Supporting Information

Remotely triggered micro-shock wave responsive drug delivery system for resolving diabetic wound infection and controlling blood sugar levels

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Materials and Methods

Materials

Spermidine (+ve) (Purity ≥ 99%), Dextran sulfate (-ve) (DS, M_w = 500 kDa) (Purity – Research grade), Ciprofloxacin (M_W = 331) (Purity ≥ 99%) and Fluorescein isothiocyanate (FITC) (Purity ≥ 97.5%) were purchased from Sigma Aldrich (Bangalore, India). Insulin (Human Mixtard®50 – Novo Nordisk).Hydrogen fluoride (HF) was obtained from Thomas Baker Ltd (Bangalore, India). Acetic acid (CH_3COOH) (Purity ≥ 99%), Sodium chloride (NaCl) (Purity ≥ 99.9%), Sodium Hydroxide (NaOH) (Purity ≥ 99.9%), Hydrochloric acid (HCl) (Purity ≥ 99%) and Citrate buffer (hipure) were obtained from Rankem, RFLC Limited (Bangalore, India). Double autoclaved de-ionized water (Millipore, Billerica, MA, USA) was used for all the experiments.

Animal experiments license

All the experiments were carried out in accordance with the approved guidelines of institutional animal ethics committee at Indian Institute of Science, Bangalore, India (Registration No: 48/1999/CPCSEA). All procedures with animals were carried out in accordance with the institutional rules for animal experimentation.

Preparation of hollow capsules

The hollow capsules were prepared by the layer by layer technique. The principle behind this process is the sequential deposition of oppositely charged polyelectrolytes onto a sacrificial template such as calcium carbonate ($CaCO_3$). The negatively charged template was incubated with the positively charged spermidine solution (1mg/ml in 1 M NaCl at pH 5.6) for about 30 min at room temperature. The unadsorbed spermidine was removed by washing the sample thrice by centrifugation at 4000 rpm for 5 min (MIKRO 200R; Hettich Zentrifugen, Tuttlingen, Germany). This was followed by deposition of dextran sulfate (1mg/ml in 1 M NaCl) by incorporating the same procedure. This process was continued till 4 bi-layers of the polyelectrolytes [$(SD)_4$] were fabricated. In order to obtain hollow capsules, the $CaCO_3$ core is leached

out from the coated particles by treatment with 0.2 M EDTA for 10 min. In order to ascertain the removal of EDTA, the samples were subjected to washing five times with water (pH 5.6) by centrifugation at 2000 rpm for 8 min. The capsules were stored at 4°C before using.

Zeta potential measurements

The LbL assembly of positively charged Spermidine (sper) and negatively charged dextran sulfate (DS) on $CaCO_3$ templates was followed by measuring the zeta potential after each deposition using Zetasizer Nano ZS (Malvern, Southborough, MA). The zeta potential values were calculated using the Smoluchowski relation between the ionic mobility and the surface charge of the particles. Each value noted was the average of three repeated measurements performed at pH 5.6.

Drug loading studies

In order to load ciprofloxacin (1 mg/ml) or insulin (40 IU/ml) into the capsules, 400µl of the drug was incubated with 200 µl (2 mg/ml) of hollow capsules for 12 h. The electrostatic interaction between the polyelectrolytes was reduced by changing the pH of the sample to 8 at room temperature by the addition of 0.1 M NaCl. By 12 h, the amount of drug loaded into the capsules would have reached the saturation limit. This was followed by locking of the capsule layers and retention of ciprofloxacin / insulin was ensured by changing the pH of the sample to 4 with the addition of 0.1 M HCl. The difference between the initial loading concentration and the drug concentration in the supernatant after retention within the capsule gave the amount of loaded drug. Ciprofloxacin concentration was determined using a fluorimeter (TEKAN, Infinite M200 Pro, Switzerland) with excitation at 280 nm and emission at 450 nm for ciprofloxacin. Insulin concentration was measured by Bradford method by taking absorption at 595 nm and using insulin standard curve as described elsewhere[1].

Drug release studies

After loading studies, the sample was washed twice by centrifugation at 2000 rpm for 5 min; to remove the unloaded ciprofloxacin / insulin. The loaded capsules were incubated in citrate buffer (pH 4.8), PBS (pH 7.4) and pH 9.0 buffer at 37°C to study the release profile of ciprofloxacin / insulin and placed on a shaker at 100 rpm/min. The supernatant was taken out at different time points, and the amount of ciprofloxacin / insulin released was quantified as mentioned before. The volume of supernatant, which has been taken out for analysis, is replaced by an equivalent volume of buffer (pre-warmed to 37°C). At 2 h, 24 h and 40 h the particles were removed from PBS and analyzed with SEM as described below.

Characterization of the capsules using SEM

The capsule samples were dropped onto a clean silicon wafer and dried overnight. These samples were processed for SEM characterization. As electrical conductivity is a pre-requisite for SEM imaging, the samples were subjected to gold sputtering (JEOL JFC 1100E Ion sputtering device; JEOL, Tokyo, Japan) and analyzed by Field emission - Scanning electron microscopy (SEM) (FEI-SIRION, Eindhoven, Netherlands).

Cytotoxicity assay

MTT assay was performed in Intestine 407 and Hela epithelial cell lines (Obtained from National Center for Cell Science, Pune, India) to assess the *in vitro* cytotoxicity of empty and ciprofloxacin loaded Sper-DS capsules. The cell line was maintained at 37°C and 5% CO_2in Dulbecco Modified Eagle Medium (DMEM; Sigma) supplemented with 10% fetal calf serum (Sigma). In a 96-well plate 5×10^4 cells were seeded and incubated for 14 h. This is followed by incubation with various concentrations of empty capsules 48 h. 20µl of MTT dye (5 mg/ml) was added in each well and kept for 4 h at 37°C. If the cells are viable, MTT will be reduced to insoluble formazan crystals which are then solubilised by DMSO (dimethyl sulfoxide) into a purple coloured solution. Spectrophotometer reading at 570 nm was taken and the percentage of cell viability was determined with reference to non-treated cells[2].

Micro-shock wave apparatus

Previously, small amount of explosives was used to generate micro-shock waves in a repeatable and controlled manner which was found to be a simple method[3-5]. The device consists of an explosive-coated polymer tube (DynoNobel, Sweden), ignition system (DynoNobel, Sweden), a copper foil with 100 µm thickness, drug loading chamber and a cavity holder. The polymer tube was ignited from one end and the drug loading chamber was placed at the other end. The metal foil was placed to transfer the micro-shock wave energy to the drug loading chamber. When the micro-shock waves pass through the chamber the pressure increases in the liquid chamber which leads to the release of drug from the Sper-DS micro particle aggregates.

Micro-shock wave responsive release

The drug loading chamber was filled with micro particle aggregates loaded with ciprofloxacin / insulin and 0.15 mm copper diaphragm was placed between the polymer tube and the micro particles. The delivery system was exposed to micro-shock waves by igniting the polymer tube at the other end. The released ciprofloxacin / insulin was measured as mentioned earlier. The micro particles were exposed to micro-shock waves different number of times and the released ciprofloxacin / insulin concentration was measured.

Disc diffusion assay

S.aureus culture was plated on LB agar and a sterile Whatman filter paper disc was placed in the middle of the plate. The disc was loaded with 10 µl of PBS, ciprofloxacin (4 µg/ml), empty capsules or capsules loaded with ciprofloxacin (equivalent to ciprofloxacin concentration). These discs were treated with or without micro-shock waves and incubated at 37°C for 12 h. The clearing zone, which indicated the death of *S.aureus* was measured to assess the shock wave responsive stimuli.

***S.aureus* skin infection model**

All procedures with animals were carried out in accordance with the institutional rules for animal experimentation. The Central Animal Facility at the Indian Institute of Science provided BALB/c mice of 6-8 week old. The mice were

anesthetized by intra peritoneal injection of 100 mg/kg body weight of ketamine and 10 mg/kg body weight of xylazine mixture. The fur was stripped from the mice with an elastic adhesive bandage. An area of 2 cm^2 was stripped with the tape to create a superficial skin infection by *S.aureus*[6]. 5 µl of overnight grown *S.aureus* (1 x 10^6) in PBS was applied on the surface of the skin and allowed to dry. The mice were treated with topical application of 10 µl ciprofloxacin (4 µg/ml) once a day for 3 days with or without shock wave exposure. An equivalent amount of sper-DS microparticles (empty or ciprofloxacin loaded) were applied on the infected surface once a day for 3 days with or without micro-shock wave exposure. After treatment for 3 days, the mice were sacrificed and checked for *S.aureus* infection in the skin by homogenization and plating.

Diabetic mice model

All procedures with animals were carried out in accordance with the institutional rules for animal experimentation. Diabetic mouse model were used for *S.aureus* skin infection due to the relevance of diabetes with delayed wound healing and biofilm infection[7]. Diabetes was induced in mice by giving intraperitoneal injection of alloxan (200mg/kg body weight, single dose). Mice with a blood glucose level more than 250 mg/dl were considered diabetic[8]. These mice were used for *S.aureus* skin infection and as diabetic model.

Insulin delivery

All procedures with animals were carried out in accordance with the institutional rules for animal experimentation. Diabetic mice were administered subcutaneously with insulin loaded micro particles (100 µl) or free insulin (1.0 IU/kg) with or without micro-shock wave exposure (once or on different days) at the site of administration. At different time points, blood glucose level was measured.

Statistical analysis

Statistical analysis was done with Student's *t* test and Mann-Whitney U test with the help of Graph Pad Prism 5 software. *P* value of <0.05 was considered as significant.

References

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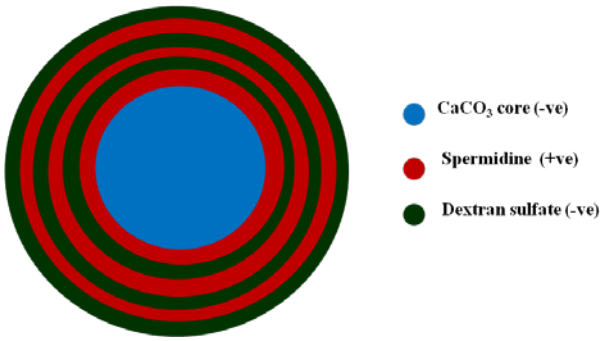
Figure S1

(a)



(b)

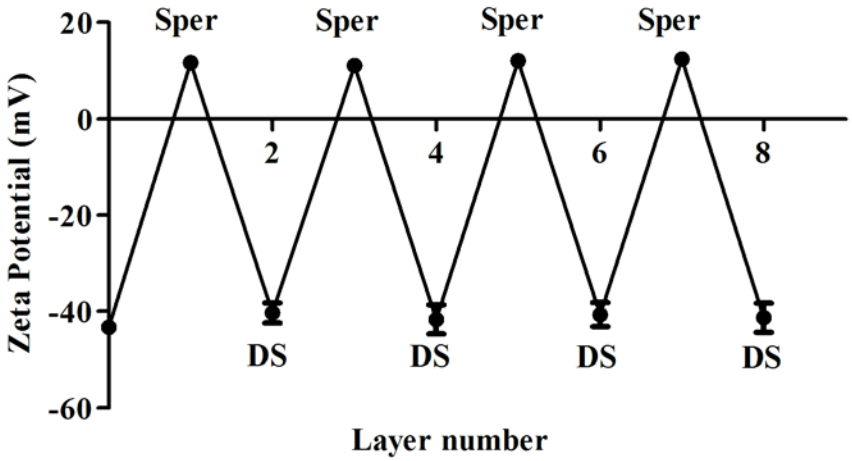
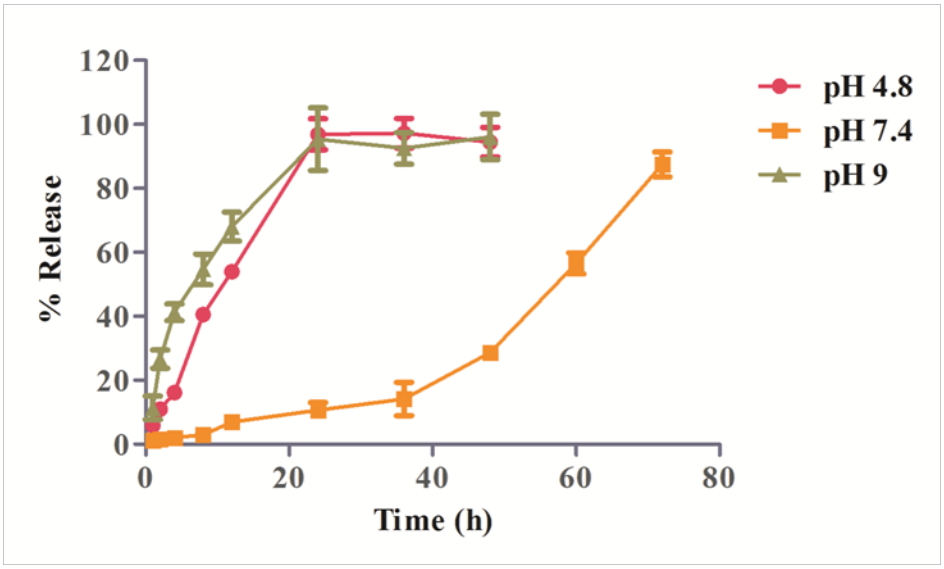


Fig. S1 (a) Schematic representation of Spermidine – Dextran sulfate capsules. (b) Zeta potential variation as a function of outermost adsorbed layer. It can be seen that Spermidine (sper) as outermost layer gave a positive zeta potential whereas dextran sulfate (DS) as in the outermost layer gave negative zeta potential value.

Figure S2

(a)



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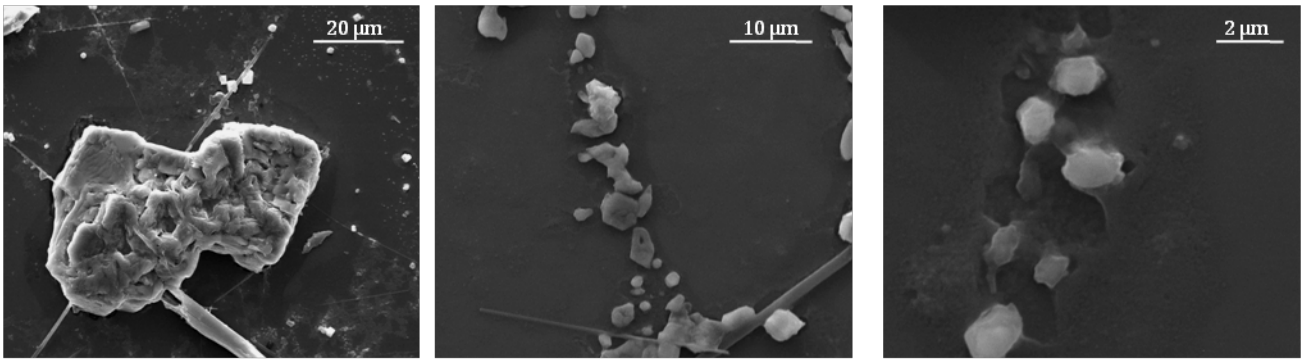

Fig. S2 (a) Ciprofloxacin loaded particles were placed in different pH solutions (pH 7.4, pH 4.8 and pH 9) and the released ciprofloxacin was measured by fluorimeter with excitation at 280 nm and emission at 450 nm. Representative data of three independent experiments. (b) SEM images of Sper-DS microparticle aggregates placed in PBS (pH 7.4) for 2h, 24 h and 40 h.

Figure S3

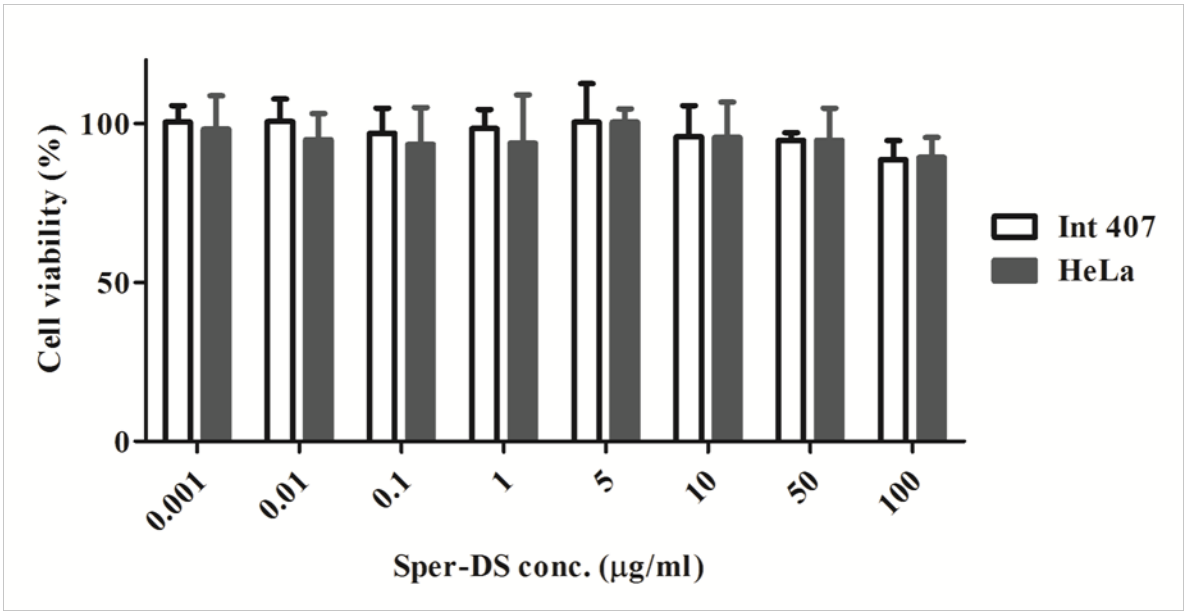

Fig. S3 Cytotoxicity effect of different concentration of empty capsules were checked using MTT assay in Intestine 407 and HeLa epithelial cell lines. Values are shown as mean±S.D. Representative data of three independent experiment.

Figure S4

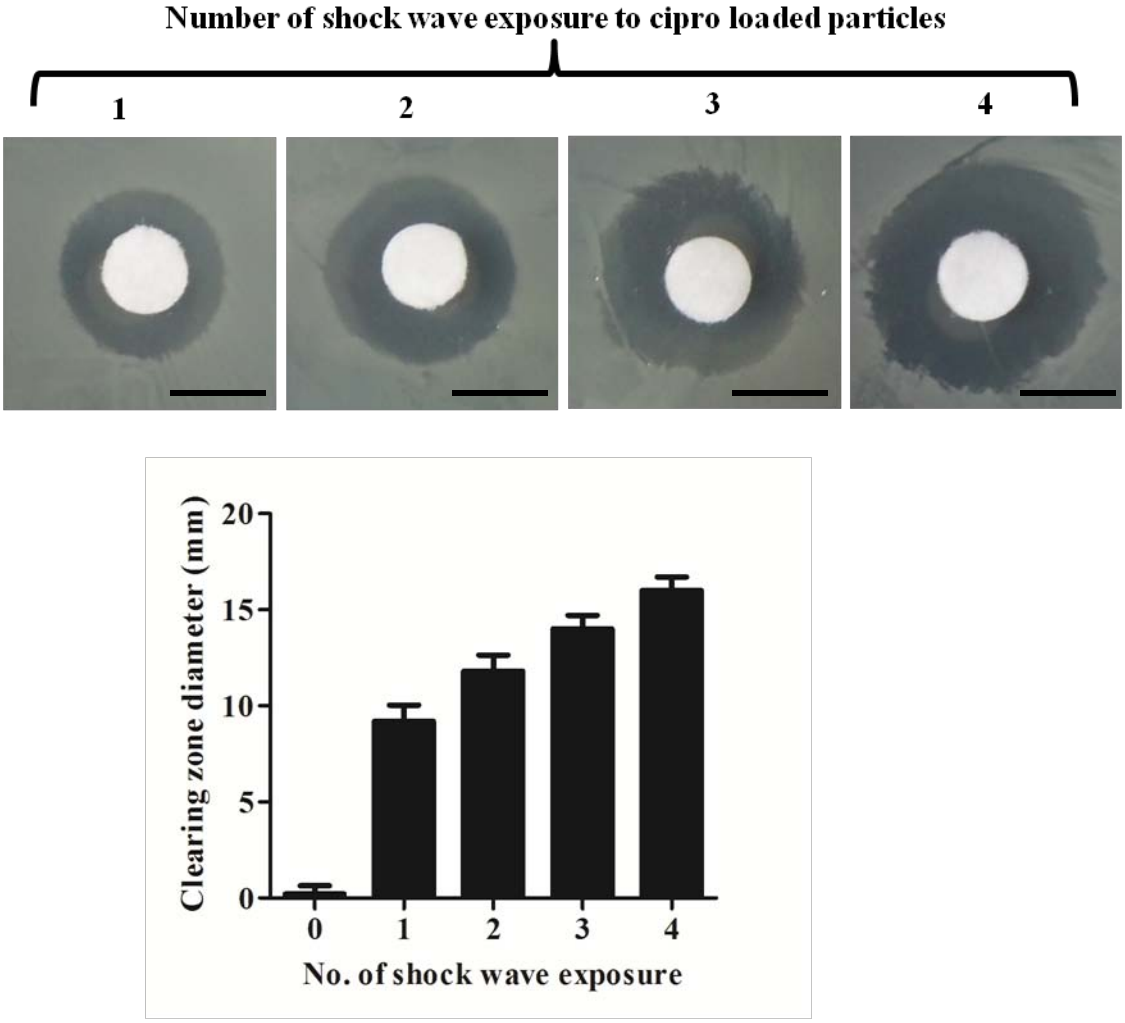

Fig. S4 A sterile filter disc was placed in *S.aureus* spread plate and the clearing zone was measured after 12 h micro-shock wave treatments. Dose dependent micro-shock wave responsive release was studied by exposing the ciprofloxacin loaded particles to shock waves with different number of times. The clearing zone was measured and plotted. Scale bar – 5 mm. Values are shown as mean±S.D. Representative data of three independent experiments.

Figure S5

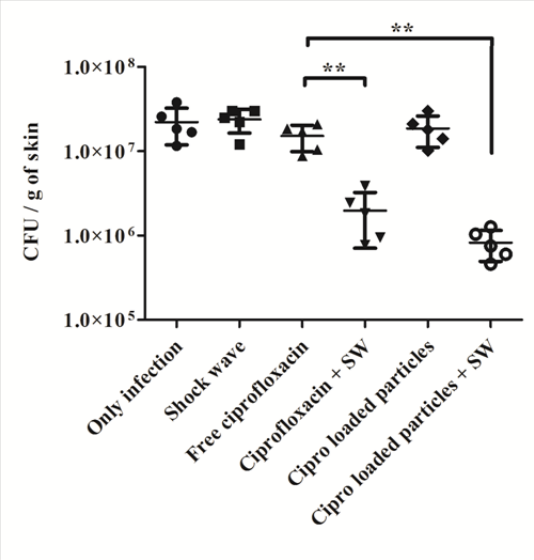

Fig. S5 The fur of BALB/c mice was stripped with adhesive tape and 5 µl of *S.aureus* ($1x10^6$ bacteria) was placed in the skin. Alloxan treated mice having blood glucose level > 250 mg/dl were considered as diabetic mice and they were provided with different treatments after *S.aureus* skin infection. The bacterial burden was assessed after 3 days of treatment.

Figure S6

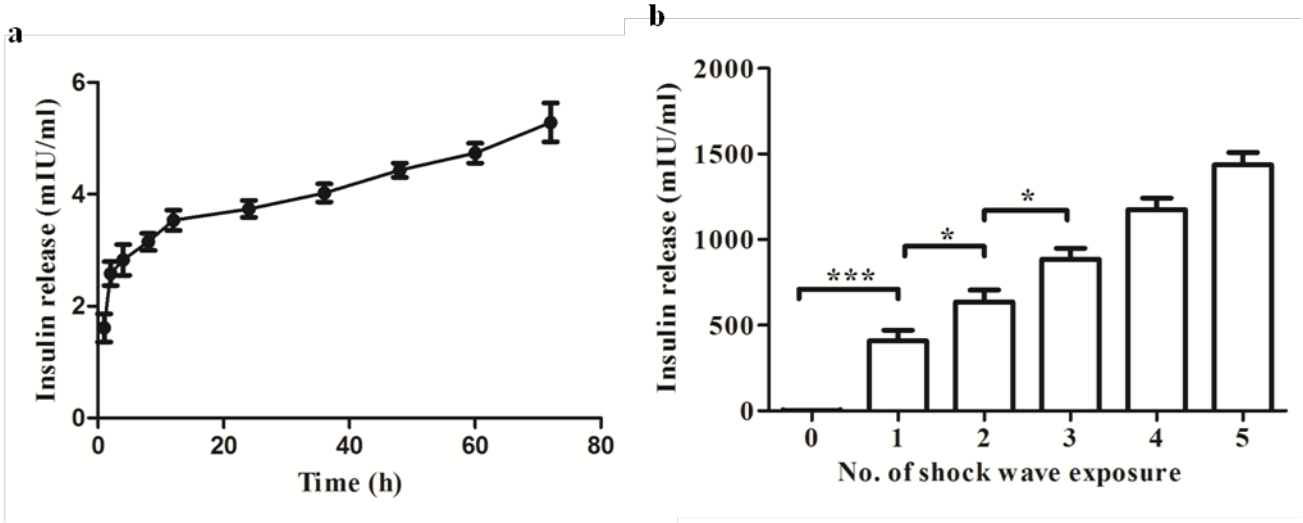

Fig. S6 (a) Insulin loaded particles were placed in PBS (pH 7.4) and the released insulin was measured by Bradford method. (b) Insulin loaded particles were exposed to shock waves with different number of times and the release of insulin was measured.

Figure S7

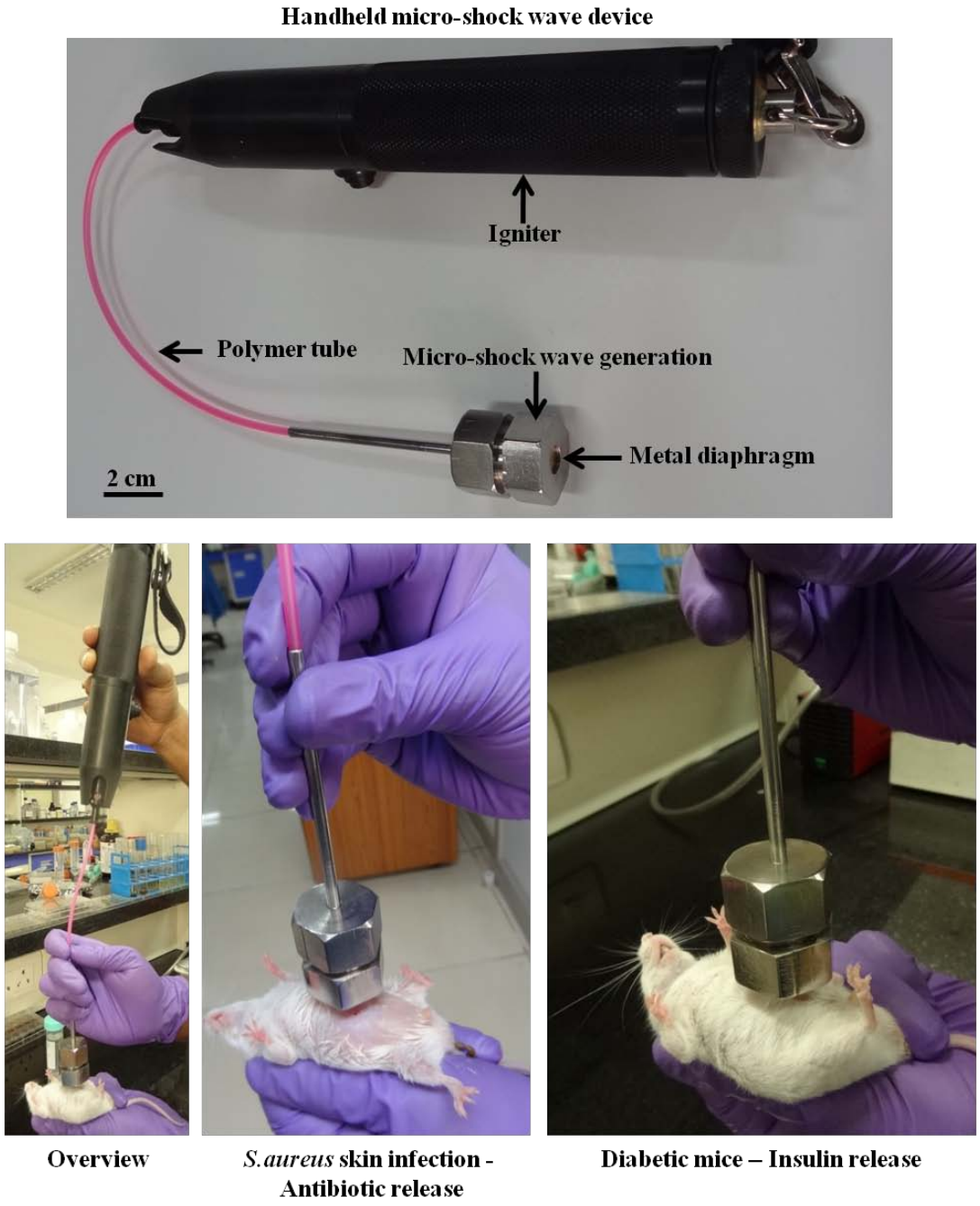

Fig. S7 Working of the micro shock wave trigger for insulin and antibiotic release. The hand held micro shock wave generator can be conveniently used to trigger the release of the insulin or antibiotic from the Sper-DS microparticles. No contact is required to trigger the release between the device and the skin of the mouse.