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Photo-Responsive Functional Gold Nanocapsules for Inactivation of Community-Acquired, Hivirulent, Multidrug-Resistant MRSA

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Reagents:

Cetyl trimethyl ammonium bromide (CTAB), methoxy polyethylene glycol thiol (mPEG-SH), curcumin, $HAuCl_4$, $NaBH_4$, sodium salicylate, 5-bromosalicylic acid, $AgNO_3$, $HAuCl_4$, ascorbic acid, CH_2Cl_2, polyethyleneimine (PEI), 2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA), 1-N-phenylnaphthylamine (NPN), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid buffer (HEPES), 3′-dipropylthiadicarbocyanine iodide (DiSC3(5)), glucose buffer, glutaraldehyde, ethanol, fluorescein diacetate (FDA), propidium iodide (PI) phosphate buffered saline (PBS), tryptic soy broth (TSB), dithiothreitol (DTT), NaOH, tryptic soy agar (TSA), all these reagents and buffers are from Sigma-Aldrich.

DNA aptamer from Aptagen, methicillin-resistant *Staphylococcus aureus* (MRSA) USA 300 is available in the Facuty of Medicine, Department of Microbiology, Medical University of Wrocław.

Methods:

Preparation of gold nanocapsules: Synthesis procedure of gold nanocapsules involves four intermediate steps these are (i) synthesis of 30 nm gold nanorods (GNRs), (ii) ligand Exchange on CTAB-Capped GNRs with mPEG-SH, (iii) adding curcumin (Cur) to mPEG-SH@GNRs and (iv) aptamers functionalization of Cur@mPEG-SH@GNRs.

(i) Synthesis of GNRs: ~30 nm in length GNRs with longitudinal surface plasmon resonance (LSPR) located at λ_{LSPR} = 800 nm were prepared by following a two-step seed-mediated method[1,2]. A seed solution was prepared by mixing 10 mL of 0.5 mM $HAuCl_4$ (5.0 mmol) with 10 mL of 0.4 M CTAB (4 mmol) in a 40 mL tube. To this solution, 0.6 mL of 0.02 M freshly prepared $NaBH_4$ (0.012 mmol) was added under stirring. The solution color observed brownish-yellow, immediately. Further, the resultant seed solution was vigorously stirred at 1200 rpm for 2 min and kept at 25 °C for further use. In the second step growth solution is prepared using 18.0 g of CTAB (49.2 mmol), 1.6 g of sodium salicylate (10 mmol) and 2.2 g of 5-bromosalicylic acid (10 mmol) in 250 mL warm water (~60 °C). The mixture was allowed to cool to 30 °C, and then 18 mL water dissolved silver nitrate ($AgNO_3$, 8 mM) was added. The solution was kept undisturbed at 30 °C for 25 min and then 250 mL of aqueous 1 mM $HAuCl_4$ solution (0.50 mmol) was added. After 20 min of slow stirring (500 rpm), 2 mL ascorbic acid solution was added (124

mM). The solution became colorless after 30 s of stirring. In the final step, freshly prepared 0.4 mL seed solution was injected gently into the growth solution and left undisturbed for 18 h. CTAB grafted GNRs were collected by centrifugation, and the supernatant was discarded and finally CTAB@GNRs were redispersed in water.

(ii) Ligand exchange with mPEG-SH: CTAB is exchanged with mPEG-SH (MW = 750 Da) by modifying the protocol reported earlier[3]. In brief, CTAB grafted GNRs are washed with water 3 times and centrifuged at 5000 rpm to remove excess CTAB. 0.5 M mPEG-SH was added to the GNR solution. The pH of the total solution was adjusted to 8 by adding NaOH and the solution further kept on stirring for 12 h. The resultant solution then washed by water and centrifuged at 5000 rcf. for 10 min. The mPEG-SH grafting was observed by significant decrease in ζ-potential, from 32 mV (CTAB@GNRs) to 7 mV (mPEG-SH@GNRs).

(iii) Curcumin functionalization: 10 mM curcumin solution were prepared in 1 mL of CH_2Cl_2, this solution is slowly added in 5 mL of GNRs@mPEG solution, and after mutual phase separation, the mixture was emulsified with a tip probe sonicator (600 W input, 2 min, 80% ampl). Afterwards residual organic solvent was evaporated under reduced pressure. The so-obtained GNRs@mPEG@Cur were then concentrated and purified with a centrifugal filter device (Amicon Ultra, Ultracel membrane with 100,000 NMWL, Millipore, USA), further washing with water and filtered on syringe filter SterivexTM-GP 0.22 μm of polyether sulfone (Millipore, USA).

(iv) Aptamer functionalization: Staphylococcus aureus targeting DNA aptamer each consisting of 18 nucleotides (5'-ATC CAG AGT GAC GCA GCA $(N)_{40}$ TGG ACA CGG TGG CTT AGT-3') was selected for conjugation [4]. The thiol group on the 5'-end was deprotected by DTT. To functionalize the Cur@mPEG-SH@GNRs with aptamer, initially solution of GNRs@mPEG-SH@Cur is suspended in 0.5 mM polyethyleneimine (PEI) for 10 min in water and then washed twice with water. GNRs@mPEG-SH@Cur assembly is then incubated with aptamer (final concentration of 500 nM) overnight at room temperature. After functionalization of the aptamers onto GNRs@mPEG-SH@Cur, the whole assembly of nanocapsules (GNRs@mPEG-SH@Cur@Apt) is formed, the solution was subjected to washing through two centrifugation cycles at 10,000 rpm for 10 min. Finally, the mixtures were re-dispersed in 2 mL of 10 mM phosphate buffered saline (PBS) for analysis, UV absorbance was used to calculate the aptamer concentrations using UV–vis spectrophotometer. The GNR concentration is estimated by using following steps: (i) measuring the Au concentration through inductively coupled plasma mass

spectrometry (ICP-MS), (ii) determining the size and aspect ratio (AR) of GNRs with TEM, (iii) calculated the average size on a population of 100 GNRs, (iv) AR is calculated from UV-Vis, (v) then the volume per nanorod by approximating the cylinder and assuming the bulk crystal structure of gold (face-centered cubic (fcc), 4.08 Å).

In vitro curcumin release kinetics: Amount of encapsulated curcumin and released kinetics was evaluated by comparing UV-vis absorbance of GNRs@mPEG-SH@Cur and GNRs@mPEG-SH@Cur@Apt samples, dissolved in methanol to standard curve of curcumin. Release over time was evaluated by dispersing individual aliquots of 100 µg/mL both samples in phosphate buffered saline (PBS, pH = 7.4) and incubating at 37 °C at 100 rpm. At 2-hour intervals, individual samples were pelleted and dissolved in methanol to solubilize unreleased curcumin. The amount released was calculated by dividing absorbance at each time point by absorbance of the estimated encapsulated maximum.

Characterization and photothermal properties of nanocapsules: The size and morphology of nanocapsule were observed under a high resolution transmission electron microscope (HRTEM) (Cs-corrected/EDS/EELS model JEM ARM 200F, JEOL, USA). PSS-NICOMP-380 ZLS (USA) particle sizing and zeta potential system were used for size distribution and zeta potentials. Photothermal conversion efficacy of nanocapsule was analyzed with an 800-nm infrared diode laser with a varying power density of 1 W/cm^2 (Changchun New Industries Optoelectronics Technology Co., Ltd., Changchun, China) and a thermometer (UNI-T 1310, UNI-T Electronic Corp., Dongguan, China).

Bacterial growth and culture conditions: Strains of methicillin-resistant *Staphylococcus aureus* (*MRSA*) USA300 were obtained from Centres for Disease Control and Prevention (CDCP), the strain deposited in BEI Resources. MRSA were grown in tryptic soy broth (TSB) media under orbital shaking at 200 rpm or on tryptic soy agar plates (TSA, 1.5% agar) at 37 °C. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}). Bactericidal activity of nanoformulations. Percentage reduction of bacterial viable count of S. aureus USA 300 is presented. The Percentage reduction of viable count was calculated over different concentrations of nanoformulations using two fold broth micro dilution method in 96-well plates.

The percentage of bacterial reduction was calculated using Equation

$$\%\ Reduction = \frac{Initial\ count\left(\frac{CFU}{mL}\right) - Test\ count\ result\left(\frac{CFU}{mL}\right)}{Initial\ count\left(\frac{CFU}{mL}\right)} \times 100$$

All experiments were performed in triplicates.

Nanocapsule anti-biofilm activity, qualitative and quantitative study: The effect of nanocapsules on biofilm formation was studied by using a standard protocol[5]. Briefly, MRSA were grown overnight and diluted 1:100 in fresh medium for the biofilm assay. Nanocapsules and other control samples at varying concentrations were added to the microtiter wells and confocal slides 24 h at 37 °C to allow for biofilm formation. After the incubation for 24 h, the old media is replaced by fresh media and plates were incubated for an additional 24 h. After final incubation, plates were gently submerged in autoclaved MilliQ water and water was removed by gentle flipping. This process was repeated twice. Biofilm quantitative and qualitative analysis is performed using crystal violet assay and confocal microscopy, respectively, methodology is reported in our recent publication[6].

Nanocapsule PTT treatment on MRSA: The initial bacterial cell number was maintained at an OD_{600} of 1 for all PTT experiments. The PTT treatment was applied to either free bacterium (without nanocapsules) and with all other controls in PBS, to estimate the nanocapsule mediated PTT killing in 10 mL PBS buffer (total, 1×10^8 cells). The untreated control cells at 25 °C and 48 °C were incubated with or without nanocapsules to evaluate the individual effects of temperature and nanocapsules on antibacterial activity. After time and temperature dependent PTT samples (with or without nanocapsules) were diluted 5 fold and the killing percentage was determined by counting the colony forming units (CFU).

***Reactive oxygen species (ROS) measurement*:** Total ROS levels were measured using 2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent dye assay technique, to determine the generation of free radicals with and without PTT. Control and PTT exposed cells at 25 °C and 48 °C with or without nanocapsules samples were washed three times in PBS after treatment. Equal cell numbers of all bacterial samples were adjusted to an OD_{600} equivalent of 0.1. The bacterial

samples were stained with 10 μM ROS-specific fluorescent dye DCFH-DA and samples kept for 30 min in the dark at the RT. Samples then washed with PBS and extra dye was washed out. Fluorescence was recorded using a microplate reader with excitation at 488 nm and emission at 525 nm (Cytation 5 Multi-Mode Reader from BioTek Instruments). The total ROS level was presented as a percent relative to the control in triplicates.

***Assessment of bacterial membrane integrity*:**

Inner membrane potential: 3,3′-dipropylthiadicarbocyanine iodide (DiSC3(5)) dye based biochemical assay were used to find the inner membrane potential disruption. Cell numbers were adjusted 0.1 OD in HEPES/glucose buffer (5 mM HEPES, 5 mM glucose, pH 7.0) in 48 well plate. 10 μL of 10 mM DiSC3(5) solution is added into each well and kept in dark at the RT for 30 min. Fluorescence at 670 nm was measured after excitation at 622 nm.

Scanning electron microscopy (SEM) of bacterial samples. Bacterial samples with nanocapsule mediated PTT and all controls were collected by centrifugation at 6000 rpm for 3 min and then washed with PBS. The cells were fixed in 2.5% glutaraldehyde for 12 h at 4 °C, after that cells were washed with PBS to remove unwanted glutaraldehyde. Finally, cells were dehydrated using ethanol gradient of 10, 20, 40, 80% and finally were re-suspended in 100% ethanol. Finally, samples were put on the silicon grid and observed under SEM.

Live/dead staining. The nanocapsule mediated PTT samples and control MRSA cells were washed in PBS, OD_{600} of all samples was adjusted to 0.1 (1×10^7 cells). For live/dead staining, 980 μ L cell samples were stained with 10 μL FDA (10 mM) and 10 μL PI (10 mM) for 10 min and washed twice using PBS to remove surface unbound dye. 100 μL stained cells were drop on glass slides, covered by a coverslip, and sealed with nail polish. Fluorescence images were taken under a confocal microscope (40X magnification Zeiss LSM 510 Meta).

Figure S1

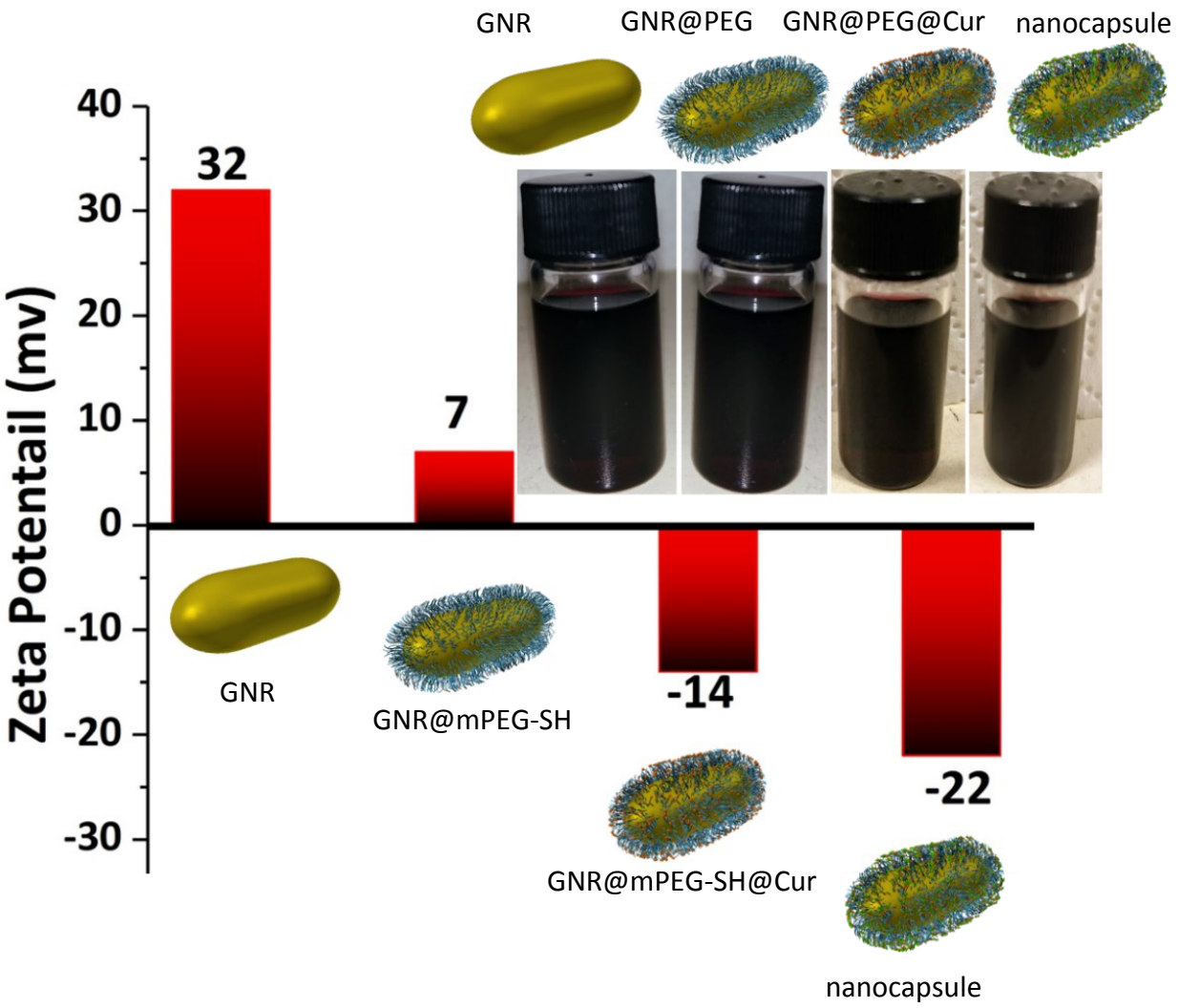

Figure S1 Zeta potential values and photographs of solutions of various nanoassemblies including nanocapsule.

Figure S2

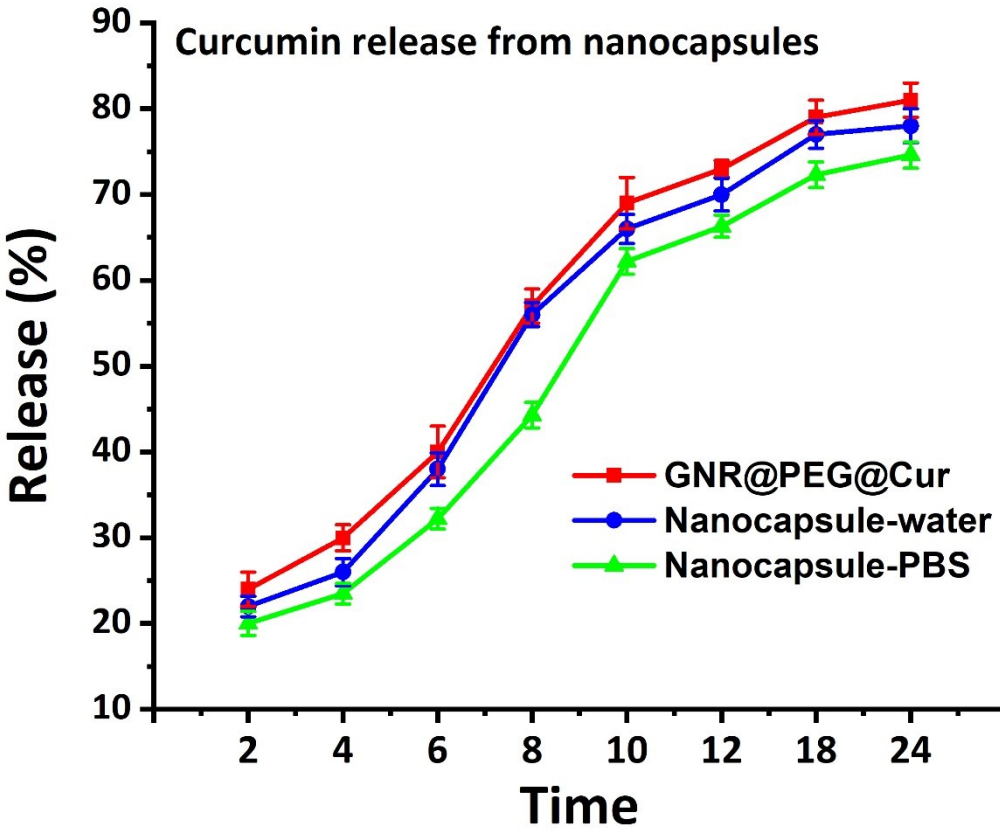

Figure S2 Curcumin release profile along the time in 24 h in aqueous medium (pH 7.4) and in PBS (pH 7.4) .

Figure S3

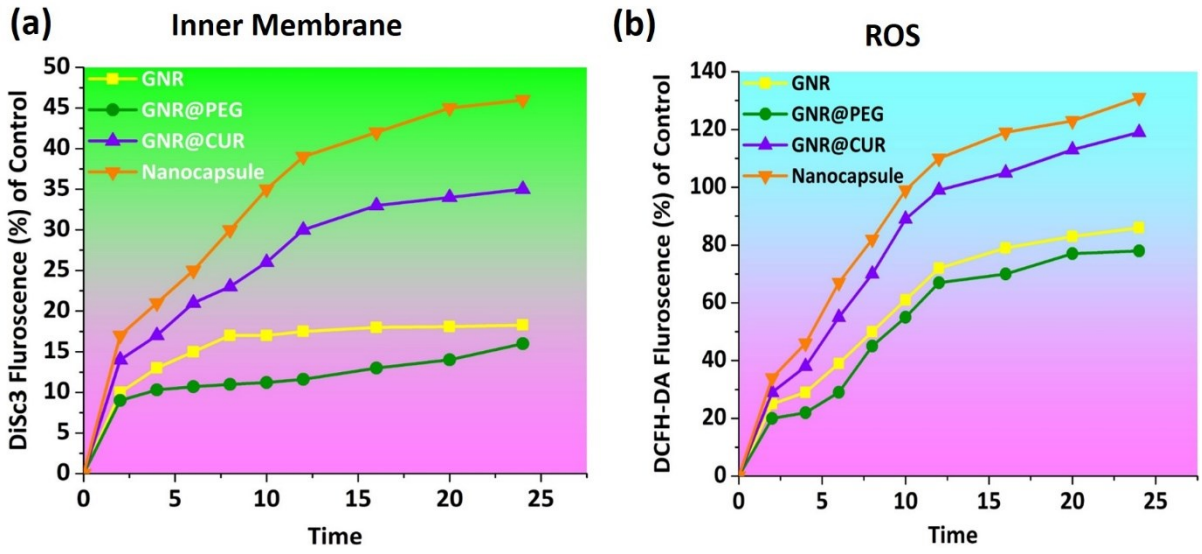

Figure S3 Membrane potential and ROS assesment of MRSA after nanocapsule PTT treatment.

Figure S4

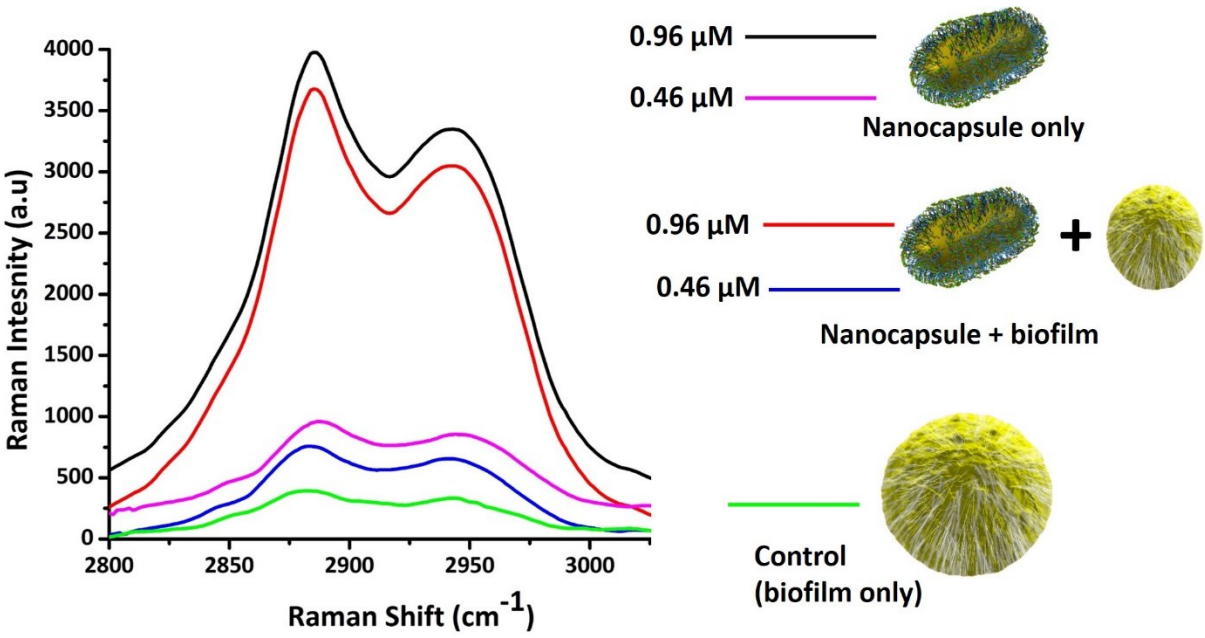

Figure S4 Surface Enhanced Raman (SERS) spectra of nanocapsule and nanocapsule incubated biofilms of MRSA with controls.

Figure S5

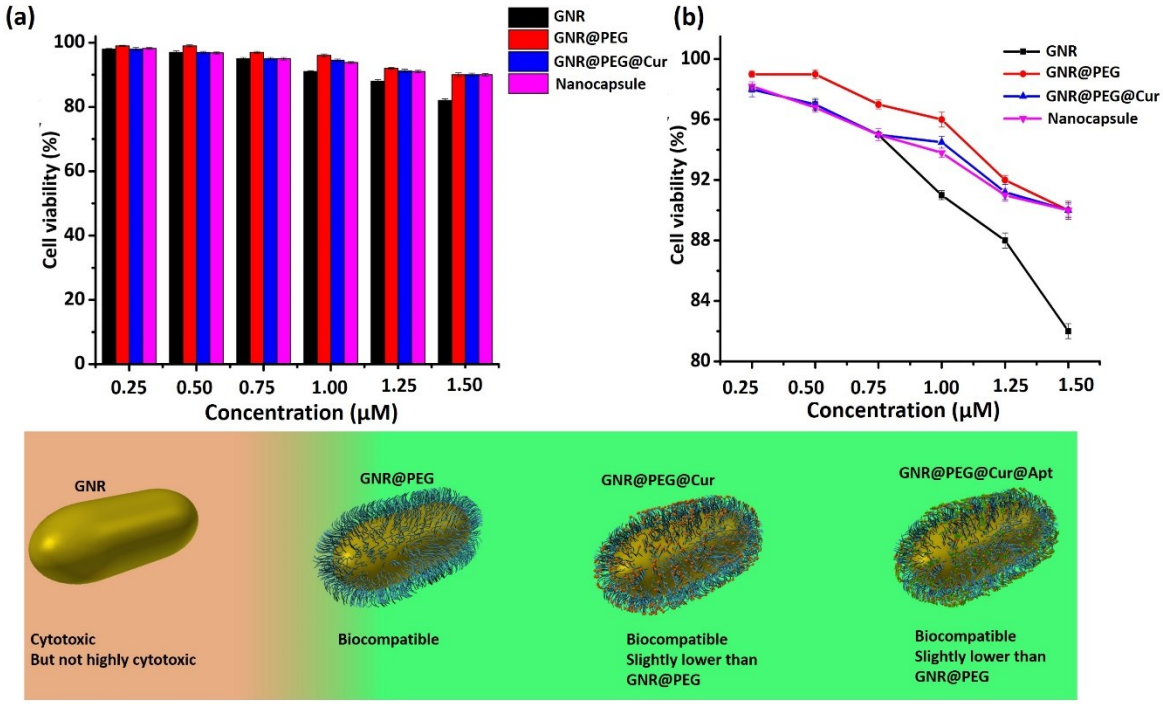

Figure S5 Cytotoxicity profile of the nanocapsule formulations on L929 cell lines. Cell toxicity is assesed to find the suitable biocompatability of these formulation for further in vivo applications.

Figure S6

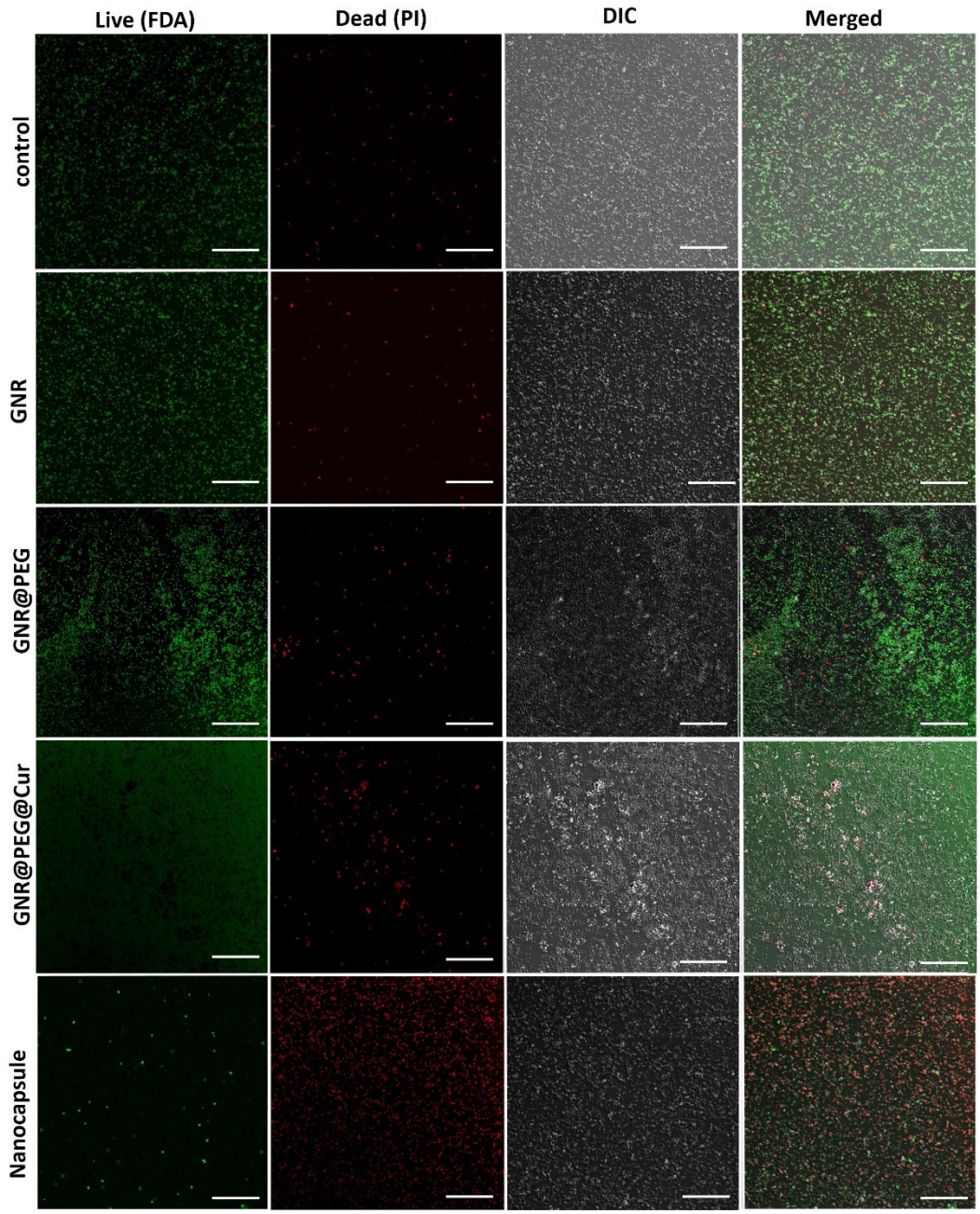

Figure S6. Representative confocal fluorescence microscopic images of bacterial biofilms on the different nanoformulations, nanoformulations were coated on glass surface and then biofilms were grown. *S. aureus* USA 300 were cultured for 10 h on the control and different nanoformulations-coated surface and stained with live/dead staining before imaging with a fluorescence microscope. The conecntration of nanofmrulations are standardized with MIC value of nanocapsule i.e. 0.92 μM and the same concentration of other nanoformulations were used to compare the synergy of antibacterial agent and aptamer on biofilm inhibition. Scale in the confocal imaging is 10 μm.

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