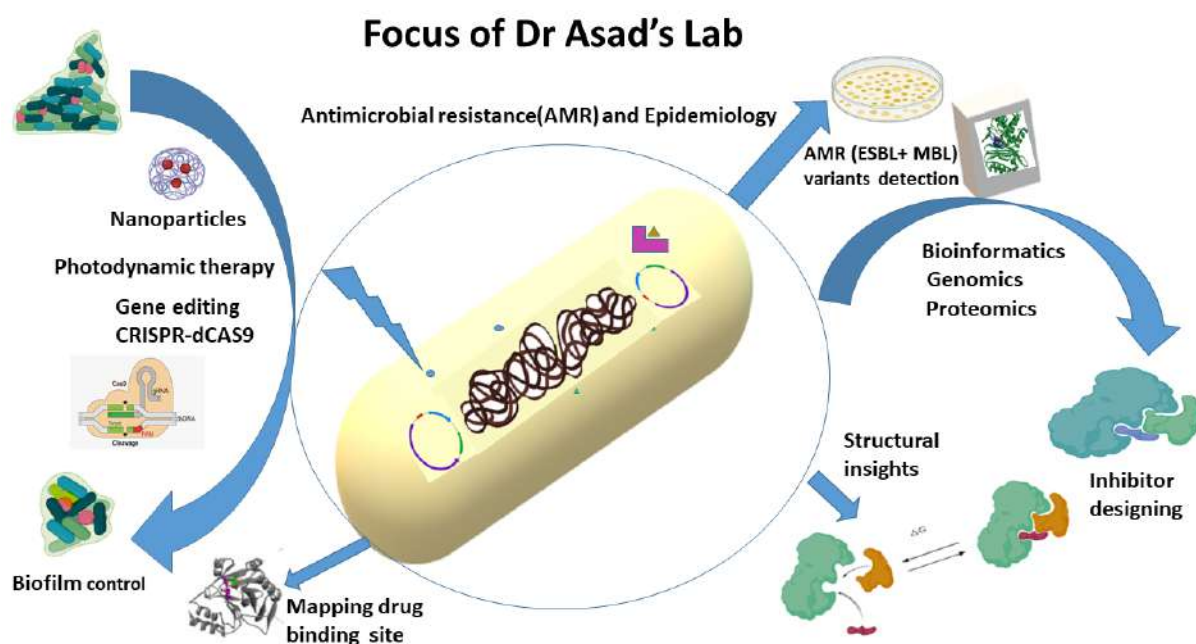


d. Detail of the research work duly signed by the applicant, for which the Sun Pharma Research Award is claimed, including references and illustrations (not to exceed 6000 words).

## **Toluidine Blue conjugated gold-silver nano-shells mediated photodynamic therapy to treat diabetic foot ulcer: A nano-phototheranostic approach**



### **Synopsis**

Diabetic foot infection caused by multidrug-resistant bacteria, is becoming serious problem. Moreover, polymicrobial biofilms contribute significantly to the persistent infections. In the present study, we investigated the effectiveness of novel toluidine blue conjugated chitosan coated gold-silver core-shell nanoparticles (TBO-chit-Au-AgNPs) mediated photodynamic therapy and demonstrate their use as a nontoxic antibacterial therapy to combat diabetic foot ulcer caused by multi-drug resistant strains both in monomicrobial and polymicrobial state of infection. In vitro efficacy of TBO-chit-Au-AgNPs mediated photodynamic therapy against polymicrobial biofilms was determined. Its therapeutic potential was validated in vivo type-2 DFU animal model. Cytokine's level was found reduced, using nano-phototheranostic approach, indicating infection control. Expression profile of growth factors confirmed both the pathogenesis and healing of DFU.

*We conclude that TBO-chit-Au-AgNPs mediated PDT is a promising anti-bacterial therapeutic approach for healing of DFU caused by MDR bacterial strains.*

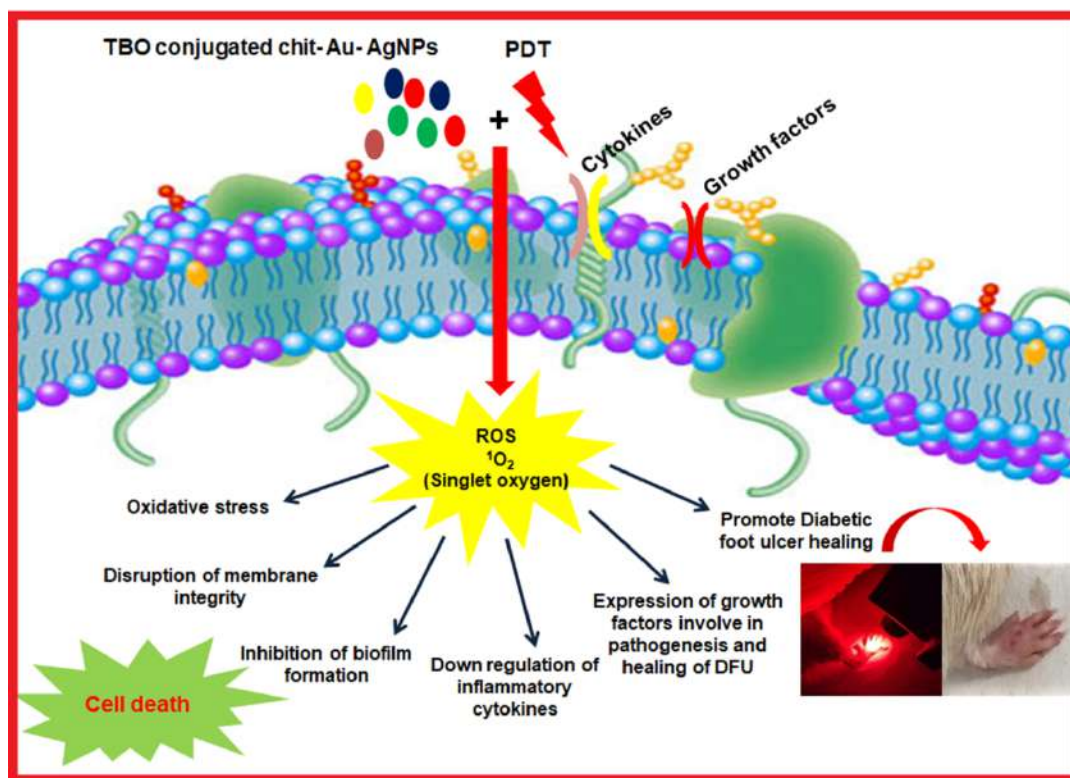
## **Background**

Type 2 diabetes mellitus (DM) has become an utmost health concern which comprised of 90-95% diabetes among worldwide population<sup>1</sup>. Nevertheless, immunocompromised patients identified with diabetes mellitus are more prone to be suffered from non-healing wounds<sup>2</sup>. It has also been reported that up to one-third of people with diabetes may develop foot ulcer during their lifetime and over 50% of these ulcers become infected. This challenge is further aggravated by the emergence of multidrug-resistance (MDR) strains induced diabetic foot infections (DFIs) which leads to increasing morbidity and mortality, and risk of lower extremity amputation (LEA) which causes low quality of life<sup>3</sup>. It has been stated earlier those biofilms intricate up to 65% of infections, leading to severe illness with a prolonged stay in hospital settings which may increase cost of treatment as well as mortality rate<sup>4</sup>.

The most commonly found microorganisms which are isolated from patients with diabetic foot ulcer were reported as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus spp.*, *Streptococcus spp.*, *Proteus mirabilis* and anaerobes<sup>5</sup>. Among them, *S. aureus* and *P. aeruginosa* are the major cause of DFIs. For instance, polymicrobial, *S. aureus* and *P. aeruginosa* infections appear frequently in deep or chronic wounds<sup>6,7</sup>. The mutualistic and parasitic interactions cause synergistic association among the two species which led to the development of infections<sup>8,9</sup>. Since no antibiotics are left to treat such infections therefore, the need of an hour is to search for alternative therapy.

These metal nanoparticles produce different kinds of intracellular reactive oxygen species (ROS) which damages microbial membrane and other cellular components, thereby causing cell death<sup>10</sup>.

The antimicrobial actions of metal nanoparticles including gold and silver have already been established. Here, we initiated to fabricate a chitosan-coated gold nanoparticle (chit-AuNPs) as a core material<sup>11</sup>, followed by the deposition of the silver shell. It is worth to mention that chitosan biopolymer act as an effective reducing and stabilizing agent as well as an external layer to provide the nanocomposites with several advantages<sup>12</sup>. Whereas, silver ions act upon various sites in microorganisms to cease their growth. Previously, it was shown that silver nanoparticles are generally liable for the contact killing of microorganisms<sup>13</sup>. We intend to conjugate chit-Au-AgNPs metal composite with a photosensitizer (TBO), which will further enhance the antibacterial efficacy of this nanocomposite and possibly eradicates infection caused by resistant bacteria. Toluidine blue O (TBO) is a cationic phenothiazine dye that has been well studied as an antibacterial photosensitizer. It departs a high quantum of cytotoxic singlet oxygen during photosensitization with 630 nm wavelength of light<sup>14</sup>. This approach is highly effective in killing MDR strains since microorganisms do not acquire resistance toward PDT<sup>15</sup>. The use of TBO-chit-Au-AgNPs mediated PDT has not been reported yet. Therefore, we initiated this study to provide a novel approach toward potential applications of nano-phototheranostic complex, as a nontoxic antibacterial agent to combat DFU caused by multi-drug resistant bacterial strains.



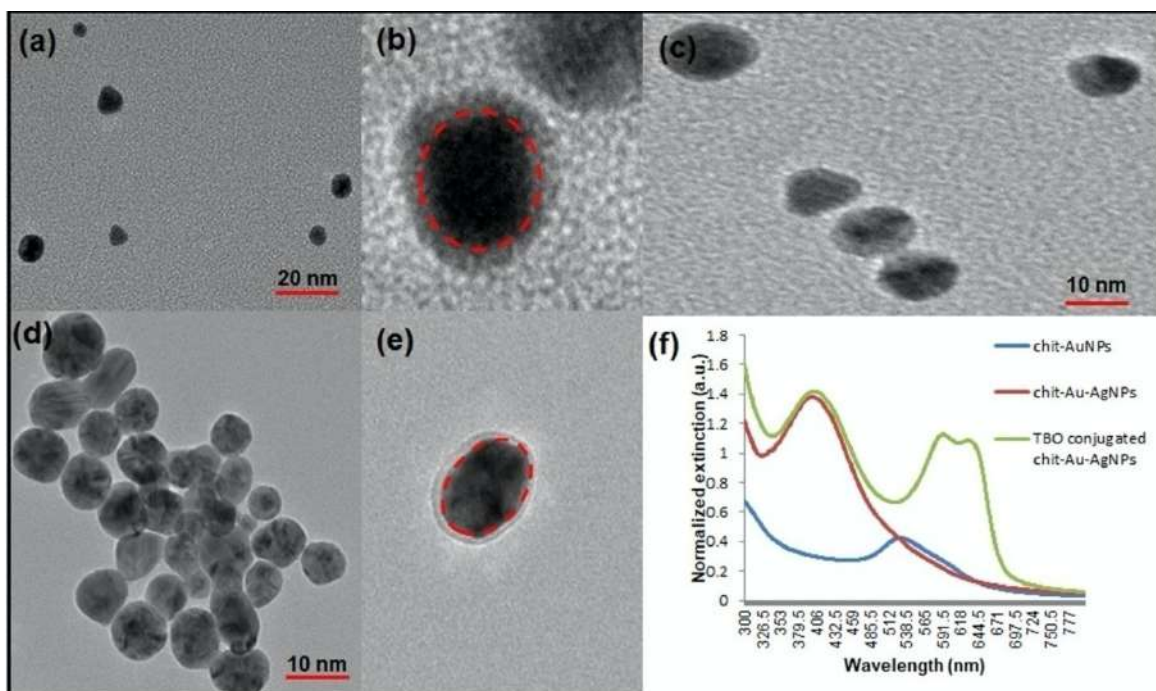
**Figure 1.** Schematic representation of nanoparticle mediated antimicrobial photodynamic therapy against diabetic foot ulcer.

## Experimental data:

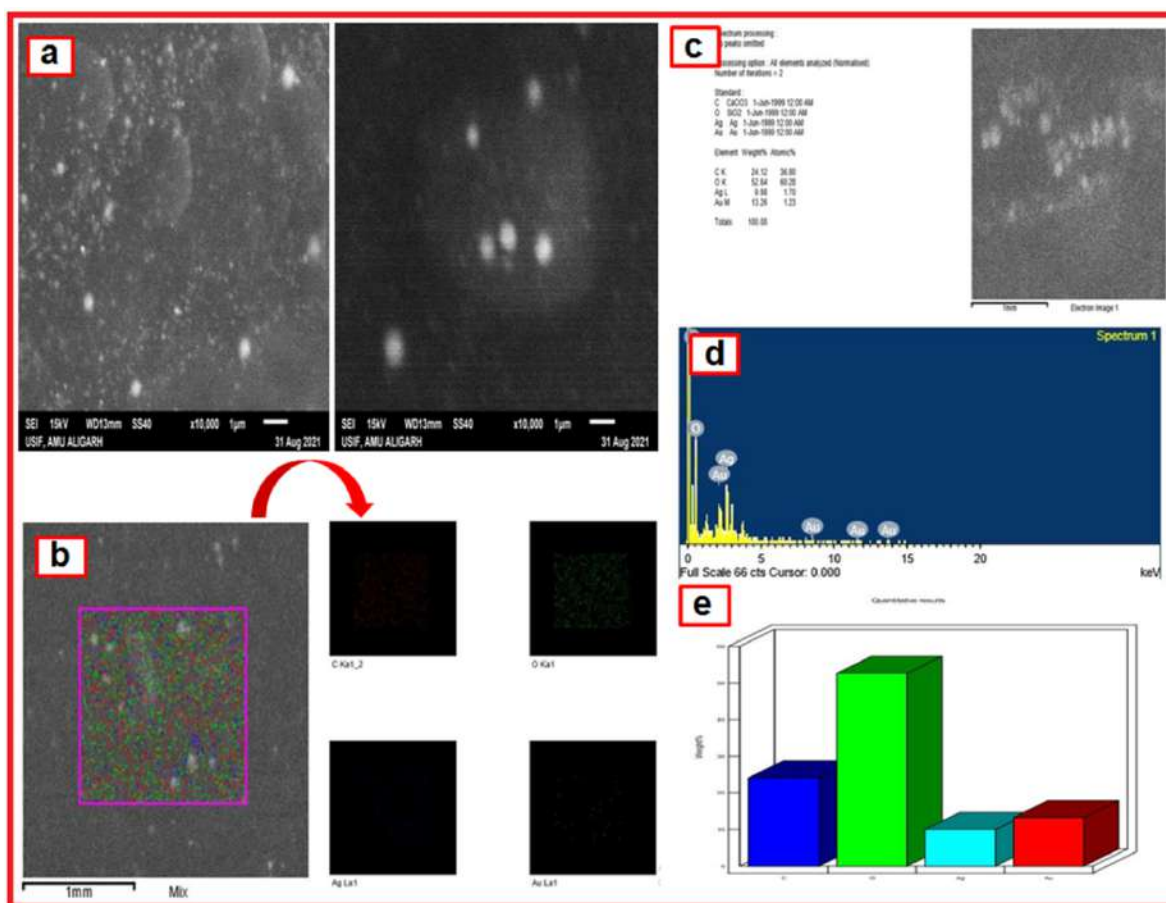
### Synthesis and characterization of TBO-chit-Au-AgNPs

Chitosan-coated gold nanoparticles (chit-AuNPs, Figure.2A (a)) were synthesized as core materials to produce biocompatible gold-silver core-shell nanoparticles (chit-Au-AgNPs). The synthesized TBO-chit-Au-AgNPs were characterized by UV-spectroscopy, TEM, DLS and Zeta potential. Furthermore, we have performed elemental mapping of chit-Au-AgNPs using scanning electron microscopy (SEM) with energy dispersive X-ray spectrometry (EDS). The addition of TBO with chit-Au-AgNPs under dark conditions has led to the formation of TBO-chit-Au-AgNPs (Figure.2A (f)-green spectrum). The UV-visible spectrum of the synthesized chit-AuNPs (Figure.2A (f)-blue spectrum) exhibits a well-defined SPR band centered at 530 nm. After 60

minutes, occurrence of a new band takes place at 405 nm (Figure. 2A (f)-red spectrum) upon first addition of AgNO<sub>3</sub> aliquots. The dark gold core and the brighter silver shell as observed in Figure.2A (b-d), was clearly distinguishable because of higher electronic density of gold than silver. Furthermore, Figure.2A (e) showed the presence of chitosan layer as a faint shadow which encapsulates the Au-AgNPs after their formation. Moreover, the result of scanning electron microscopy (SEM) with energy dispersive X-ray spectrometry (EDS) analysis (EDX) clearly shows the presence of Au, Ag and chitosan in pure form with weights of 13.26% Au, 9.98% Ag, 24.12% C and 52.64% O in the sample (Figure.2B-a, b, c, d and e). Besides, the average size of chit-AuNPs, chit-Au-AgNPs and TBO-chit-Au-AgNPs were found to be 129.4 nm, 131 nm, and 134 nm, respectively, as analyzed by dynamic light scattering (DLS) (Table.1). Changes in the polydispersity index (PDI) were also measured over this time. The PDI of chit-AuNPs, chit-Au-AgNPs and TBO-chit-Au-AgNPs remained low, indicating that there is least or no aggregation of particles. In addition, the result of zeta potential clearly illustrates chit-Au-AgNPs synthesized through stepwise addition of AgNO<sub>3</sub> retains their positive zeta potential of +37.6 mV, which shows that the polymeric coating is well-preserved all through the formation of core-shell nanoparticles (chit-Au-AgNPs).. Even after the addition of TBO, chit-Au-AgNPs exhibited strong positive zeta potential of +41.2 mV, suggesting the stability and biocompatibility of synthesized TBO-chit-Au-AgNPs (Table.1).



**Figure 2. Characterization of nanoparticles:** A. (a) TEM image of spherical chit-AuNPs. (b)-(d) TEM images of chit-Au-AgNPs after additions of  $\text{AgNO}_3$  aliquots. The interface between the Au-core and the Ag-shell was marked by a red dashed line in figure 1(b). (e) A closer view of an Au-AgNP coated by a chitosan layer. (g) UV-Vis absorption spectra of chit-AuNPs, chit-Au-AgNPs and TBO-chit-Au-AgNPs.

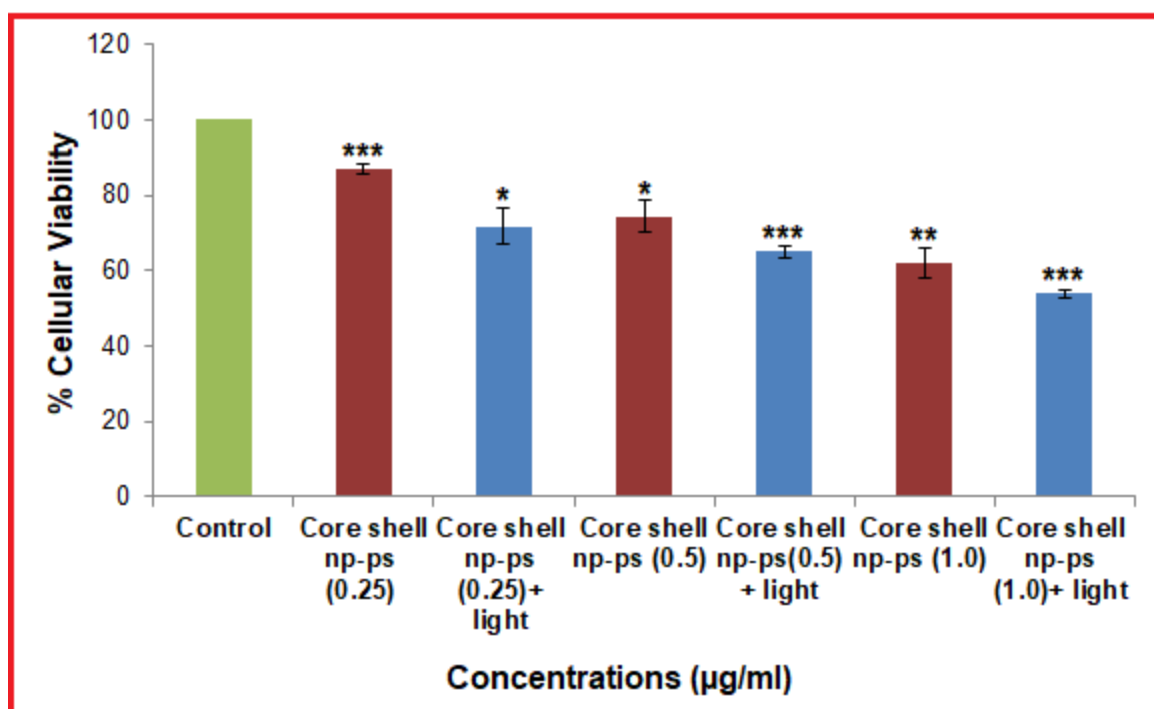


**Figure 2B.** (a) Scanning electron microscopy (SEM) images of chit-Au-AgNPs. (b)-(e) Energy dispersive X-ray spectrometry (EDS) profile of chit-Au-AgNPs and quantitative analysis.

### Effect of TBO-chit-Au-AgNPs mediated photodynamic therapy on cellular toxicity

The relative cellular viability of L929 fibroblast cells in the presence of various concentrations (0.25 mM, 0.5 mM and 1 mM) of TBO-chit-Au-AgNPs with and without laser irradiation is shown in Figure.3. Almost 65.12 % and 74.32 % viability were observed at our tested concentration (0.5 mM) with and without laser irradiation respectively. Thus, the concentration of the TBO-chit-Au-AgNPs and exposure time ( $100 \text{ J/cm}^2$ ) of red laser light used in this study was found to be non-toxic.





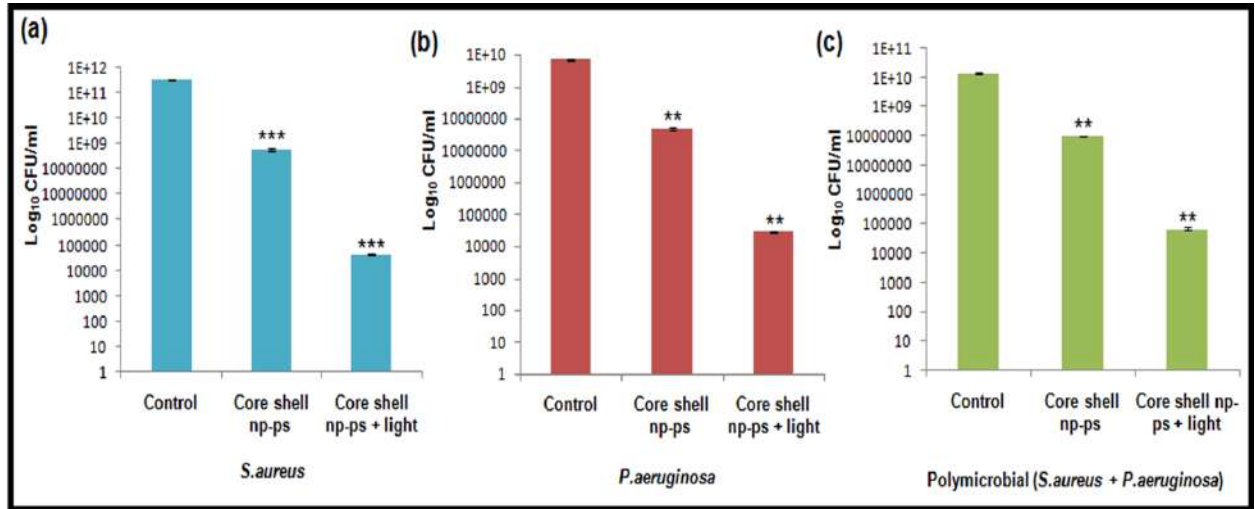
**Figure 3. Cytotoxicity assay:** Percentage viable cells as measured after 24 hrs, after treatment with TBO-chit-Au-AgNPs in absence and presence of laser irradiation.

In vitro anti-bacterial activity of TBO-chit-Au-AgNPs mediated PDT on monomicrobial and polymicrobial biofilms

After incubation for 24 h, the resulting monomicrobial biofilms showed 2.76 log<sub>10</sub> CFU/mL reduction of *S.aureus* and 2.17 log<sub>10</sub> CFU/mL reduction of *P. aeruginosa* in the group treated with TBO-chit-Au-AgNPs only. While, 2.15 log<sub>10</sub> CFU/mL reduction was seen in polymicrobial biofilms (Figure.4). A substantial reduction in bacterial load was achieved when monomicrobial and polymicrobial biofilms of *S.aureus* and *P. aeruginosa* treated with TBO-chit-Au-AgNPs and subsequently to 100 J/cm<sup>2</sup> of laser irradiation. A 6.86 log<sub>10</sub>CFU/mL and 5.4 log<sub>10</sub> CFU/mL reduction was found in the monomicrobial biofilm of *S.aureus* and *P.aeruginosa*, respectively, whereas 5.31 log<sub>10</sub> CFU/mL reductions were observed in the polymicrobial biofilms of both the species (**Figure 4**). The bacterial load reduction in the



polymicrobial biofilms of *S.aureus* and *P. aeruginosa* was found to be lower than monomicrobial biofilms of both the species after nano-photodynamic therapy



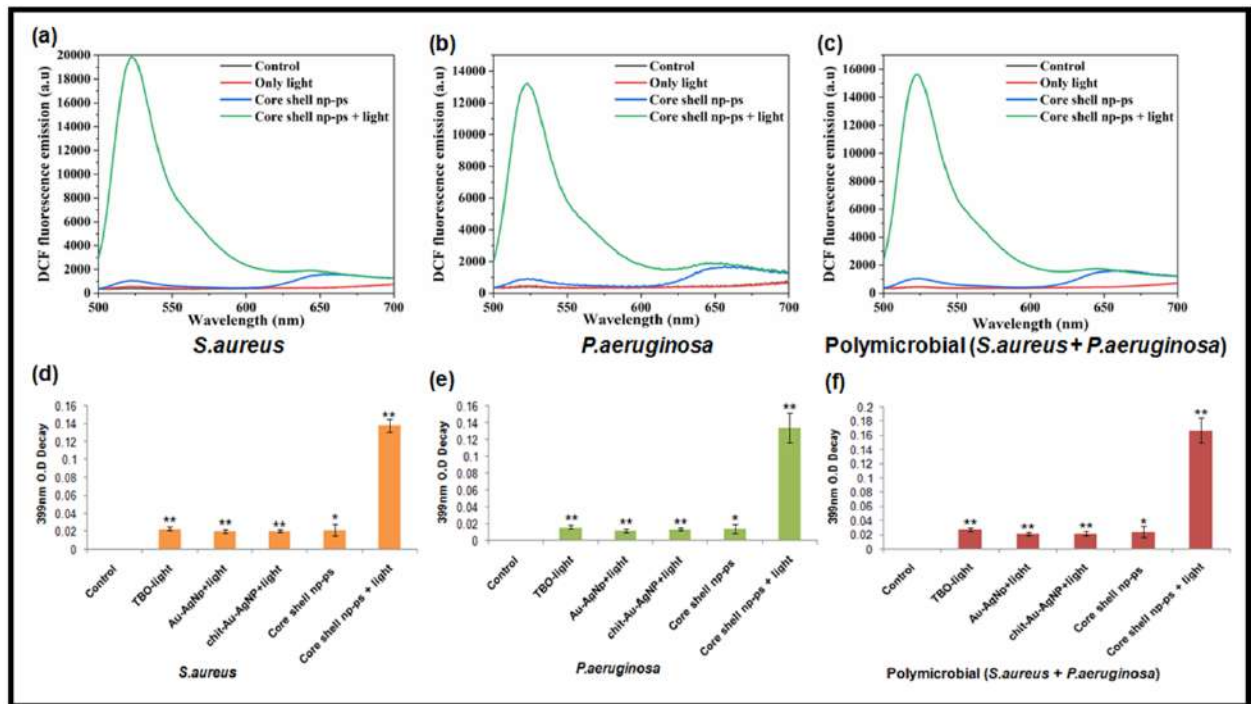
**Figure 4. In vitro colony formation:** (a) Monomicrobial *S. aureus*, (b) Monomicrobial *P. aeruginosa* and (c) Polymicrobial *S. aureus* + *P. aeruginosa* colonies after incubation with TBO-chit-Au-AgNPs or TBO-chit-Au-AgNPs and/or irradiation with 630 nm laser for 12 min and 50 seconds which corresponds to 100 Jcm<sup>-2</sup>.

#### Generation of reactive oxygen species and singlet oxygen quantification

Our data showed increase production of intracellular ROS in TBO-chit-Au-AgNPs mediated photodynamic therapy treated group as compared to TBO-chit-Au-AgNPs alone, both in monomicrobial as well as in polymicrobial biofilms. Whereas, no ROS production was seen in the group treated with light only. Thus, we conclude that the potentiation of killing or enhanced antibacterial activity is dependent upon the generation of ROS (**Figure.5 a,b,c**). However, the photoinactivation was found to be more pronounced in monomicrobial biofilm than polymicrobial biofilms of both the species.

Furthermore, to confirm the type of phototoxicity, we have measured the degradation rate of AMDA. Since, the amount of ROS produced is directly proportional to the bacterial cell death;

our data revealed enhanced production of  $1O_2$  in TBO-chit-Au-AgNPs mediated PDT treated group as compared to control and only TBO-chit-Au-AgNPs treated groups (Figure.5 d,e,f). This confirmed that type II photochemistry is the major photochemical reaction involved in TBO-chit-Au-AgNPs mediated photodynamic therapy on monomicrobial and polymicrobial biofilms.

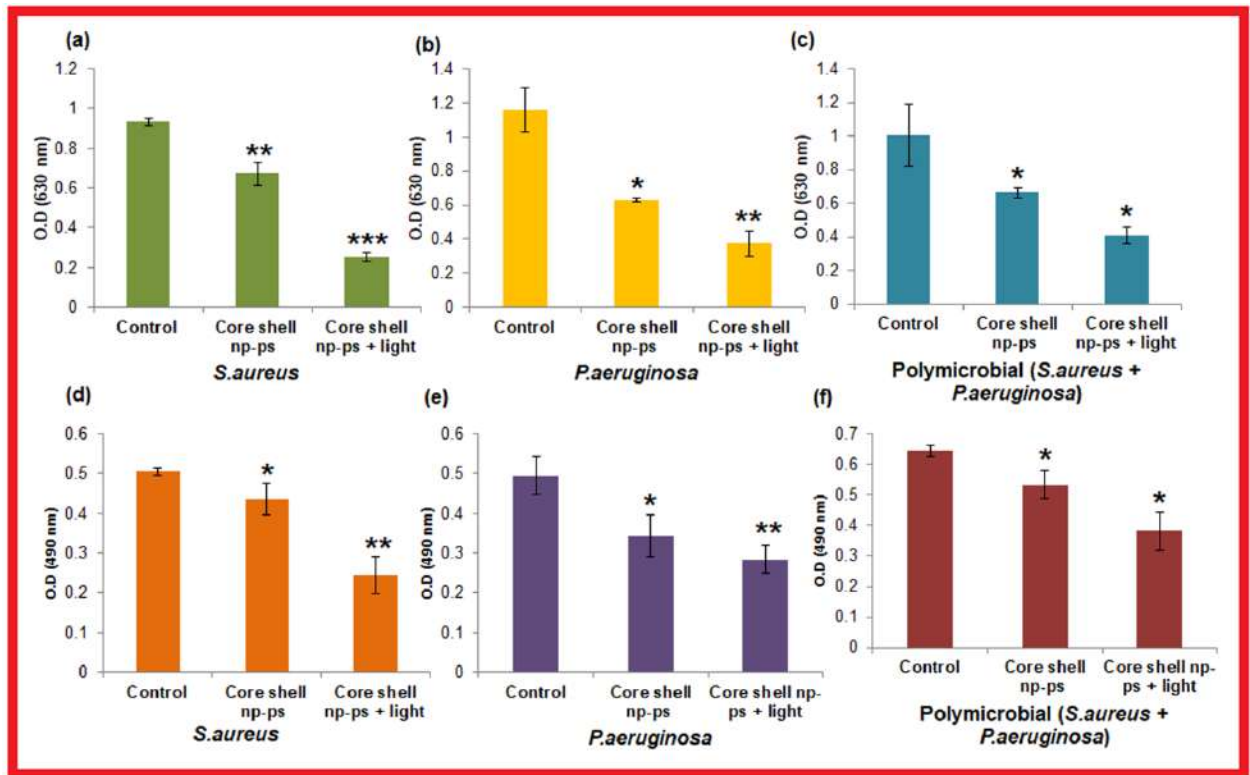


**Figure 5.** Detection of total reactive oxygen species (a)-(c) and quantification of singlet oxygen (d)-(f) in monomicrobial *S. aureus*, monomicrobial *P. aeruginosa* and (c) polymicrobial *S. aureus* + *P. aeruginosa* biofilms in the groups of control, TBO-chit-Au-AgNPs and TBO-chit-Au-AgNPs + laser. 630 nm laser (100 Jcm<sup>-2</sup>, 12 min and 50 seconds) were used in the corresponding laser group.

### Anti-biofilm effect of TBO-chit-Au-AgNPs mediated photodynamic therapy

The result of crystal violet assay showed 28.02 % and 34. 04% reduction in the monomicrobial *S. aureus* and *P. aeruginosa* biofilm formation, respectively, after treatment with, exclusively TBO-chit-Au-AgNPs, whereas 45. 89% of polymicrobial biofilms were found to be reduced.

Moreover, significant decrease in microbial biomasses was found in TBO-chit-Au-AgNPs mediated photodynamic therapy treated group as compared to control and exclusively TBO-chit-Au-AgNPs treated groups. Our data showed 72.82% and 59.15 % reduction in the monomicrobial biofilm of *S.aureus* and *P.aeruginosa*, respectively, while 67.69% reduction was found in the polymicrobial biofilms after treatment with TBO-chit-Au-AgNPs and subsequently to 100 J/cm<sup>2</sup> of laser irradiation (Figure.6 a,b,c). Besides this, EPS production was reduced by 13.65% and 30.71% in monomicrobial biofilm of *S.aureus* and *P.aeruginosa*, whereas 17.16 % in polymicrobial biofilms after being treated with exclusively TBO-chit-Au-AgNPs. While, 51.55% and 42.71% EPS reduction was achieved in monomicrobial biofilm of *S.aureus* and *P.aeruginosa*, respectively, after being photoinactivated by TBO-chit-Au-AgNPs. However, 40.9% reduction was seen in polymicrobial biofilms as shown in Figure.6 d,e,f.

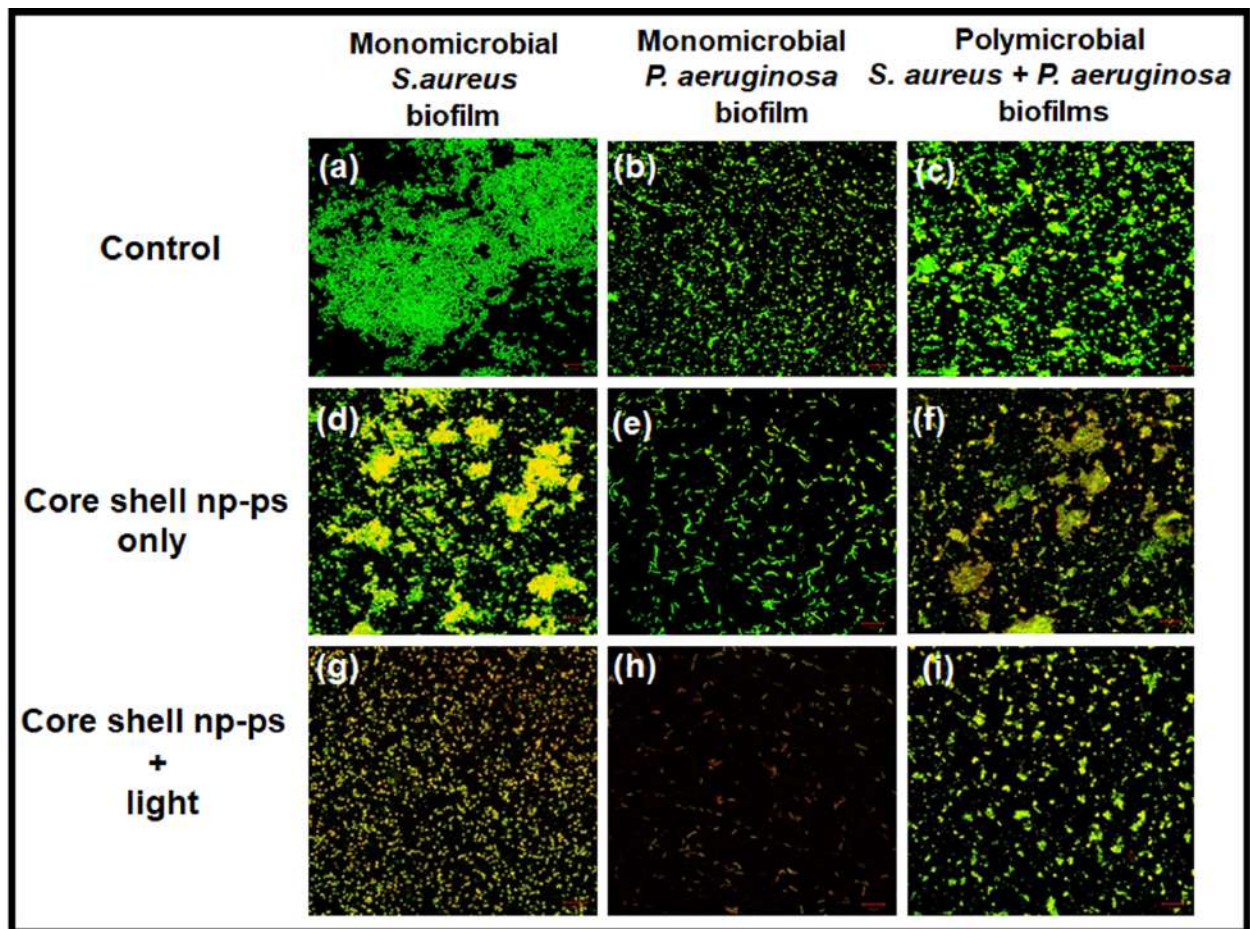


**Figure 6. Antibiofilm forming ability of TBO-chit-Au-AgNPs mediated photodynamic therapy:** (a)-(c) represents inhibitory effect of TBO-chit-Au-AgNPs mediated PDT on monomicrobial *S. aureus*, monomicrobial *P. aeruginosa* and polymicrobial *S. aureus* + *P. aeruginosa* biofilms, respectively as quantified by Crystal violet assay. Absorbance was measured at 630 nm. Figure (d) - (f) showed effect of TBO-chit-Au-AgNPs mediated PDT on extracellular polysaccharide substance (EPS) reduction as quantified by Congo-red assay.

**Visualization of biofilms architect** after TBO-chit-Au-AgNPs mediated photodynamic therapy

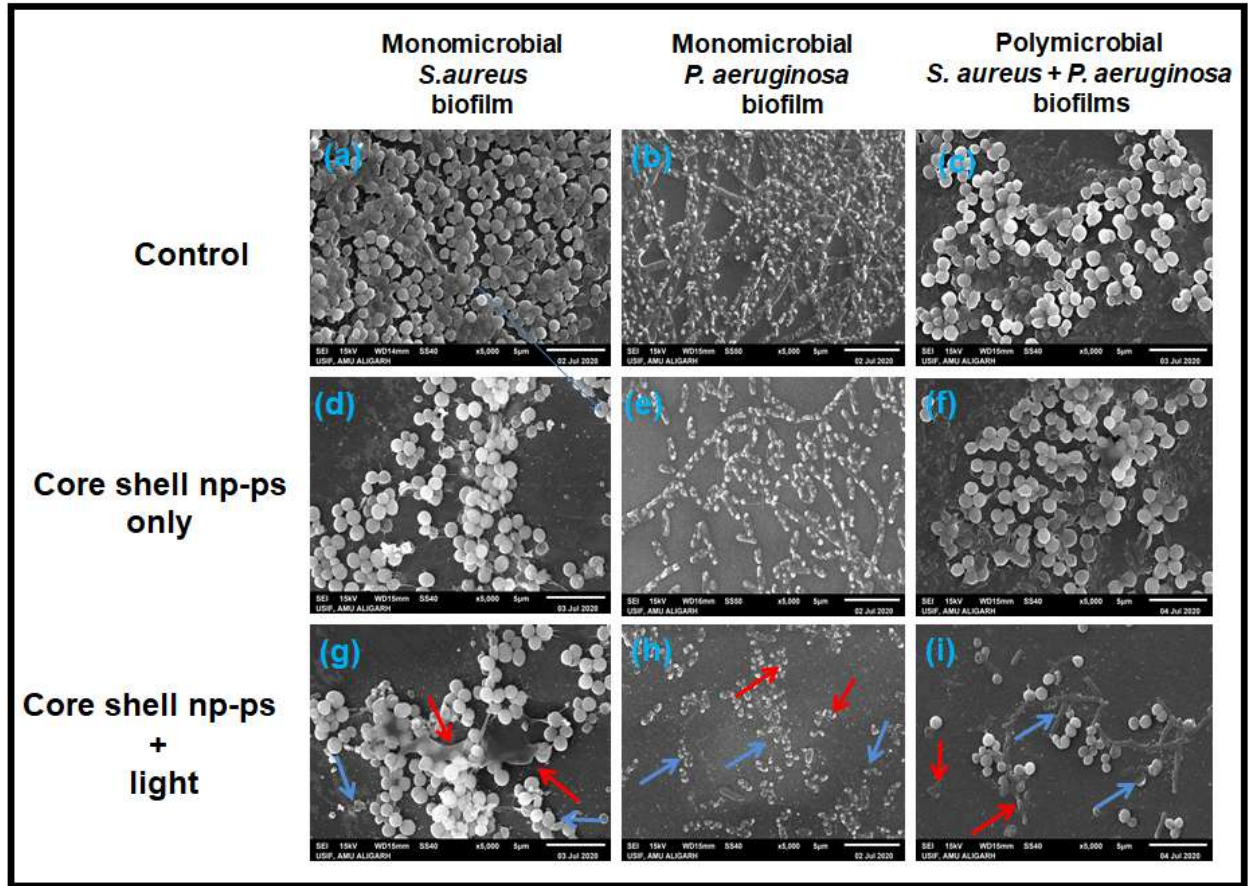
Confocal and scanning electron microscopy was performed to examine the morphological changes in bacteria after TBO-chit-Au-AgNPs mediated photodynamic therapy, and to elucidate the underlying anti-bacterial mechanisms. CLSM micrographs illustrate greater disruption of polymicrobial biofilms of *S. aureus* and *P. aeruginosa* in TBO-chit-Au-AgNPs mediated photodynamic therapy treated group (Figure.7i) as compared to control (Figure.7c) and exclusively TBO-chit-Au-AgNPs (Figure.7 f). Moreover, the monomicrobial biofilms of *S. aureus* and *P. aeruginosa* was more severely disrupted, almost all the bacterial cells in the biofilms were found dead in TBO-chit-Au-AgNPs mediated PDT group (Figure.7-gand h), as compared to control (Figure. 7-a and b) and exclusively TBO-chit-Au-AgNPs treated group (Figure.7-d and e). Thereby, demonstrating strong antibiofilm action of TBO-chit-Au-AgNPs mediated photodynamic therapy. TBO-chit-Au-AgNPs treatment reduced the thickness of the biofilm to approximately 4  $\mu\text{m}$  and 3  $\mu\text{m}$  in monomicrobial biofilm of *S. aureus* and *P. aeruginosa*, respectively, as compared to control (6  $\mu\text{m}$  and 4  $\mu\text{m}$ , respectively). However, the thickness was found to be 2.5  $\mu\text{m}$  and 2  $\mu\text{m}$ , respectively in TBO-chit-Au-AgNPs mediated PDT treated group, suggesting prevalence of dead cells throughout the biofilm. Likewise, polymicrobial biofilm of *S. aureus* and *P. aeruginosa* treated with TBO-chit-Au-AgNPs mediated PDT decreases the thickness of the biofilm to approximately 3  $\mu\text{m}$  as compared to control (8  $\mu\text{m}$ ) and TBO-chit-Au-AgNPs alone (6  $\mu\text{m}$ ). This observation was further supported

by the scanning electron microscopy. The control groups of monomicrobial as well as polymicrobial *S. aureus* and *P. aeruginosa* biofilms displayed highly dense and compact microbial cells (Figure. 8-a, b, c). While, after treatment with TBO-chit-Au-AgNPs followed by exposure to 100 J/cm<sup>2</sup> of laser light, the density of the microbial cells in the monomicrobial as well as in the polymicrobial biofilms of both the species decreases significantly (Figure.8-g, h, i). Less reduction was observed in only TBO-chit-Au-AgNPs treated groups (Figure.8-d, e, f). Furthermore, TBO-chit-Au-AgNPs mediated PDT treated groups showed detrimental effects on cell wall with significant dispersion of the cells leading to leakage of the cellular content, thus obliterating the structural integrity of the biofilm.





**Figure 7.** Fluorescence-based live/dead analysis of monomicrobial *S. aureus*, monomicrobial *P. aeruginosa* and polymicrobial *S. aureus* + *P. aeruginosa* biofilms: Representative fluorescence images of SYTO 9 (live, green) and PI (dead, red) stained bacteria in the groups of control (a)-(c), TBO-chit-Au-AgNPs treated (d)-(f) and TBO-chit-Au-AgNPs + laser treated (g)-(i). 630 nm laser (100 Jcm<sup>-2</sup>, 12 min and 50 seconds) were used in the corresponding laser group. Scale bar = 10  $\mu$ m.

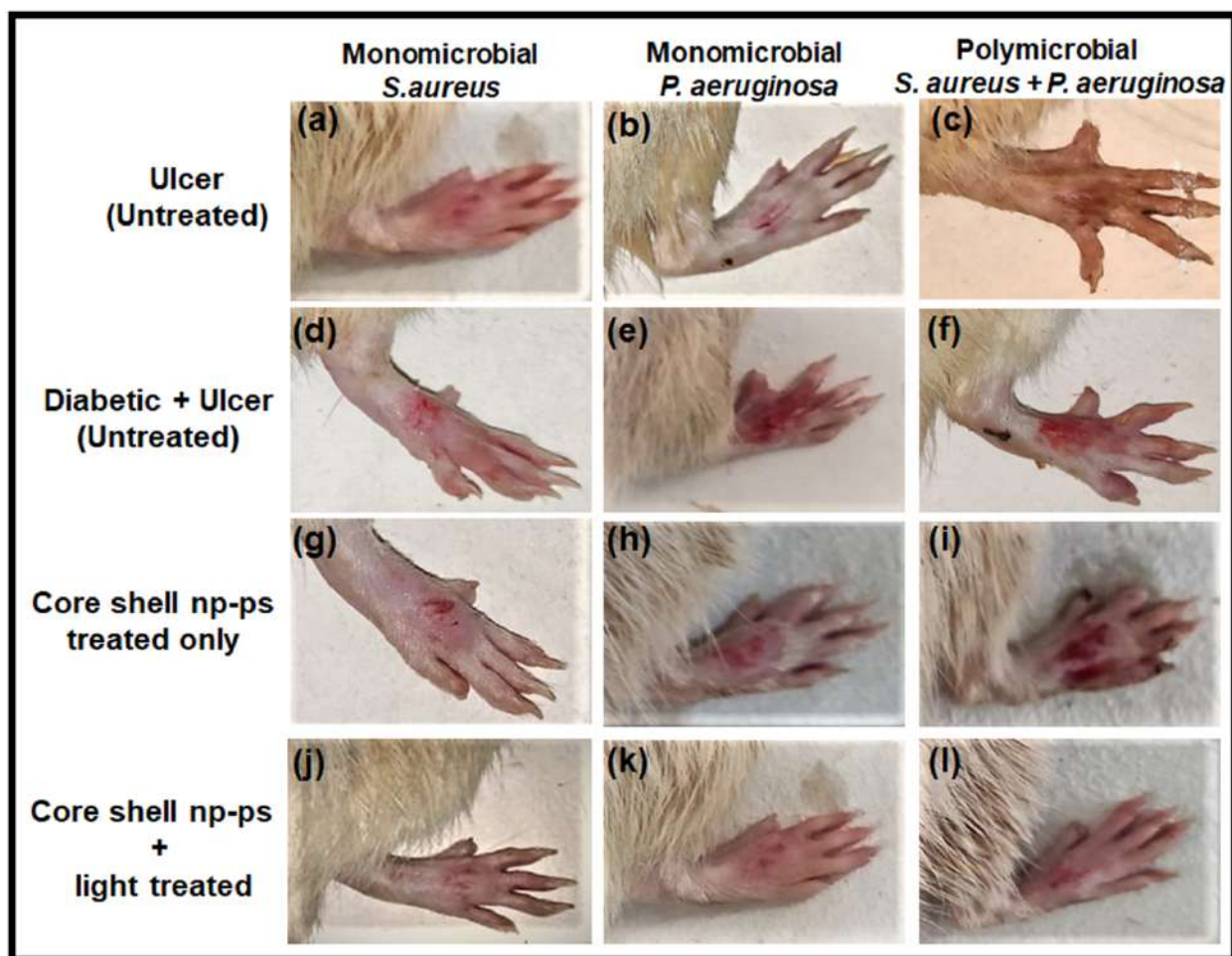


**Figure 8.** Scanning electron microscopy (SEM) images of monomicrobial *S. aureus*, monomicrobial *P. aeruginosa* and polymicrobial *S. aureus* + *P. aeruginosa* biofilms of control (a)-(c), TBO-chit-Au-AgNPs treated (d)-(f) and TBO-chit-Au-AgNPs + laser treated (g)-(i). 630 nm laser (100 Jcm<sup>-2</sup>, 12 min and 50 seconds) were used in the corresponding laser group. Red arrow indicates bursting and release of cellular constituents while blue arrow indicates complete rupturing of the cells.

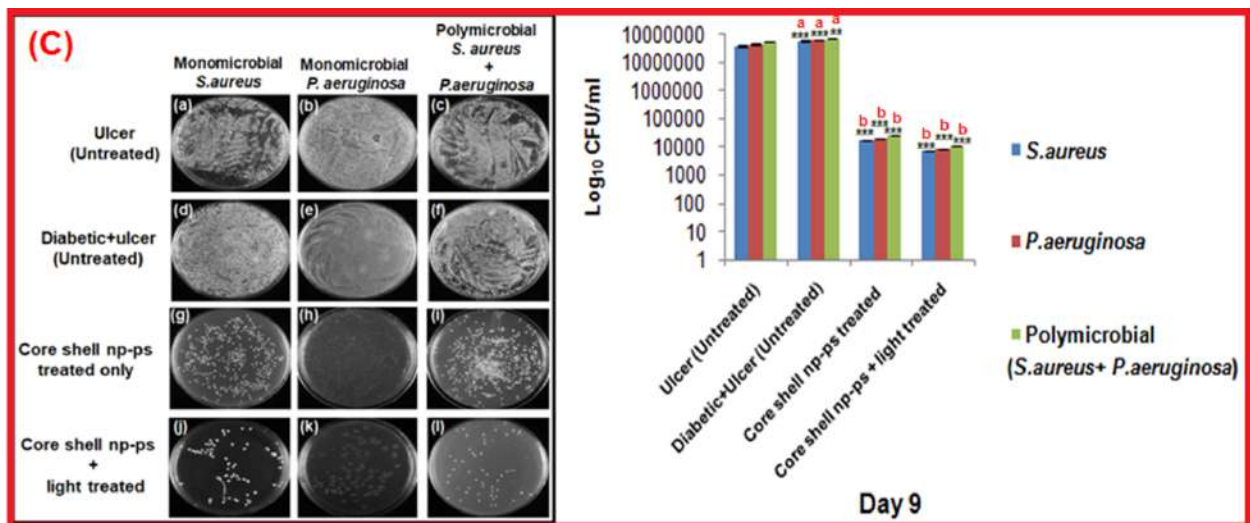
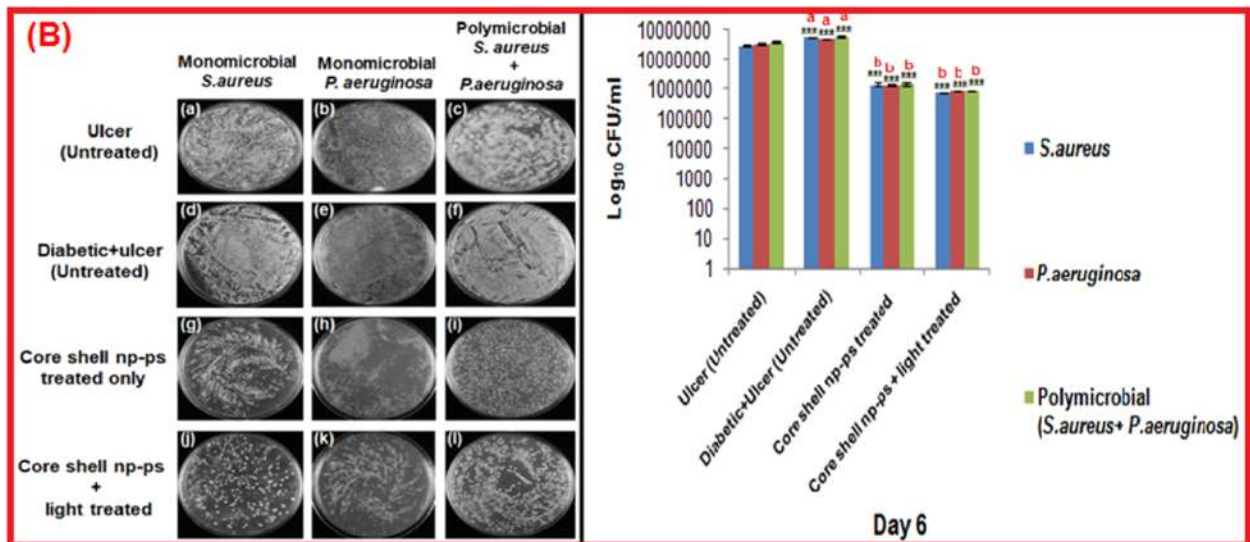
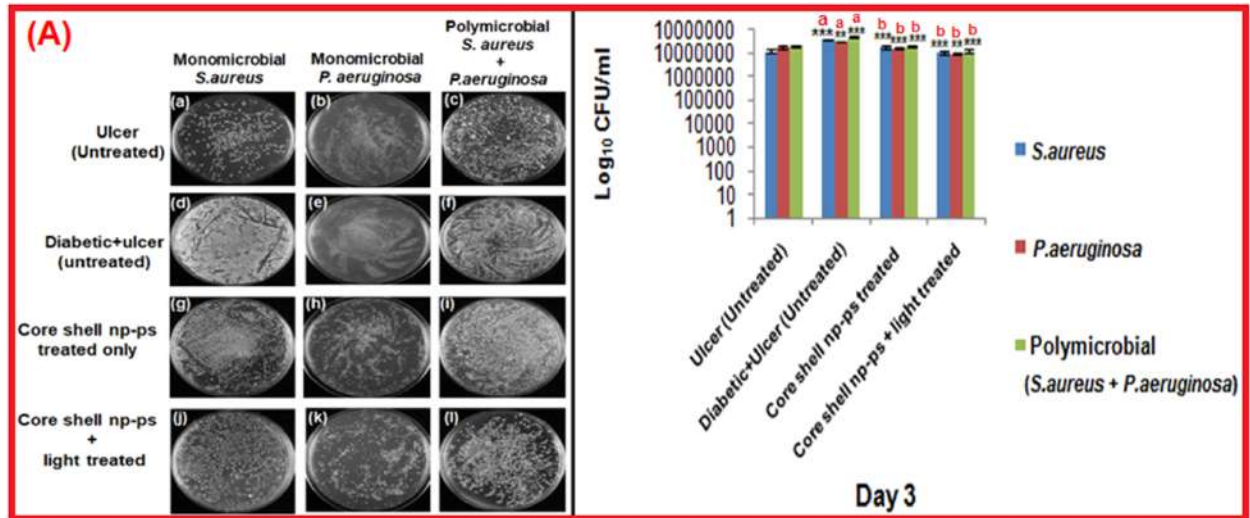
**In vivo efficacy of TBO-chit-Au-AgNPs mediated photodynamic therapy in the treatment of Diabetic Foot Ulcer**

The efficacy of TBO-chit-Au-AgNPs mediated photodynamic therapy in healing of diabetic foot ulcer in male wistar rats was checked. Streptozotocin (STZ)-induced type 2 DM rats with blood glucose level higher than 300mg/dl were developed and used for further study (Table.2). Our result showed that daily topical exposure of TBO-chit-Au-AgNPs followed by light irradiation led to the marked reduction of *S. aureus* and *P. aeruginosa* colonization in diabetic foot ulcer rats within 7 days, starting from the day 3 post infection (**Figure.9 and Figure.10 A, B, C**). Furthermore, histopathological analysis of untreated monomicrobial and polymicrobial DFU (**Figure.11B-d, e, f**) revealed dense population of inflammatory cells with focal neutrophilic infiltrate attached to the stratified squamous epithelium as compared to control, diabetic (**Figure.11A**) and ulcerated groups (**Figure.11B-a,b,c**). In addition, the ulcerated squamous epithelium in untreated DFU rats showed decrease collagenization with few basal thin capillaries and mild fibrosis. This confirms the incidence of an ongoing infection. However, the ulcer sites of the TBO-chit-Au-AgNPs treated group showed acanthotic (thickened) squamous epithelium with moderate fibrosis. Besides this, restorative ulcer was observed with focal moderately thick collagen fibers and few compressed capillaries (Figure.11B-g, h, i). In comparison, TBO-chit-Au-AgNPs mediated photodynamic therapy treated DFU showed intact stratified squamous epithelium with marked neo-angiogenesis and collagenization (**Figure.11B-j, k, l**).

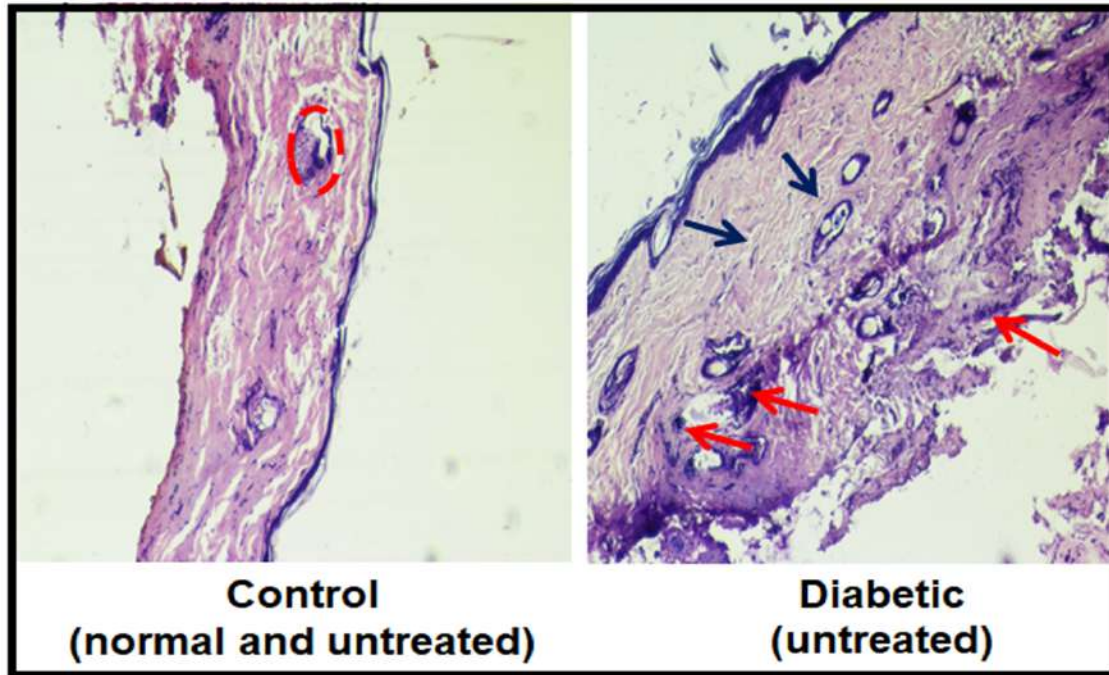




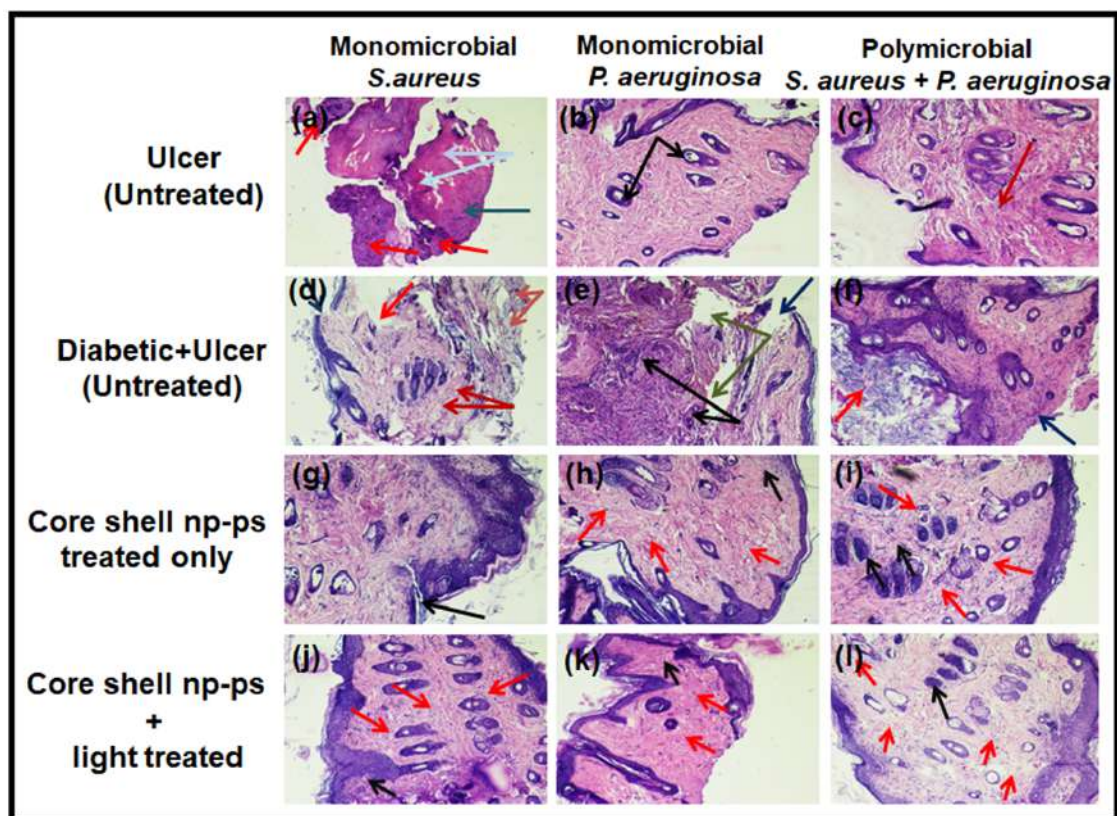
**Figure 9. Photographic image of wistar rats:** Untreated foot ulcer (a)-(c), untreated foot ulcer with diabetes (d) - (f), diabetic foot ulcer after applying TBO-chit-Au-AgNPs (g)-(i) and diabetic foot ulcer after treatment with TBO-chit-Au-AgNPs mediated PDT.



**Figure 10. In vivo antibacterial effect of TBO-chit-Au-AgNPs mediated photodynamic therapy in DFU rats: (A) CFU/ml at day 3 post ulceration, (B) CFU/ml at day 6 post ulceration and treatment, (C) CFU/ml at day 9 post ulceration and treatment.**







**Figure 11. Hematoxylin and Eosin (H& E) (x 10X) staining to examine response to microorganism of:** (A) Control and diabetic rats, (B) Ulcerated rats (untreated):figure (a)-(c), DFU rats (untreated): figure (d)-(f), DFU rats treated with TBO-chit-Au-AgNPs: figure (g)-(i) and DFU rats treated with TBO-chit-Au-AgNPs + laser: figure (j)-(l).

### **Effect of TBO-chit-Au-AgNPs mediated photodynamic therapy on growth factors and inflammatory cytokines**

The expression levels of growth factors and cytokines in control (normal) rats were compared with diabetic and non-diabetic rats with healed (treated) and unhealed (untreated) ulcers.

We have found that failure of an ulcer to heal in diabetic rats was linked with an increased level of pro-inflammatory cytokines, such as IL-6 and TNF- $\alpha$ . Furthermore, the expression levels of pro-inflammatory cytokines were significantly higher in DFU rats with polymicrobial infections as compared to DFU rats with monomicrobial infection. However, decreased levels

of these cytokines were found in TBO-chit-Au-AgNPs mediated photodynamic therapy treated groups as compared to control, untreated and only TBO-chit-Au-AgNPs treated groups, implying restoration of immunosuppression (Figure.12-a and b). In addition, our data demonstrated significantly higher levels of EGF and VEGF in all those groups of rats whose ulcers healed as compared to those groups whose ulcers did not healed (**Figure.12-c and d**). However, their expression level was found to be lower in DFU rats with polymicrobial infections than that of monomicrobial infection, as the severity of infection was found to be more in polymicrobial state of infection. Besides this, elevated level of TGF- $\beta$ -1 was found in all diabetic groups (with and without foot ulcer) (**Figure.12e**). Moreover, we have found expression levels of TGF- $\beta$ -1 were significantly higher in diabetic rats with polymicrobial infections as compared to monomicrobial infection. In addition, IGF-1 level was found to be lower in diabetic rats as compared to non-diabetic and control rats. However, considerable decrease was found in DFU rats with polymicrobial infections (Figure.12f). Hence, the data suggest that TBO-chit-Au-AgNPs mediated PDT promotes healing in DFU rats through significantly reducing cytokine production while elevating EGF and VEGF levels by regulating the expression of TGF- $\beta$ -1 and IGF-1.

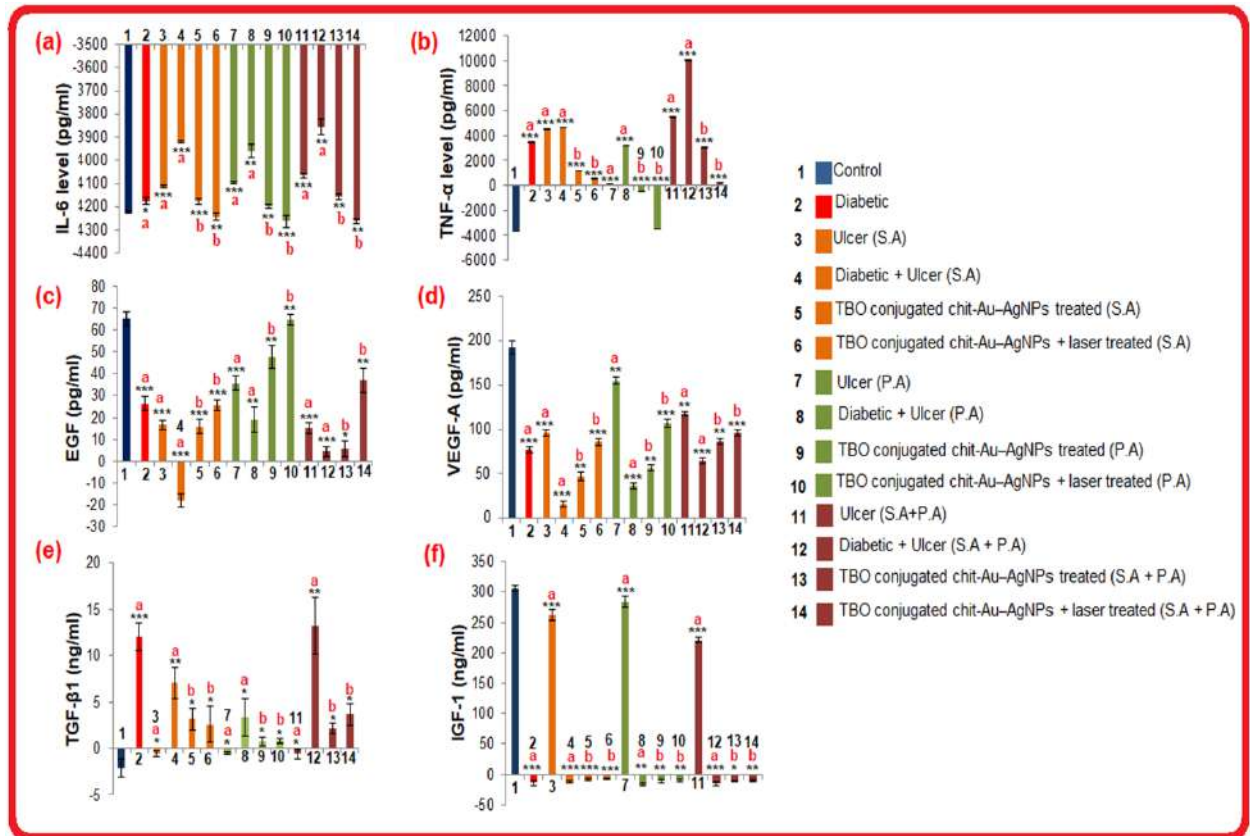


Figure 12. Effect of TBO-chit-Au-AgNPs mediated photodynamic therapy on pro-inflammatory cytokines and growth factors levels in Control rats, STZ induced diabetic rats (untreated), Ulcerated rats (untreated), DFU rats (untreated), DFU rats treated with TBO-chit-Au-AgNPs, DFU rats treated with TBO-chit-Au-AgNPs + laser: (a) IL-6 level, (b) TNF- $\alpha$  level, (c) EGF level, (d) VEGF-A level, (e) TGF- $\beta$ 1 level and (f) IGF-1 level. Each bar represents mean  $\pm$  SD of 4 rats.

## Discussion

Type 2 diabetes mellitus has become an utmost health concern which comprised of 90-95% diabetes among worldwide population<sup>1</sup>. It has also been reported that up to one-third of people with diabetes may develop foot ulcer during their lifetime and over 50% of these ulcers become infected. This challenge is further aggravated by the emergence of multidrug-resistance (MDR) strains induced diabetic foot infections (DFIs) which leads to increasing morbidity and mortality, and risk of lower extremity amputation (LEA) which causes low quality of life<sup>3</sup>.

Majority of these infections are now being caused by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus spp.*, *Streptococcus spp.*, *Proteus mirabilis* and anaerobes<sup>5</sup>.

*S. aureus* and *P. aeruginosa* are two versatile bacterial pathogens that are frequently found together in chronic wound infections<sup>6</sup>. Polymicrobial (mixed microbial culture) *S. aureus* and *P. aeruginosa* infections are more virulent and/or result in worse outcomes than the single infections caused by either species<sup>5</sup>, leading to the formation of more resistant biofilms, which are difficult to be eradicated. In this regard, nanoparticle mediated photodynamic therapy emerged as an ideal candidate to meet such requirements.

Recently, chitosan coated gold-silver core-shell nanoparticles (chit-Au-AgNPs) was developed and labeled with para-mercaptobenzoic acid (4MBA) to demonstrate their ability to perform as SERS nanotags inside of human ovarian adenocarcinoma cells (NIH: OVCAR-3) under multiple excitation wavelengths<sup>12</sup>. Here, chit-Au-AgNPs were used for the first time in association with TBO.

Earlier studies have shown that photosensitizer activity is significantly improved when combine with nanoparticles<sup>15</sup>. Our study was planned to investigate the effectiveness of novel toluidine blue conjugated gold-silver core-shell nanoparticles (TBO-chit-Au-AgNPs) mediated photodynamic therapy and demonstrate their use as a nontoxic antibacterial therapy to combat diabetic foot ulcer (DFU) caused by multi-drug resistant strains both in monomicrobial and polymicrobial state of infection.

The synthesized TBO-chit-Au-AgNPs were characterized by UV-spectroscopy, TEM, scanning electron microscopy (SEM) with energy dispersive X-ray spectrometry (EDS), DLS



and Zeta potential. The UV-visible spectrum of the synthesized chit-AuNPs (Figure.2A (f)- blue spectrum) exhibits a well-defined SPR (surface plasmon resonance) band centered at 530 nm. For instance, occurrence of a new band takes place at 405 nm (Figure.2A (f) - red spectrum) upon first addition of AgNO<sub>3</sub> aliquots. This spectral profile has already been reported in previous studies and it confirms the successful synthesis of a thin silver layer on the surface of the gold core<sup>16</sup>. The morphological progression of core-shell nanoparticles (chit-Au-AgNPs) was further confirmed by TEM. The dark gold core and the brighter silver shell as observed in Figure.2A (b-d), was clearly distinguishable because of higher electronic density of gold than silver. Furthermore, Figure.2A (e) showed the presence of chitosan layer as a faint shadow which encapsulates the Au-AgNPs after their formation. This foremost result demonstrated not only the effective synthesis of Au-AgNPs, but also their steadiness and biocompatibilization, which are required essentially for the use of this nanoconjugate in biological systems. We next performed elemental mapping to verify the presence of gold and silver using scanning electron microscopy (SEM) with energy dispersive X-ray spectrometry (EDS). The result confirms the formation of chit-Au-Ag nanocomposites with no additional impurities as detected in the EDS (Figure.2B- a, b, c, d and e). Changes in the polydispersity index (PDI) were also measured over this time. The PDI of chit-AuNPs, chit-Au-AgNPs and TBO-chit-Au-AgNPs remained low, indicating that there is least or no aggregation of particles (**Table 1**). We have also measured the surface zeta potential to further confirm about the stability of nanoparticles. Our data demonstrated strong positive zeta potential of chit-Au-AgNPs (+41.2 mV) even after the addition of TBO, suggesting the stability and biocompatibility of synthesized gold-silver core-shell nanoparticles (chit-Au-AgNPs) with TBO.

As presented in Figure.3, the synthesized TBO-chit-Au-AgNPs in the presence of laser irradiation did not exhibit any obvious cytotoxic effects at our tested concentration (0.5 mM). This could be attributed mainly to the biological and chemical properties of chitosan, as well as low toxic effect of TBO on fibroblast cells<sup>17</sup>.

One of the main factors responsible for the resistance of *S.aureus* and *P. aeruginosa* to both antibiotics and immune cells is their propensity to form biofilms. We therefore assessed the antibacterial activity of TBO-chit-Au-AgNPs in the presence as well as in the absence of laser irradiation using colony formation assay against polymicrobial biofilms and compared with that of monomicrobial biofilms. A substantial reduction in bacterial load was achieved when monomicrobial and polymicrobial biofilms of *S.aureus* and *P. aeruginosa* treated with TBO-chit-Au-AgNPs and subsequently to 100 J/cm<sup>2</sup> of laser irradiation (**Figure.4**). The results from the present study illustrated synergistic interaction between *S.aureus* and *P. aeruginosa* which is in concordance with previous studies<sup>13</sup>. Moreover, we have found Gram-positive, *S. aureus* as more sensitive towards nanoparticle mediated photodynamic therapy as compared to Gram-negative, *P. aeruginosa*. Since, it has been reported in the literature that teichoic acid found within the cell wall of Gram-positive bacteria is the main binding site of some small molecules and nanoparticles<sup>18</sup>.

In order to illustrate the above outcomes, we further analyzed the mechanism behind the antibacterial activity of TBO-chit-Au-AgNPs mediated photodynamic therapy using DCFH-DA and quantified the production of total reactive oxygen species (ROS) under laser irradiation. Our data showed increase production of intracellular ROS in TBO-chit-Au-AgNPs mediated photodynamic therapy treated group as compared to TBO-chit-Au-AgNPs alone, both in monomicrobial as well as in polymicrobial biofilms. Additionally, bacterial cells have

sufficient amount of scavengers such as catalase, peroxidase and superoxide dismutase to thwart the free radical mediated bactericidal activity, however they have no remedy against the singlet oxygen molecule, as a result,  $^1O_2$  leads to maximum cell damage<sup>19</sup>. Therefore, to confirm the type of phototoxicity, we have used AMDA. Our data revealed enhanced production of  $^1O_2$  in TBO-chit-Au-AgNPs mediated PDT treated group as compared to control and only TBO-chit-Au-AgNPs treated groups (**Figure.5 d,e,f**).

Furthermore, the ability of bacterial adherence and exopolysaccharide production which are significantly important for the formation of biofilm architecture, were analyzed by Crystal violet (CV) and Congo red (CR)-binding assays, respectively. Our data revealed polymicrobial biofilm was more impervious and difficult to eradicate than monomicrobial biofilm<sup>20</sup> (**Figure.6**). This may be due to the presence of more than one type of EPS formed by the bacteria which resulted into more viscous matrix<sup>21</sup>.

CLSM micrographs illustrate disruption of polymicrobial biofilms of *S.aureus* and *P. aeruginosa* in TBO-chit-Au-AgNPs mediated photodynamic therapy treated group as compared to control and exclusively TBO-chit-Au-AgNPs treated groups. Moreover, the monomicrobial biofilms of *S. aureus* and *P. aeruginosa* was found to be more severely disrupted, almost all the bacterial cells in the biofilms were found dead in TBO-chit-Au-AgNPs mediated PDT group, as compared to control and exclusively TBO-chit-Au-AgNPs treated group (**Figure.7**). Thereby, demonstrating strong antibiofilm action of TBO-chit-Au-AgNPs mediated photodynamic therapy. This observation was further supported by the scanning electron microscopy.

Our result showed that daily topical exposure of TBO-chit-Au-AgNPs followed by light irradiation led to marked reduction of *S. aureus* and *P. aeruginosa* colonization in diabetic foot ulcer rats (Figure.9). Previous study has shown that foot ulcers in the diabetic rats healed slower than those of the non-diabetic rats, however in the present study, TBO-chit-Au-AgNPs mediated PDT remarkably healed foot ulcer in diabetic rats within 7 days, after daily treatment (Figure.10, 11 A, B, C). Such fast recovery might obviate the chances of having further bacterial infection and consequent mortality. Furthermore, the result of histopathological investigation of monomicrobial and polymicrobial DFU revealed that TBO-chit-Au-AgNPs mediated PDT promotes healing and reduces inflammation in DFU rats.

Foot ulcers in diabetic patients take a long time to heal due to a series of cellular and molecular mechanism employed in the process of healing, such as neuropathy, high probability of infection, non-physiological inflammatory response, lack of neoangiogenesis, oxidative stress, insufficient concentrations of growth factors, cellular abnormalities, etc.<sup>1, 32</sup>. We have shown decreased levels of IL-6 and TNF- $\alpha$  in TBO-chit-Au-AgNPs mediated photodynamic therapy treated groups as compared to control, untreated and only TBO-chit-Au-AgNPs treated groups, implying restoration of immunosuppression (Figure. 12-a and **b**). Furthermore, we have found higher levels of EGF and VEGF in all those groups of rats whose ulcers healed as compared to those groups whose ulcers did not healed (**Figure. 12-c and d**). This might be, because EGF promote ulcer healing by stimulating cell growth, proliferation and differentiation whereas, VEGF stimulates vasculogenesis and angiogenesis<sup>23,24</sup>. Besides this, elevated level of TGF- $\beta$ -1 was found in all diabetic groups (with and without foot ulcer) (**Figure.12e**). This is because TGF- $\beta$ -1 is a systemic marker of type-2 diabetes and is positively associated with hyperglycemia. In addition, IGF-1 level was found to be lower in diabetic rats as compared to

non-diabetic and control rats. However, considerable decrease was found in DFU rats with polymicrobial infections (**Figure.12f**). IGF-1 contributes in cell granulation during wound healing; its expression decreases in diabetic patients which resulted into anomalies in cell granulation<sup>25</sup>. Thus, the findings of present study clearly indicate pathogenesis, healing of DFU and infection control.

## **Methodology:**

### **Nanoparticles synthesis**

Chitosan coated gold nanoparticles termed as 'core' was synthesized by mixing 15 ml of  $10 \times 10^{-3}$  M (10 mM)  $\text{HAuCl}_4$  and 90 ml chitosan solution under magnetic stirring at  $50^\circ\text{C}$ . The appearance of a red color indicates the formation of spherical gold nanoparticles. The next step involves 3 consecutive additions of 140  $\mu\text{l}$  of  $10 \times 10^{-1}$  M of  $\text{AgNO}_3$  to a solution containing 70 ml of chit-AuNPs and 560  $\mu\text{l}$  of  $10 \times 10^{-1}$  M of ascorbic acid, the latter used as reducing agent in this mixture.

### **Characterization**

The chit-Au-AgNPs (0.5 mM) with TBO ( $1 \text{ mg ml}^{-1}$ ) were characterized by Transmission electron microscopy and Scanning electron microscopy (SEM) with energy dispersive X-ray spectrometry (EDS) were carried out to verify the presence of gold and silver. Hydrodynamic size and zeta potential of chit-AuNPs, chit-Au-AgNPs and TBO-chit-Au-AgNPs were recorded.

### **Photosensitization and light source**

Red diode laser was used for photosensitization.

The samples acquire the shape of a hemisphere; hence the irradiated area was  $2\pi r^2$ , where  $r$  is the radius of the laser beam exposed, which is equal to 0.35 cm. The beam height from the

base was 24.8 mm. Thus, the value of applied PD was  $0.1300 \text{ Wcm}^{-2}$  and the energy fluency was set to  $100 \text{ Jcm}^{-2}$  (12 minutes and 50 seconds) based on above mentioned formula.

#### **Estimation of *in vitro* antibacterial activity by colony forming assay**

*Staphylococcus aureus* and *Pseudomonas aeruginosa* were grown overnight in BHI broth supplemented with 1% sucrose. Thereafter, the cell densities of the suspensions were adjusted to approximately  $10^8 \text{ CFU/mL}$  using spectrophotometer. 100  $\mu\text{l}$  of the diluted bacterial suspension were added into 96 well plates and incubated for 24 hours at  $37^\circ\text{C}$ . Subsequently, the preformed biofilm was incubated with TBO-chit-Au-AgNPs in the dark for 30 min and then irradiated with  $100 \text{ J/cm}^2$  (12 minutes and 50 seconds). Control (matured biofilm in phosphate buffer saline) and only TBO-chit-Au-AgNPs treated wells were not exposed to laser light. Finally, the resulting biofilm was then disrupted by vortexing followed by 10-fold serial dilution. 100  $\mu\text{l}$  of the diluted suspension was spread onto BHI agar plate and then incubated at  $37^\circ\text{C}$  for 24 h.

#### **Total ROS detection inside the cells**

2', 7'- dichlorofluorescein-diacetate (DCFH-DA) was used to quantify endogenous ROS production in biofilms. Monomicrobial and polymicrobial *S. aureus* and *P. aeruginosa* biofilms were grown for 24 h as described above. After incubation, the cultures were centrifuged at  $10,000 \times g$  for 15 min. Pellets were washed two times with PBS and finally re-suspended in it by adjusting the cell density to  $10^8 \text{ CFU/ml}$  followed by incubation for 10 minutes with  $10 \mu\text{M}$  DCFH-DA. At the end of incubation, the cells were treated with TBO-chit-Au-AgNPs and then irradiated with or without laser light. In, only light treated group, cells were exposed to  $100 \text{ J/cm}^2$  of laser light. Thereafter, the fluorescence intensity produced from DCFH-DA was estimated by excitation at 485 nm using slit width 1.5 nm<sup>35</sup>.

### **Quantification of biofilm formation by crystal violet assay**

Crystal violet assay was performed to quantify the biomass of the monomicrobial and polymicrobial biofilms after treatment with TBO-chit-Au-AgNPs mediated photodynamic therapy. Preformed monomicrobial and polymicrobial biofilms of *S. aureus* and *P. aeruginosa* were grown for 24 hours as described in colony forming assay. Thereafter, the biofilm was incubated with TBO-chit-Au-AgNPs mediated for 30 mins and then exposed to laser light (100 J/cm<sup>2</sup>). Subsequently, the control and treated (only TBO-chit-Au-AgNPs treated, TBO-chit-Au-AgNPs + laser light treated) wells of microtiter plates were fixed with formalin (37%, diluted 1:10) and 2% sodium acetate. Biofilms in different groups were stained with 200µl of crystal violet (0.1%, 15 min). Thereafter, plates were shaken for 10 min to allow full release of the dye and the absorbance was recorded at 630 nm<sup>35</sup>.

### **Live/Dead Staining by CLSM**

Confocal laser scanning microscopy (CLSM) was performed to analyze the consequence of nano photodynamic therapy on monomicrobial and polymicrobial biofilm formation by *S. aureus* and *P. aeruginosa*.

### **Structural imaging of bacteria in biofilms**

Scanning electron microscopy (SEM) was executed to observe the morphology of *S. aureus* and *P. aeruginosa* monomicrobial as well as polymicrobial biofilms after treatment with TBO-chit-Au-AgNPs followed by exposure to laser light.

### **Cytotoxicity Assay**

A cytotoxicity assay was performed on L929 fibroblast cells. Cells (~10<sup>5</sup>cells/well) were seeded in 96-well plates, left overnight to adhere and then treated with various concentrations (0.25 mM, 0.5 mM and 1 mM) of TBO-chit-Au-AgNPs in the presence as well as in the



absence of laser irradiation. After 24 h, cell viabilities were determined by methyl thiazolyl tetrazolium (MTT) assay. The medium was removed, and 100 µl of the mixture solution containing fresh medium and MTT (5 mg/ml) solution was added into each well, followed by incubation for 4 h at 37°C.

### ***In vivo* study**

A total of 56 adult male wistar rats, weighing, 250-300g, were used in this study. Different groups of treated and untreated were made to analyse the data.

### **Experimental induction of diabetes and Foot Ulceration**

The animals were fasted overnight and diabetes was induced by a single intra-peritoneal injection of a freshly prepared solution of streptozotocin (40 mg/kg b. wt) in 0.1 M citrate buffer (pH 4.5)<sup>1</sup>. Induction of foot ulcer was based on an excisional model.

### **Treatment of DFU by nano-photodynamic therapy**

The infected foot ulcers were irradiated daily in morning time for 1 week, starting on the 3rd day post infection induction. TBO-chit-Au-AgNPs was added in the middle and spread over the whole infected area. Thirty minutes after the addition, irradiation was carried out with 630 nm of laser light for 12 minutes and 50 seconds which corresponds to 100 J/cm<sup>2</sup>.

### **Determination of bacterial load reduction (CFU/ml)**

In order to confirm the absence of monomicrobial and polymicrobial *S. aureus* and *P. aeruginosa* colonization, bacterial load was measured for every sample by CFU counting on BHI agar plates and expressed as CFU per ml.

### **Histopathological analysis**

Fresh skin biopsies of control, untreated and treated rat groups were incised and fixed in 10 % phosphate buffered formalin (pH 7.4). The tissues were then dehydrated in ascending grades of ethyl alcohol, cleared in xylene and mounted in molten paraplast at 58-62°C. Thereafter, 5- $\mu$ m histological sections were cut and stained with haematoxylin and eosin (H/E) to examine microorganism response.

### **Effects of nano-photodynamic therapy on growth factors and inflammatory cytokines involved in the pathogenesis of diabetic foot ulcers**

To determine the production level of growth factors and inflammatory cytokines, blood samples were drawn under anesthesia and collected into sterile blood collection tubes from the eyes of controls rats, untreated rats, only TBO-chit-Au-AgNPs treated rats and TBO-chit-Au-AgNPs + laser light treated rats. Rats were immediately sacrificed as per the guidelines.

### **Conclusion**

We have developed a stable and biocompatible chitosan coated gold-silver core shell nanoparticles conjugated with TBO. The synthesized TBO-chit-Au-AgNPs mediated photodynamic therapy was used for the first time to eliminate multi-drug resistant Gram-positive, and Gram-negative, monomicrobial as well as polymicrobial biofilms. Furthermore, this novel nano-phototheranostic complex proved as a nontoxic antibacterial agent to combat DFU caused by multi-drug resistant bacterial strains. Therefore, this approach holds a promising potential to be validated and implemented in clinical translation.

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**Table 1.** Hydrodynamic diameter (size by number) obtained from DLS, Polydispersity index (PDI) and zeta potential values of chit-AuNPs, chit-Au-AgNPs and TBO-chit-Au-AgNPs.

<b>Sample</b>	<b>Size (d.nm)</b>	<b>Polydispersity index (PDI)</b>	<b>Zeta potential (mV)</b>
<b>chit-AuNPs</b>	<b>129.4</b>	<b>0.107</b>	<b>49</b>
<b>chit-Au-AgNPs</b>	<b>131</b>	<b>0.067</b>	<b>37.6</b>
<b>TBO-chit-Au-AgNPs</b>	<b>134</b>	<b>0.085</b>	<b>41.2</b>

**Table 2.** Experimental induction of type-2 diabetes by streptozotocin (STZ) injection.

S.No.	Groups	Blood sugar concentration (mg/dL) in overnight fasting rats before the injection of Streptozotocin (STZ)	Blood sugar concentration (mg/dL) after the injection of Streptozotocin (STZ)
1.	Control (normal rats)	i. 102 ii. 92 iii. 87 iv. 108	-
2.	Diabetic without ulcer (untreated rats)	i. 98 ii. 90 iii. 89 iv. 99	i'. 368 ii'. 239 iii'. 366 iv'. 261
3.	<i>Pseudomonas aeruginosa</i> Diabetic with ulcer (untreated rats)	i. 110 ii. 102 iii. 94 iv. 87	i'. 325 ii'. 338 iii'. 302 iv'. 319



4.	<i>Pseudomonas aeruginosa</i> Diabetic with ulcer (TBO-chit-Au-AgNPs treated rats)	i. 95	i'. 380
		ii. 130	ii'. 397
		iii. 94	iii'. 394
		iv. 102	iv'. 382
5.	<i>Pseudomonas aeruginosa</i> Diabetic with ulcer (TBO-chit-Au-AgNPs +light treated rats)	i. 108	i'. 376
		ii. 100	ii'. 265
		iii. 80	iii'. 277
		iv. 98	iv'. 264
6.	<i>Staphylococcus aureus</i> Diabetic with ulcer (untreated rats)	i. 138	i'. 309
		ii. 112	ii'. 386
		iii. 98	iii'. 287
		iv. 104	iv'. 312
7.	<i>Staphylococcus aureus</i> Diabetic with ulcer (TBO-chit-Au-AgNPs treated rats)	i. 139	i'. 299
		ii. 87	ii'. 310
		iii. 94	iii'. 264
		iv. 93	iv'. 317
8.	<i>Staphylococcus aureus</i>	i. 130	i'. 385
		ii. 123	ii'. 305
		iii. 117	iii'. 377

	Diabetic with ulcer (TBO-chit-Au-AgNPs +light treated rats)	iv. 103	iv'. 338
9.	Polymicrobial ( <i>Staphylococcus aureus</i> + <i>Pseudomonas aeruginosa</i> ) Diabetic with ulcer (untreated rats)	i. 104 ii. 110 iii. 96 iv. 117	i'. 291 ii'. 271 iii'. 359 iv'. 331
10.	Polymicrobial ( <i>Staphylococcus aureus</i> + <i>Pseudomonas aeruginosa</i> ) Diabetic with ulcer (TBO-chit-Au-AgNPs treated rats)	i. 109 ii. 106 iii. 103 iv. 122	i'. 362 ii'. 358 iii'. 376 iv'. 321
11.	Polymicrobial ( <i>Staphylococcus aureus</i> + <i>Pseudomonas aeruginosa</i> ) Diabetic with ulcer (TBO-chit-Au-AgNPs +light treated rats)	i. 106 ii. 114 iii. 73 iv. 111	i'. 319 ii'. 375 iii'. 332 iv'. 345



Asad Ullah Khan