Supplementary Information:

Tailored smart bioactive glass nanoassembly for the dual antibiotic in vitro sustained release against osteomyelitis

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S1. Discussion on methodology adopted for bioactivity test, cell culture studies and bacterial viability Test

S1.1 Bioactivity Test

In brief, the powder sample were pelleted of equal shape and size of 10 mm diameter and dipped in SBF. The ratio of sample to SBF was maintained at 1 mg into 1 ml SBF solution for 3 days in an ORBITEK incubator (Scigenics Biotech) at 37°C under sterile conditions. Once removed from the incubation solution, first the samples were rinsed gently with Milli-Q water and then in acetone. Subsequently, the samples were dried in air at room temperature and stored in a desiccator for further characterizations by XRD, FTIR and SEM.

S1.2 Cell studies

The osteoblast like osteosarcoma cell lines were maintained in DMEM high glucose medium with 10% heat-inactivated fetal bovine serum and 0.1% streptomycin at 37 °C with 5% CO₂. The cells were detached from the culture plate at 80–85% confluence and further used for evaluating the biomaterial cytocompatibility.

Initially, elution test (ISO 10993-5) was carried out wherein, different stages of the bioglass nanoassemblies at a concentration of 50, 100 and 200 µg/ml were incubated with the Saos2 cells 24 h and subjected to MTT assay. In MTT assay, the viability of cells grown in the presence of the different stages of bioglass nanoassembly was determined using the colorimetric MTT assay. MTT assay measures the reduction of the tetrazolium component (MTT) into formazan crystals by viable cells reflecting the level of cell metabolism. For the assay, cells were then seeded onto 96-well plates with a density of $10^4$ cells per well and were incubated under standard culturing conditions. In order to carry out the assay, initially, 100mL of 10% MTT reagent was added and the plate was covered with foil leaving some free space to allow air in, and incubated for 2 h at 37°C under cell culture conditions. After 2 h the plate was taken out and then 100mL of dimethyl sulfoxide was added by removing the cell culture medium and incubated for 20 min at cell culture conditions to dissolve formazan crystals. After incubation, the plate was taken and read with 570 nm and 630 nm wavelengths using a TECAN multimode plate reader. In addition to this, cells were also observed under an optical microscope Floid Cell Imaging Station for visible signs of toxicity in response to the test. The cells cultured in DMEM medium were used as
positive control. All experiments were run with five samples and the data was represented as means standard deviation. The statistical difference was analyzed using Students' GraphPad and a P value of <0.05 was obtained.

For Ki67 Immunostaining analysis, U2OS (5000 cells per well) cells were seeded in a 12 well plate. When the cells became 70% confluent/adhered to the wells, they were treated with 1 mg/ml nBGA1 and nBGA2 nanoassemblies. Untreated pure U2OS cells were considered as a control. Subsequently, cell-seeded pellets were kept at 37°C in a humidity incubator under standard culturing conditions. After 3 days, the culture medium was changed with a fresh media. Cells were left to proliferate for next 24 hours after which Ki67 immunostaining analysis was carried out to evaluate the u2os cellular proliferation rate. In order to perform Ki67 immunostaining, the cells were washed with 2% paraformaldehyde, permeabilized for 5 min with 0.1% triton X100 followed by blocking with 0.3M glycine in 0.1% BSA for 1 h. It was then incubated with primary antibody (Anti-Ki67 antibody (ab15580)) for overnight at 4 °C followed by secondary antibody (Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647) (ab150079)) for 1 h at room temperature. Further, FACS was recorded to evaluate the U2OS cells proliferation rate. The experiment was carried out in triplicate.

**S1.3 Bacterial viability Test**

Bacterial viability test was performed as per the procedure mentioned in the backlight Live/Dead assay kit. In brief, 30 mL cultures of either *E. coli* or *S. aureus* or mixed culture (1:1) was grown to late log phase in LB media. Concentrate it at 10,000 × g for 10–15 minutes. Remove the supernatant and resuspend the pellet in 2 mL of 0.85% NaCl. Add 1 mL of this suspension to each of two 50 mL centrifuge tubes containing 20 mL of 0.85% NaCl (for live bacteria) and 20 mL of 70% isopropyl alcohol (for killed bacteria). Incubate both samples at room temperature for 1 hour, mixing every 15 minutes. Pellet both samples by centrifugation at 10,000 × g for 10 minutes. Resuspend the pellets in 20 mL of 0.85% NaCl and centrifuge again. Resuspend both pellets in separate tubes with 10 mL of 0.85% NaCl each. Determine the optical density at 670 nm. Adjust the *E. coli* suspensions (live and killed) to 1 × 10^8 bacteria/mL (~0.03 OD 670) and the *S. aureus* suspensions (live and killed) to 1 × 10^7 bacteria/mL (~0.15 OD 670). Mix five different proportions of the bacterial suspensions. The total volume of each of the five samples was kept 3 mL. To this add 1.5 µl of PI. Mix it thoroughly by pipetting up and down several
times. Incubate at room temperature in the dark for 15 minutes. The stained samples were analyzed through both Flow cytometer FACS-Accuri™ (Becton Dickinson, USA) using BD Accuri™ software and fluorescence microscope Leica TCS SP8. Average of three independent experiments was considered.

**S1.4 Cellular Proliferation**

Both the pellets of bioactive glass assembly (nBGA1 and nBGA2) were subjected to UV light illumination for 15 minutes prior to cell seeding for sterilization purpose. Saos2 cells were then seeded on sterilized pellets at a concentration of 20,000 cells per sample in the presence of DMEM culture medium. In another set of study, both Saos2 and mixed bacterial strains (*E.coli* and *S.aureus*) culture (1×10⁴ cells per ml) was seeded in the presence of both DMEM culture medium and bacterial LB media i.e. the cell seeded bioglass nanoassembly were challenged with bacterial strains. Such type of experiment was designed to mimic the natural infectious site condition. All the samples were kept at 37 °C in an atmosphere of 5% CO₂ for 6 days incubation period. Further cell-cultured samples were fixed in 2% paraformaldehyde at 48 °C overnight, then dehydrated in a graded series of alcohols and placed in a desiccator overnight. After 24 h the samples were then gold coated and observed by HITACHI-S-3700N SEM at 15 keV.

Even, the U2OS cells proliferation quantification was carried out in the absence and presence of bacterial (both *E.Coli* and *S.aureus*) challenge using Lactose dehydrogenase (LDH) assay. Herein, LDH assay kit of CytoTox-ONE™ Homogeneous Membrane Integrity Assay (G7890) was used. Standard protocol was followed as mentioned by Promega manufactures. Average of three independent experiments was considered.

**S1.5 Chemical analyses of Chitosan**

Three drops of the compound to be tested is dissolved in 2 ml water. To this, 2% KMnO₄ solution (a purple solution) is added dropwise and the solution is shaken. If the purple color of the KMnO₄ solution disappears and a precipitate of MnO₂ is formed then it is an indicative of chitosan presence in the test sample. This was then visually observed.
**Fig. S1** FTIR spectra of nBG (A) before and (B) after interaction with SBF for 3 days at various concentration of CTAB (a) 0.4, (b) 0.6, (c) 1.2, (d) 1.4, (e) 10, (f) 27 mM.
<table>
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<th>Concentration (mM)</th>
<th>Before SBF Interaction</th>
<th>After SBF Interaction</th>
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<td>(g)</td>
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<td>0.6 mM</td>
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<td>27 mM</td>
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<td></td>
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Fig. S2 SEM micrographs of nBG before (a-f) and after (g-l) interaction with SBF.
Fig. S3 TEM Particle size distribution histograms obtained by Image J analysis.
Fig. S4 BET isotherm for as mentioned different CTAB concentration templated bioactive glass nanoparticles. (Where  

- Adsorption Curve;  
- Desorption Curve)
Fig. S5 FTIR spectra for the various stages during the fabrication of (a) nBGA1 and (b) nBGA2.
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**Fig. S9** SEM image of nBGA1 (a) and nBGA2 (b) bioglass nanoassembly interaction with SBF after 8 h.
Fig. S10 XRD graph for bioglass nanoassembly (nBGA1/nBGA2) after SBF soaking.
**Fig. S11** SEM image of bacteria interacted nBGA1 and nBGA2 bioglass nanoassembly. (Here, arrow marks depicts the hydroxyapatite nodules. G-: Gram negative; G+: Gram positive)