres demonstrated the TiO_2 NPs (figure S2-a), as core and shell respectively as indicated by the arrow. This suggested that some core-shell particles have been formed. While the results showed the diameter distribution of the as prepared Ag/TiO_2 NP colloid (figure S2- c and d). From this result it is clear that majority of the size of the NPs falls in the range of 10 nm for the PBS Ag/TiO2 nanoparticle core-shell. The PVA/Ag/TiO_2, demonstrated the Ag NP dark spheres were homogeneous in their shape (figure S2- b). While the TiO_2 NP were not obvious in the TEM image. The bright spots around Ag NP represented that these NP were coated by PVA, and the coating of PVA to Ag NP has reduced its size diameter to less than 7.9 nm (figure S2- d).

The Uv-Visible spectrum of the as prepared Ag/TiO_2 nanoparicle core-shell colloid in both PBS and D.W. was illustrated in Figure S3. In figure S3, we can see the material has absorption at the II-IR window 1060 nm despite the intensity of the uv spectrum at the specified wavelength was not high but it was enough to raise the temperature of the solution above 40º for PBS Ag/TiO_2 nanoparticle core-shell upon laser exposure.

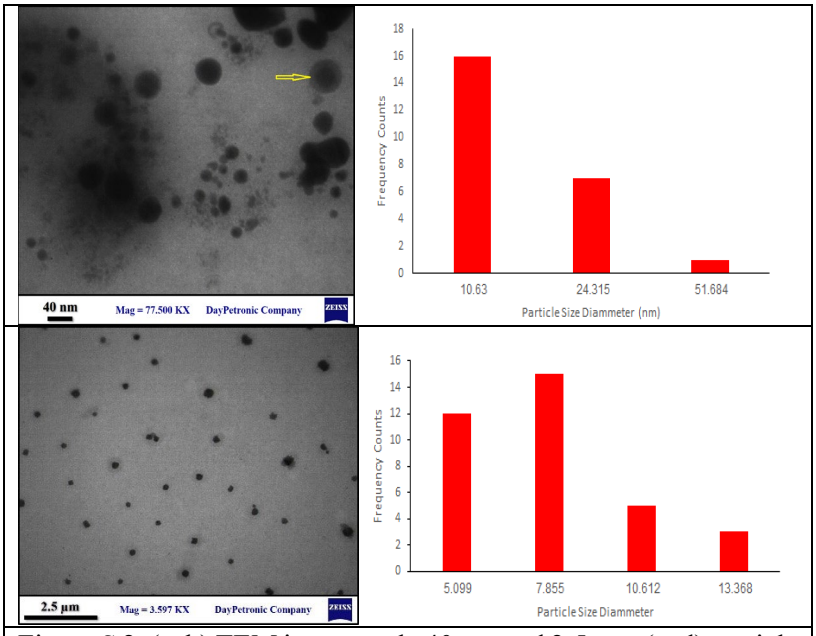


Figure S 2. (a, b) TEM image scale 40 nm and 2.5 µm, (c, d) particle size frequency with particle size diameter for Ag/TiO_2 and $PVA/Ag/TiO_2$ respectively.

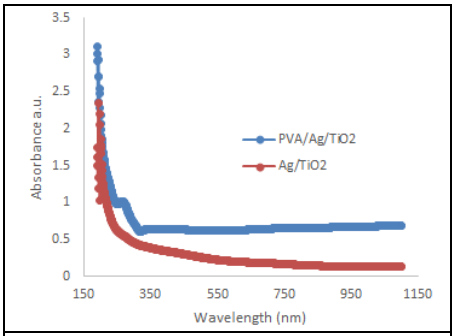


Figure S 3. The Uv-Vis spectrum of the as-prepared colloids

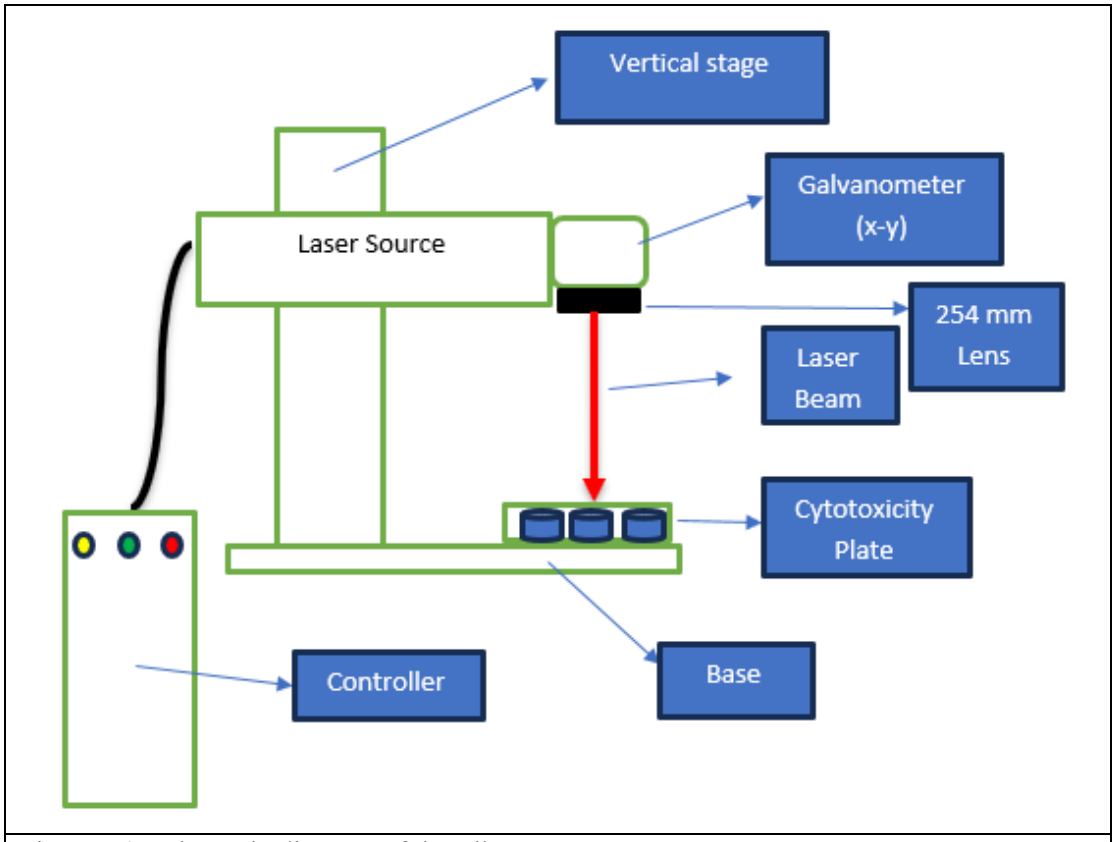


Figure S4. Schematic diagram of the Fiber Laser

Table S2. The laser parameters used in the PTT

Laser Power (Watts)	Frequency (kHz)	Pulse Duration (ns)	Laser spot diameter at the focal point (µm)	Exposure Time (min)	Laser Wavelength (µm)	Scanning speed (mm)
5	20	120	0.5	5	1064	100

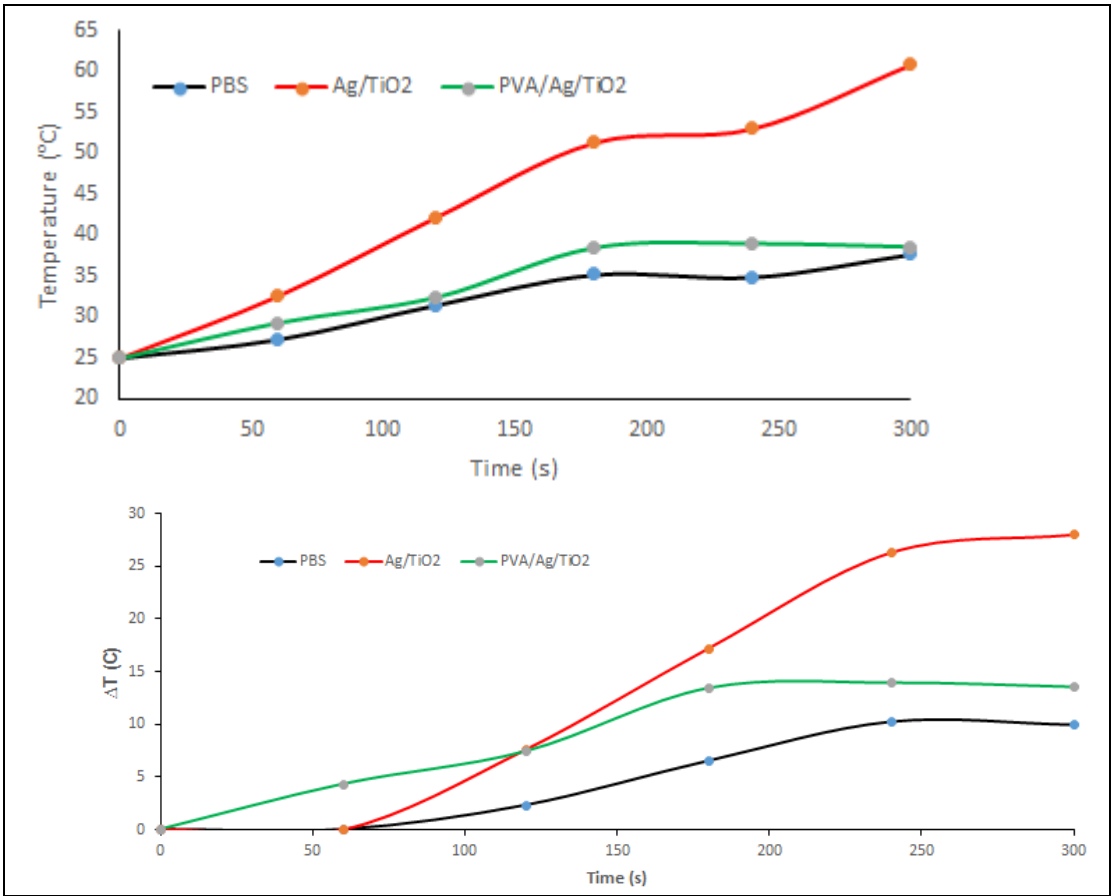
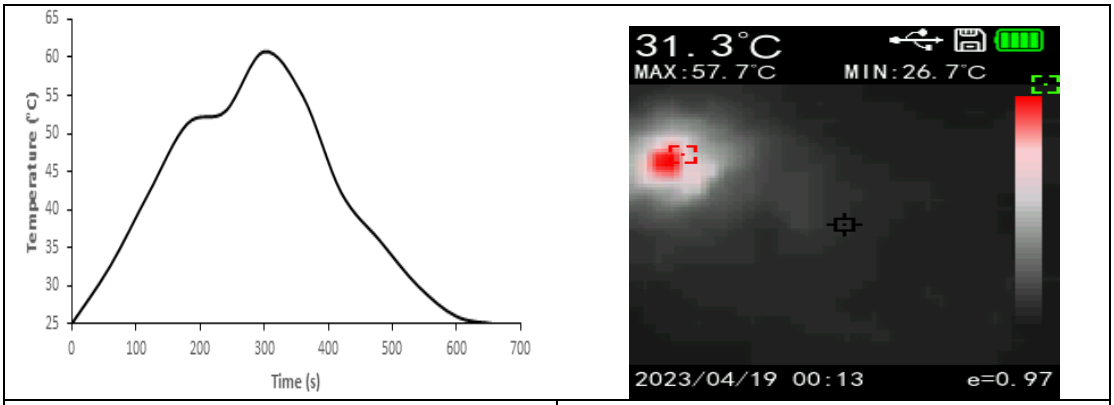
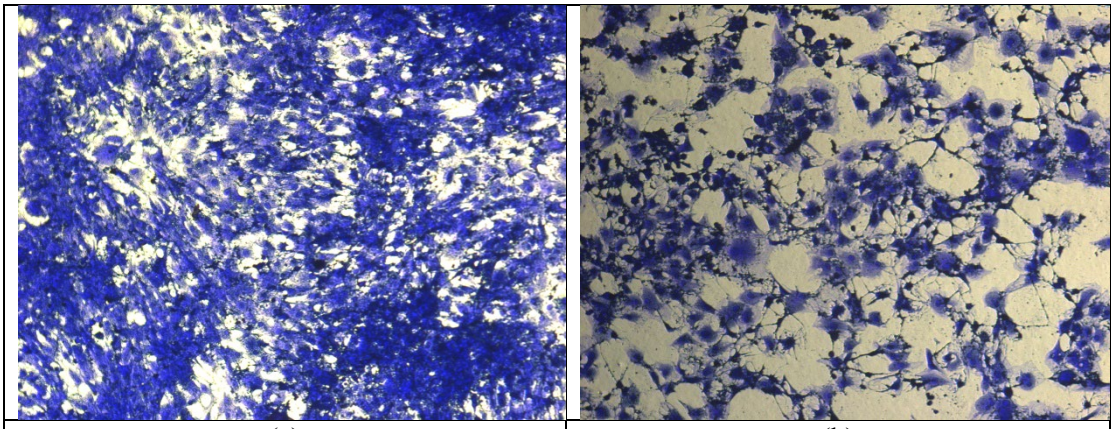


Figure S 5. (a) Temperature profile (b) Temperature changes of the PBS, Ag/TiO_2 NPs (266 ppm) and PVA/Ag/TiO_2 with laser irradiation of power 5W, 5 min.



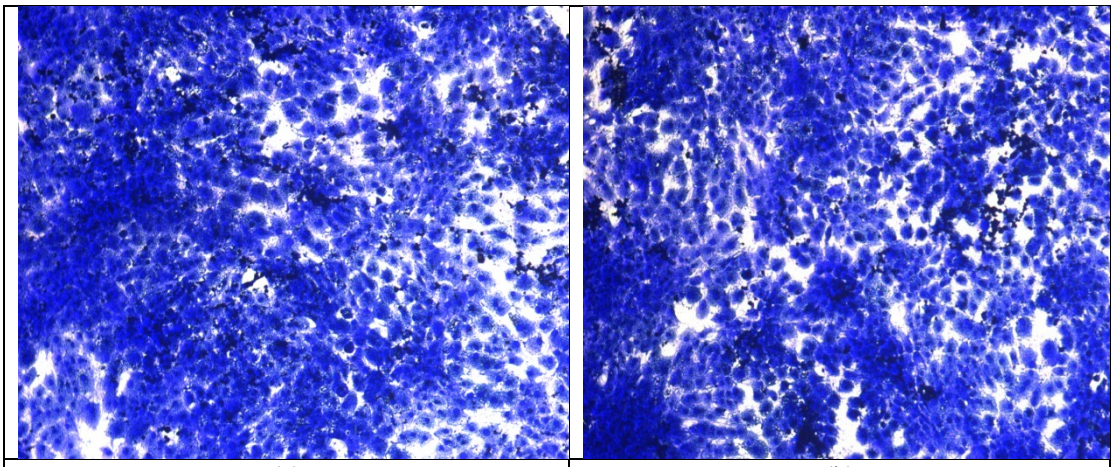
(a) (b)

Figure S 6. (a) Temperature profile of Ag/TiO_2 NPs (Laser On-Off) at (266 ppm) (b) Thermal images of Ag/TiO_2 under 1060 nm laser irradiation at a concentration of (266 ppm)



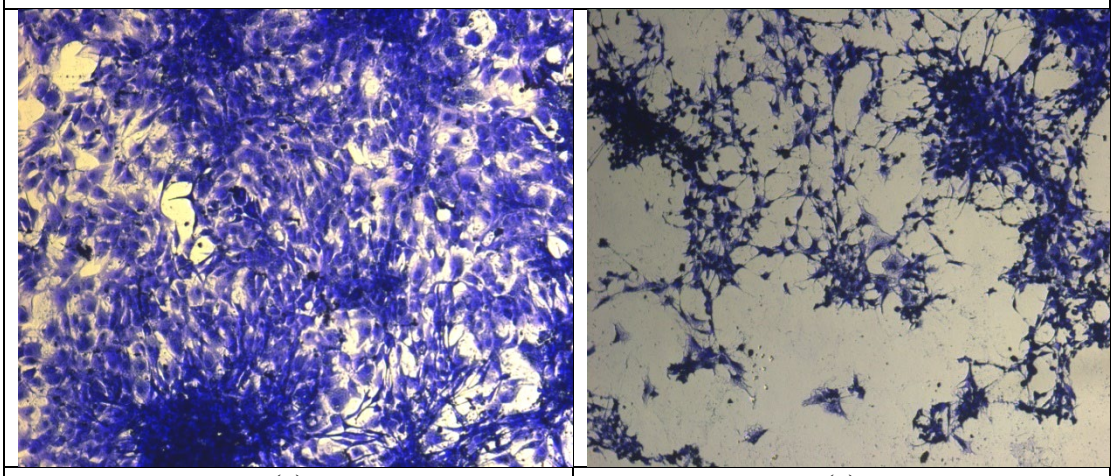
(a) (b)

Figure S 7. Microscope images of cytotoxicity of breast cancer cell line AMJ13 after exposed to Ag/TiO_2 (for 24 hrs.) (a) control and (b) 266 µg/ml. that showed a cytotoxic effect as shown by the inverted microscope at 10X.



(a) (b)

Figure S 8. Microscope images of cytotoxicity of normal cell line REF after been exposed to Ag/TiO_2 (for 24 hrs). (a) control and (b) 266 µg/ml. that showed no cytotoxic effect as shown by the inverted microscope at 10X.



(a) (a)

Figure S 9. Microscope images of Cell Viability of AMJ13 without and with Ag/TiO_2 using laser power of 5 W, irradiation time of 5 Min and Incubation Time 24 hrs., (a) control (b) 266 µg/ml that showed a cytotoxic effect as shown by the inverted microscope at 10X.

Measurement of the photothermal effect:

To evaluate the photothermal effect of Ag/TiO_2 NPs, 200 µL of as-prepared with 266 µg/ml was irradiated with 1064 nm fiber laser (Raycus, China). The photothermal conversion efficiency of the nanoparticle (NP) was calculated according to the following the equation:

$$\eta = \frac{hS\,(T_{max,PBS} - T_{surr}) - Q_{diss}}{I(1-10^{-A_{1064}})} \quad \text{……………………………………(1)}$$

Where $I(1-10^{-A_{1064}})$ is the nanoparticle (NP) input energy

I was referred the laser incident power (W), A was the absorbance of the nanoparticles at the wavelength of the laser (λ=1064 nm)

hS could be calculated when the laser was turned off

Q_{diss}= was the heat dissipated by the container

h was the heat transfer coefficient; S was the surface area of the container which can be calculated according to the equation:

$$\tau_s = \frac{m_D C_D}{hS} \quad \text{…………………………………………………………(2)}$$

Where m_D and C_D were the mass and the heat capacity of the PBS solvent

τ_s was the system time constant which can be calculated when the laser was turned off

$$\tau = \frac{-t}{\ln(\theta)} \quad \text{…………………………………………………………(3)}$$

Where $\theta = \frac{(T - T_{surr})}{T_{max} - T_{surr})}$ ……………………………………………..(4)

Equation (1) can be calculated by substituting equations (2,3,4), so the photothermal conversion efficiency calculated was 16.1%, τ_s was the slope of the fitted curve of equation 3 which had a value of 163.08 (s). Figure S 9

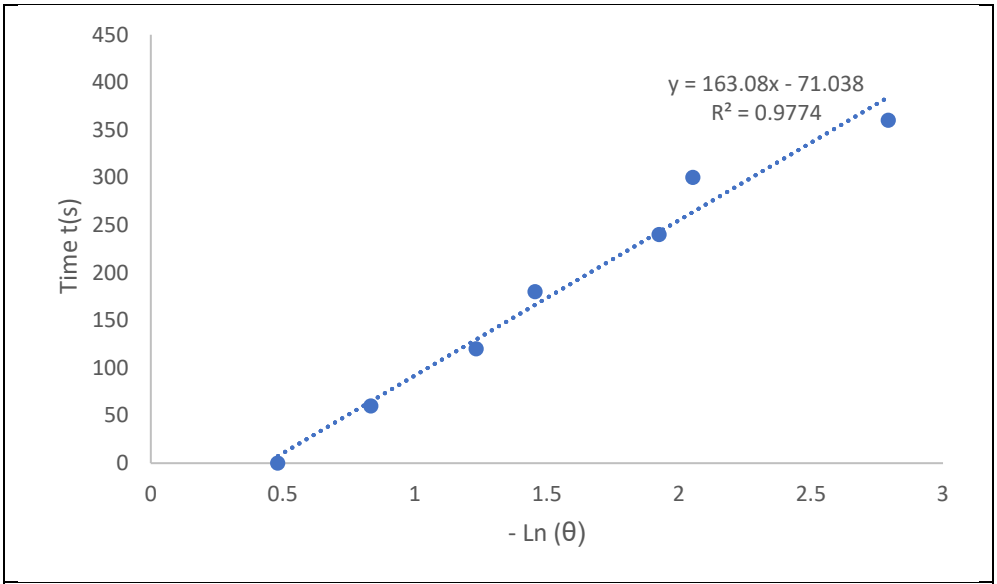


Figure S 10. The time constant τ_s of the heat transfer from the system

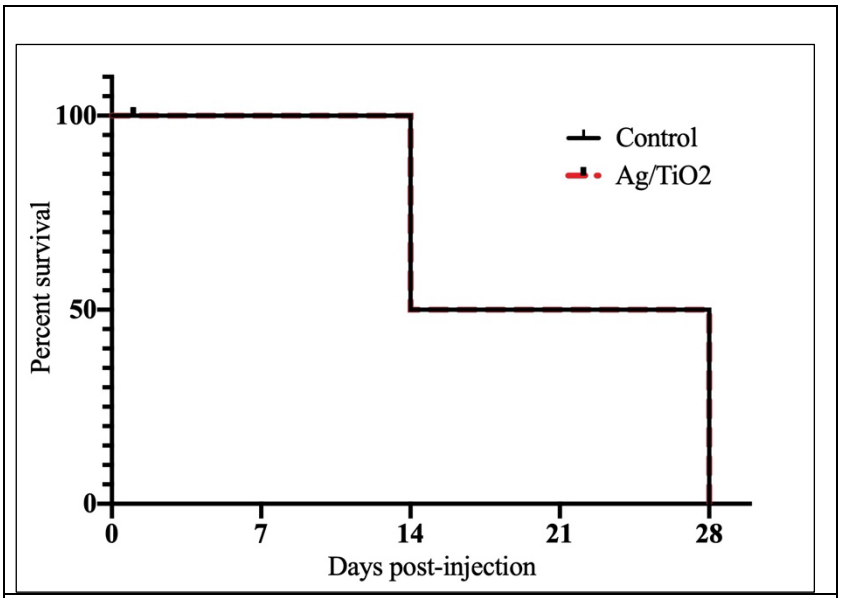


Figure S 11. Survival profile of male albino mice. Mice were injected intraperitoneally with either a single dose of PBS (control) or Ag/TiO_2 at 266 mg/ml (500 µl/mouse) and half mice were sacrificed at 14 days, while the remaining were sacrificed at 28 days post-injection have showed 100% of survival rate.[8,9]

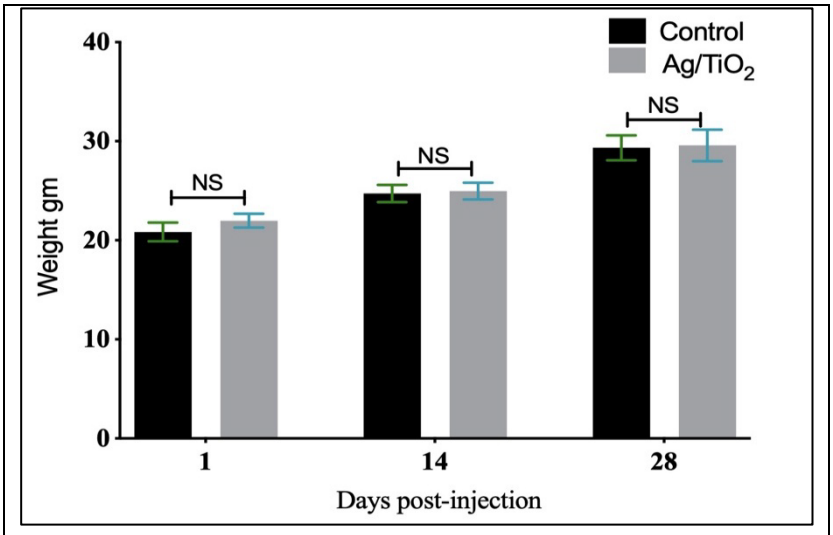


Figure S 12. Body weight profiles of male albino mice injected intraperitoneally with either a single dose of PBS (control) or Ag/TiO_2 at 266 mg/ml (500 µl/mouse) and half mice were sacrificed at 14 days and the remaining were sacrificed at 28 days post-injection have showed no significant differences in the body weight between control and treated group throughout the study.

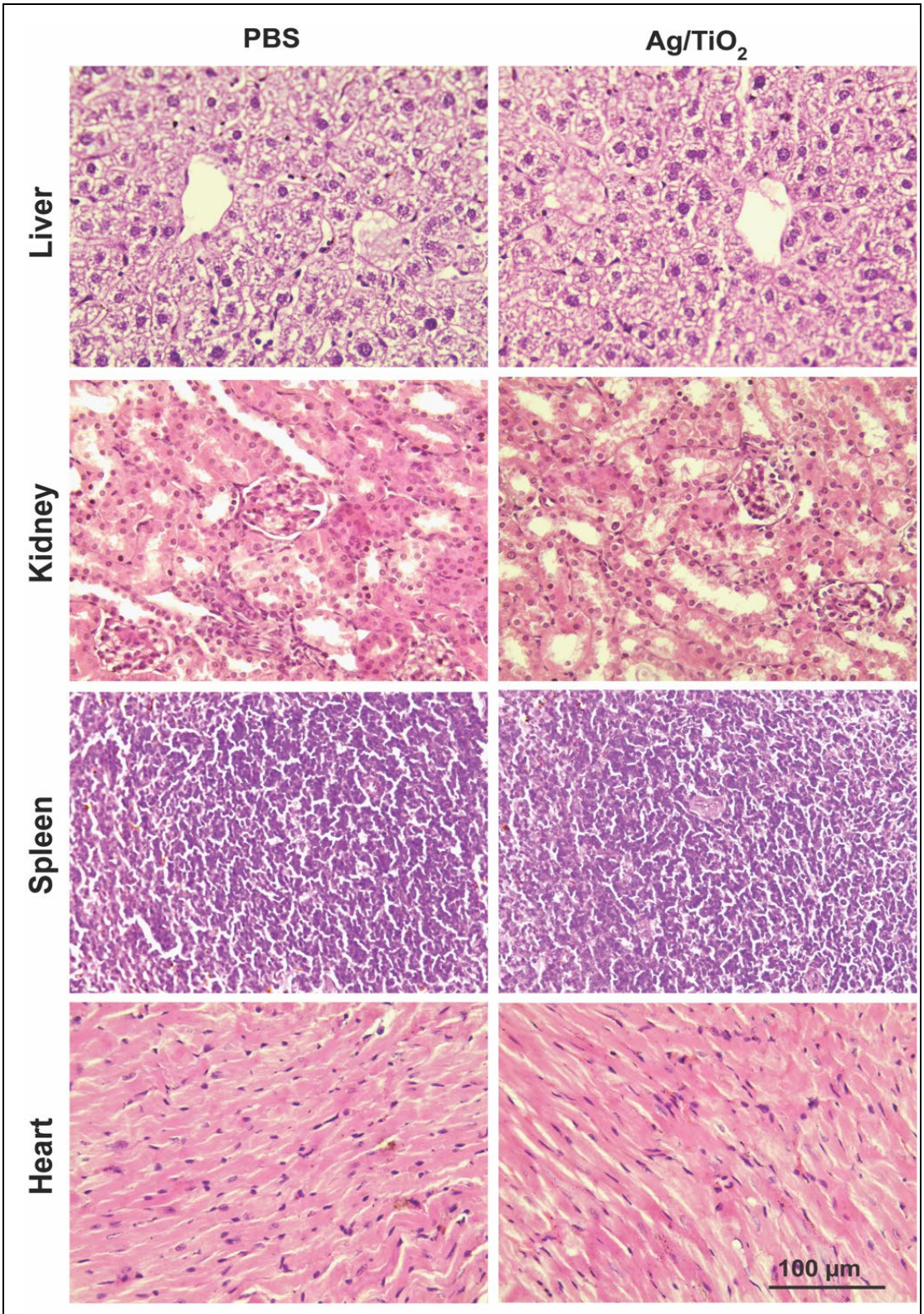


Figure S 13: Representative histopathological sections of different tissues of mice following a single injection of PBS (control left panel) or Ag/TiO_2 at 266 mg/ml (500 µl/I.P/mouse) (right panel) and sacrificed 2 weeks later. Tissue sections of liver, kidney, spleen, and heart of both injected groups showed no significant lesions detected, H&E.

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