Supplementary Information for:

Smart active antibiotic nanocarriers with protease surface functionality can overcome biofilms of resistant bacteria

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Contents

Mechanism of PenG and oxacillin susceptibility and resistance

Penicillin's all contain a thiazolidine ring (sulphur-containing with a carbonyl group), a beta-lactam ring (cleaved by beta-lactamases at C-N bond) and a variable side chain attached through an amide linkage.[1] Penicillin G (benzylpenicillin) is part of a first-generation fermentation-derived family, oxacillin is a second-generation semi-synthetic antibiotic with beta-lactam resistance.[2] Additional ring structures on the variable group of semi-synthetic penicillin's acts as a steric hindrance and inhibits beta-lactamase (penicillinase) activity, i.e the isoxzolyl antibiotic oxacilling.[3]

Bacteria can quickly become resistant to antibiotics due to genetic mutations, formed from environmental pressures such as antibiotic treatments, partially at sub-lethal concentrations.[4] Horizontal gene transfer can allow the creation of resistance strains which can then only be killed with alternative antibiotics are at much higher concentrations.[5] However, methicillin-resistant S. aureus (MRSA) is resistant to all second-generation penicillin's due to the production of a low-affinity penicillin PBP2a.[6] MRSA is a considerable public health risk due to this mechanism .[7] Beta lactamase inhibitors such as clavulanic acid and sulbactum are commonly administered synergistically with penicillin antibiotics for their ability to bind to beta lactamase broadening the effectiveness of the antibiotic.[8]

Gram-positive cell walls are primarily made of peptidoglycan monomer comprised of two covalently joined amino sugars, N-acetylmuramic acid (*NAM*) and N-acetylglucosamine (*NAG*), bound using transpeptidases enzymes called penicillin binding proteins (PBPs).[9] To maintain the rigidity and support this cross-linked NAM-NAG matrix is constantly remodelled to allowed cell growth and replication.[10] Penicillin antibiotics bind to PBPs serine residues in the active site via acetylation and inhibit their peptide bridge cross-linking function preventing new cell wall formation.[11] A lack of cell wall quickly results in cell death, leaving humans cells unharmed due to a lack of peptidoglycan in their cellular structure.[12] Bacteria have also evolved modified PBPs which have a low penicillin binding affinity and therefore can evade inactivation.[6]

Materials and Methods

Materials

The shellac formulation was kindly provided as a gift by Stroever Schellack Bremen (SSB) and is commercially available as SSB® AquaGold, an aqueous solution of ammonium shellac salt (25 wt%). Poloxamer 407 (P407) (>99%) was purchased from Sigma-Aldrich, UK. Alcalase 2.4 L FG EC number; 3.4.21.62 was kindly provided as a sample by Novozymes, Denmark. Alcalase 2.4 L FG is a serine endo-peptidase (mainly subtilisin A), which performs stereo-selective hydrolysis of amino esters and selective esters. Alcalase also efficiently hydrolyzes amino esters, which include heterocyclic amino esters. Optimal conditions for usage are 30–65 °C and pH 7–9. Its enzymatic activity was 2.4 AU A g^{-1}. The protein concentration of Alcalase 2.4 L FG was 55 mg mL^{-1}, determined by a NanoDrop Lite Spectrophotometer (Thermo Scientific, U.K.). *Staphylococcus aureus* subsp. *aureus* Rosenbach (sensitive to Oxacillin) - ATCC® 29213™ was purchased from ATCC. Benzylpenicillin and Oxacillin were supplied by Sigma-Aldrich, UK. Mueller-Hilton Broth (MHB), Mueller-Hilton Agar (MHA), benzylpenicillin (10 U) and oxacillin (5 μg) Antimicrobial Susceptibility Disk were supplied by Oxoid, UK. Dey-Engley neutralising broth was provided by Sigma-Aldrich, UK. A Pierce™ BCA protein assay kit was used to determine protein concentration. Crystal Violet (CV), dye content, ≥90% and Acridine Orange (AO) base (≥75%) and resazurin sodium salt (75%) were purchased from Sigma-Aldrich, U.K. Concanavalin A, Alexa Fluor™ 488 Conjugate was purchased from ThermoFisher Scientific, UK. The Beta lactamase Activity Assay Kit (Colorimetric) was purchased from Abcam, UK. HaCaT cells were obtained from AddexBio, T0020001 and HEP G2 cells from the European Collection of Authenticated Cell Cultures (ECACC) at Public Health England, 85011430. Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (EMEM) and foetal bovine serum (FBS) were obtained from Gibco, U.K., L-glutamine and trypsin ethylenediaminetetraacetic acid (EDTA) by BioWhittaker, U.K. A 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric survival and proliferation kit (Millipore Corp.) was used for HaCaT and HEP G2 cell viability experiments. Deionized water purified by reverse osmosis and ion exchange with a Milli-Q water system (Millipore) was used in all our studies. Its surface tension was 71.9 mN m^{-1} at 25 °C, with measured resistivity higher than 18 MΩ cm^{-1}. Consumable plasticware used in the study was purchased from Sarstedt (U.K.), Thermo Scientific (U.K.), or CytoOne (U.K.), unless otherwise stated.

Preparation of PenG/Oxa-loaded P407-stabilised shellac NPs

A stock solution of 25 wt% shellac-ammonia salt was diluted to 0.2 wt% and made up to a volume 50 mL in deionized water, the pH was then raised to 10 using droplets of 0.25 M NaOH and chilled to 4 ^{o}C for 1 hour. P407 and PenG/Oxa powders were added at various concentrations and mixed for 30 seconds. Afterwards, the pH was quickly reduced to 4 using 0.25 M HCl to allow the components to precipitate into NPs (temperature kept at 4 ^{o}C). The final concentration of 0.2 wt% Shellac-0.25 wt%

P407-0.1 wt% PenG/Oxa NPs (pH 5.5) was used as a stock preparation and used for further experimentation.

Enzyme surface functionalisation of the 0.2 wt% Shellac-0.25 wt% P407-0.02 wt% AmpB NPs

The stock NP formulation was functionalised with Alcalase 2.4 L FG to create a coating of immobilised protease on the corona of the NPs. The stock solution of Alcalase 2.4 L FG was diluted in deionized water to 0.2 wt% to a volume of 50 mL and sonicated for 15 min to prevent aggregation. 25 mL of this solution was added to 25 mL of AmpB-loaded P407-stabilized shellac NPs dispersion mixed for 30 mins at pH 5.5 to allow an effective coating of the NPs with the cationic protease. Afterwards, the mixture was centrifuged at 8000 rpm for 30 mins to pelletise the NPs. The supernatant was retained for analysis spectroscopic analysis. The pellet was redispersed into 25 mL of deionized water buffered to pH 5.5 (acetate buffer) yielding a stock solution of 0.2 wt% Shellac-0.25 wt% P407-0.02 wt% AmpB-0.2 wt% Alcalase NPs which were used further for NPs characterisation and antimicrobial experimentation.

Shellac NPs hydrodynamic diameter, zeta-potential and TEM characterisation

Particle size distribution and zeta potential were measured using Malvern Zetasizer Nano ZS. The refractive index (RI) of shellac was found to be 1.512 as measured by an Abbe 60 refractometer, and this index and absorbance of 1.000 was used for all measurements. Measurements were repeated three times at 25 ^{o}C using a quartz cuvette and data represented as the mean. The size of the NPs was examined by TEM. TEM photographs of 0.2 wt% Shellac-0.25 wt% P407-0.02 wt% AmpB-0.2 wt% Alcalase NPs. Particles were prepared and redispersed in deionised water at pH 6. A droplet of the suspended sample was pipetted onto Carbon coated Copper grids (EM Solutions, UK) and allowed to adhere for 2 min. The grid was quickly rinsed with deionised water and negatively stained with 1 wt% aqueous uranyl acetate. This was quickly rinsed with deionised water and allowed to dry in air. The sample was then imaged with a Gatan Ultrascan 4000 digital camera attached to a Jeol 2010 TEM 2010 electron microscope running at 120kV.

Encapsulation efficiency and release kinetics of PenG/Oxa from the shellac NPs

The encapsulation efficiency of PenG/Oxa (formulated as 0.1 wt% PenG/Oxa into 0.25 wt% shellac-0.2 wt% P407 NPs) was examined as a function of concentration. The nanoparticles were centrifuged for 30 min at 8500 rpm to pelletize leaving the supernatant with unencapsaulted PenG/Oxa. The absorbance of the supernatant was measured using a spectrophotometer to determine encapsulation efficiency. The absorbance of the supernatant was compared to a standard curve of PenG/Oxa to calculate the PenG/Oxa retained in the NPs pellet. The supernatant sample and standard curve samples were read at 250 nm, pH 5.5 and room temperature.

Elemental analysis of the NPs pellet was performed using CHN Analyzer (Carlo Erba 1108). The pellet was washed with deionized water three times and dried overnight at 60 ^{o}C to remove any remaining water. This was compared with samples of dried individual components of the NPs (shellac, P407 and PenG/Oxa).

HPLC chromatograms of 0.2 wt% Shellac, 0.25 wt% P407, 0.2 wt% Shellac-0.25 wt% P407-0.1 PenG/Oxa NPs, and 0.1 wt% PenG/Oxa (at various concentrations) were obtained.
Data were interpolated from PenG/Oxa peaks in the NP samples compared to PenG/Oxa calibration curves. 20 mL of 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs was pelletised and dissolved into 20 mL of acetate buffer (0.1 M, pH 5.5). The supernatant was discarded. 2-fold serial dilutions were made into acetate buffer (0.1 – 0 wt% PenG/Oxa). The samples were sonicated for 5 mins. 20 μL of each sample was injected into a 5 μm C18 column (Phenomenex, UK) and pumped at 1300 psi, 1 mL/min. The mobile phase was ammonium acetate (10 mM, pH 4.5) and acetonitrile in the ratio 75:25 (V:V) under isocratic elution. UV-absorbance at 220 nm was used for sample detection (Perkin-Elmer 785 A UV/VIS Detector, UK). The peak area was measured using Azur software. The same process was used to in PenG/Oxa antibiotic calibration curves

Release kinetics were also performed as a function of time and temperature over 24 h using the same formulation. 10 mL of the 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NP formulation was placed into a 10-12 kDa MWKO dialysis bag. The dialysis bag was placed into 250 mL of pH 5.5 acetate buffer. 1 mL aliquots were taken at specific time points on the absorbance of the aliquot equated into the concentration of PenG/Oxa in the buffer using a standard calibration curve. Absorption was measured at 250 nm.

Fourier transform infrared (FTIR) and UV-vis spectroscopy analysis

Fourier transform infra-red (FTIR) spectra were taken of Shellac, P407, 0.25 wt% Shellac-0.2 wt% P407 NPs, free PenG/Oxa, and 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs. Samples were prepared by removing water using a silica gel desiccant in a vacuum chamber for 1 day, then dried at 60 ^{o}C for 2 days. The spectra were measured between 4000-600 cm-1 using PIKE ATR diamond settings. A spectrum was obtained with a blank of just air to reduce transmittance interference with the samples. Absorbance spectra of 0.1 wt% PenG and 0.1 wt% Oxa measured between 220 and 1000 nm. Absorbance measurements were taken 250 nm, pH 5.5 and room temperature using a quartz cuvette. Measurements were taken using a PerkinElmer Lambda 25 UV-Vis spectrophotometer.

Bacterial culture

Overnight (O/N) cultures were prepared by incubating a single colony scraped from the MHA stock plates into 10 mL of MHB for 16 hours at 37 °C with 140 rpm (Stuart Orbital Incubator S1500). For all bacterial assays, O/N cultures were adjusted to 0.5 McFarland standard by diluting the O/N culture into 0.85 w/v% sterile saline until an optical density of 0.08–0.12 at 625 nm was obtained using a spectrophotometer (FLUOstar Omega spectrophotometer, BMG Labtech). These adjusted bacterial saline suspensions were then diluted 1 : 150 into MHB to yield starting concentrations between 5×10^5 and 1×10^6 colony forming units per mL (CFU mL^{-1}).

Time Kills

Killing curves were constructed against planktonic *S. aureus* to determine the time taken for the active agent in influencing total bacterial cell populations. O/N cultures were prepared yielding 10 mL of MHB aliquots with 5×10^5 –1×10^6 cells per mL. 10 mL of 0.25 wt% Shellac-0.2 wt%P407-0.1 wt% PenG/Oxa-0.2 wt% Alcalase NPs were added to each tube and incubated for 24 hours at 37 °C with 140 rpm. Experiments with other constituents of the NPs and free PenG/Oxa antibiotics at equivalent concentrations was performed for comparison. At time points 0, 0.5, 1, 2, 3, 4, 6, and 24 hours, a 100 µL sample was removed from each treatment tube and added to 900 µL of Dey-Engley Neutralising broth. 100 µL aliquots from each serial dilution tube were plated onto MHA plates and incubated for 24 hours at 37 °C and enumerated. The time-kill assay was repeated in three independent experiments.

Minimum inhibitory and bactericidal concentration (MIC/MBC)

The bacteriostatic and bactericidal properties of the 0.25 wt% Shellac-0.2 wt%P407-0.1 wt% PenG/Oxa-0.2 wt% Alcalase NPs and its spectrum of activity were measured in comparison to equivalent concentrations of free PenG and Oxa. Briefly, O/N cultures of *S. aureus* were prepared as described above. 100 µL of each strain suspension was added to the wells of a 96-well microtiter plate yielding 5×10^4 –1×10^5 cells per well. Aliquots of 0.25 wt% Shellac-0.2 wt%P407-0.1 wt% PenG/Oxa-0.2 wt% Alcalase NPs were prepared. This was centrifuged at 4000 rpm for 5 minutes and re-suspended into 10 mL of MHB. 2-Fold dilutions of this suspension were prepared in separate aliquots and 50 µL added to descending rows of bacteria on the microtiter plate. Equivalent 2-fold dilutions of the free ABX were added to separate columns for comparison (50 µL per well). The plates were incubated for 24 hours at 37 °C. 20 µL of resazurin sodium salt (0.15 mg mL^{-1} in DPBS) was added to each well and incubated at 35 °C for 2 hours. The absorbance was recorded using a spectrophotometer (FLUOstar Omega spectrophotometer, BMG Labtech). The resazurin in the presence of viable cells is reduced to resorufin by the bacterial co-enzyme NAHD, indicating that the cells remain metabolically active and viable. The MIC was determined to be the lowest concentration of active antimicrobial agent which inhibited the growth of each strain. The same procedure was performed to obtain the MBC, minus the addition of resazurin. Instead, 100 µL from each well was streaked onto MHA and incubated for 24

hours at 37 °C. The MBC concentration was determined from samples in which no CFU was detected. The MIC/MBC assay was repeated in three independent experiments.

Antimicrobial susceptibility testing (AST)

A VITEK® 2 (bioMérieux, FRA) microbial identification system was used to profile the susceptibility and resistance of *S. aureus* against a range of antibiotics. VITEK 2 GP (21342) and VITEK 2 AST-P635 (416911) Gram-positive identification and AST cards were used. A suspension of *S. aureus* was created by placing a single colony into 3 mL of 0.45 wt% saline and briefly vortexed. A DensiCHEK™ was used to measure the optical density of the suspension, the suspension was adjusted with 0.45 wt% saline until a 0.5 McFarland was obtained (in accordance with the VITEK2 protocol). The adjusted suspension was placed into the machine and a capillary tube inserted. The analysis was left to run overnight (typical analysis time of 6 to 12 h). The procedure was performed on 3 separate occasions to confirm the results. Post analysis, the isolates were subcultured on to Columbia blood agar plates to check purity. For further confirmation of the susceptibility and resistance results of PenG and Oxa specifically, disk diffusion AST was performed. Briefly, a single colony of *S. aureus* was isolated and placed into 10 mL of MHB and grown overnight at 37 °C. The bacteria suspension was then diluted to a 0.5 McFarland standard in MHB by measuring the turbidity at 625 nm yielding 1–2 × 10^8 CFU mL^{-1}. A cotton swab was used to streak an MHA plate lawn. 4 ± 0.5 mm deep MHA plates were prepared by adding 25 mL of molten MHA to a 9 cm round plate and left to solidify at room temperature. PenG (10 U) and Oxa (5 μg) antibiotic disks (both Oxoid, UK) were applied to the MHA plates using a disk dispenser (Oxoid, UK), and the plates were incubated for 18 hours at 35 ± 1 °C following EUCAST guidelines. The zone of inhibition diameters were illuminated using a lightbox and the images were measured in mm using a ruler; the diameter was measured across 3 lines and the mean was determined to be the zone of inhibition.

SEM imaging of the treated cells

S. aureus cells were removed from their media by centrifugation at 2000 rpm for 5 mins. The Cells were then washed thrice and resuspended in PBS buffer. A 1 wt% glutaraldehyde PBS buffer solution, applied for 1 hour, was used to fix the cells. The centrifugation and washing process was repeated to remove excess glutaraldehyde. The cells were dehydrated in 50%/75%/90% and absolute ethanol solutions for 30 mins per ethanol concentration. A sterile swab was used to transfer cell to a glass slide and place into absolute ethanol and dried using liquid CO_2 at its critical point using an E3000 Critical Point Dryer (Quorum Technologies, UK) and then coated in 10 nm Carbon. The samples were imaged using an Ultra-High-Resolution Scanning Electron Microscope using cold field emission (Hitachi SU8230, Japan).

TEM and EDS imaging of the treated cells

TEM images of untreated S. aureus, and S. aureus treated with 0.1 wt% PenG and 0.1 wt% PenG-NPs for 1 hour were obtained by placing a droplet of the suspended sample onto carbon-coated copper grids (EM Solutions, U.K.) and allowed to adhere for 2 min. The grid was quickly rinsed with deionized water. Negative staining was omitted to reduce interference during EDS analysis. The sample was left to dry in air then imaged with a Gatan UltraScan 4000 digital camera attached to the Jeol 2010 TEM 2010 electron microscope (Jeol, Japan) running at 120 kV. Energy-dispersive X-ray (EDS) data was collected via an Oxford Instruments Nanoanalysis X-Max 65-T detector and the INCA software.

Beta Lactamase Activity

The ability of *S. aureus* to produce beta lactamase was first verified. Beta lactamase strips were used for acidimetric detection of penicilloic acid, produced when benzylpenicillin (PenG) us hydrolysis by beta lactamase enzymes. The plastic strip contains an active zone saturated with penicillin G and an acid-basic indicator. Three colonies of *S. aureus* and *S. epidermidis* (positive control) were placed into 1 mL of moistening solution (from the kit) and a test strip was added. The bacterial suspension was incubated at room temperature for 10 minutes with shaking. A colour change from pink to yellow indicates the presence of penicilloic acid. A beta lactamase activity kit was used to analysis *S. aureus* after treatments. *S. aureus* samples were obtained after no treatment, or after treatment with either 0.1 wt% PenG, 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG-0.2 wt% Alcalase NPs, or 0.2 wt% Alcalase at 1, 2 and 3 hour time points. No treatment was used a control (3 hours). The experiment was capped at 3 hours due to lack of remaining cells to analyse. Treatments were administered at 37 ^{o}C to planktonic *S. aureus* with constant shaking. Post treatment, *S. aureus* samples were obtained by centrifugation at 10000 *g* for 10 mins in a pre-weighed centrifuge tube. The supernatant was removed (used as contaminated media measurements) and the pellet weighed. The pellet was resuspended into beta lactamase buffer using 5 µL of buffer per mg of bacteria. The resuspended bacteria were sonicated for 5 mins and the sample kept chilled on ice. Further insoluble material was removed by centrifugation at 16000 *g* at 4 ^{o}C for 20 mins. The supernatant (typically 5 µL) was added to the wells and adjusted to a final volume of 50 µL/well using beta lactamase assay buffer. A positive control was made by 5-fold dilution of 2 µL of positive control to 8 µL of beta lactamase buffer. 5 µL was added to the well and adjusted to a volume of 50 µL/well using beta lactamase assay buffer. To measure the beta lactamase activity of the contaminated media, 30 µL of the bacterial supernatant was added to a well and adjusted to a volume of 50 µL using beta lactamase buffer. All assays were performed using a reaction mix comprised of 2 µL nitrocefin and 48 µL beta lactamase assay buffer. 50 µL of the reaction mix was added to each well, including the positive control and gentle mixed with the pipette tip. This yielded a final volume of 100 µL/well for all samples. The absorbance of the samples was taken kinetically from 0 to 60 mins and room temperature protected from light (within the spectrophotometer) at 490 nm and

calibration graphs plotted showing absorbance over time. The beta lactamase activity of *S. aureus* (PenG, PenG-NPs, or Alcalase after 1 h, 2 h or 3 h treatment, or untreated after 3 hours) was expressed as mg of protein and calculated as;

$$Beta\ Lactamase\ Activity = \left(\frac{B}{T2 - T1 * V}\right) * D$$

= nmol/min/mL = mU/mL

where:

B = Amount of hydrolysed nitrocefin from calibration curve

T1 = Time of first absorbance reading in mins

T2 = Time of second absorbance reading in mins

V = Volume added into the reaction well (mL)

D = Sample dilution factor

Unit definition: 1 Unit Beta Lactamase activity = amount of enzyme that generates 1 μmol of nitrocefin per minute at pH 7 at 25 ^{o}C.

Preparation of microtiter *S. aureus* biofilms

100 μL of (5×10^5) - (1×10^6) CFU mL^{-1} O/N *S. aureus* culture (MHB) was pipetted into 96-well tissue culture (TC)-treated plates and incubated at 37 °C for 24 h in static conditions to allow biofilm formation. Afterwards, the media was aspired away by pipetting from the side of the well so reduce potential damage from the pipette tip. The biofilms were washed three times using 300 μL of sterile PBS to remove any detached planktonic cells (leaving only sessile cells behind).

Crystal Violet staining, biofilm weight and biofilm protein concentration after treatments

Samples of *S. aureus* biofilms grown for 24 h at 37 ^{o}C were treated with 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% PenG/Oxa-0.2 wt% Alcalase NPs and their individual components for a further 24 h. Three 96-well plate techniques were used to assess biofilm degradation after treatment; Crystal Violet (CV) staining of the biofilm mass, dry biofilm mass weight and protein concentration. Biofilms were prepared as described above. 100 μL of the stock treatment was added to the wells and incubated at 37 ^{o}C for 24 h. Afterwards, the treatment was aspirated away, and the plate washed twice by submersion in deionized water. The plate was gently shaken to remove water and left to air dry for 15 mins in an inverted position. For CV staining 125 μL of 0.1 wt% CV solution was added to each well and incubated at room temperature for 15 mins. The plates were then again washed by water submersion to remove excess dye and then left to air dry for 2 h. 30 wt% acetic acid was used to solubilise the CV and 100 μL of this solution was transferred to a new plate and the absorbance read at 570 nm. The readings were blanked against 30 wt% acetic acid and the results compared to normalized controls. For biofilm dry mass measurements, the treatment was again aspirated away. The plates were then left to dry overnight

at 60 °C. After drying the plate was weighed on a balance and the weight subtracted from the empty plate weight to give a measurement of dry mass biofilm remaining. A Protein Assay Kit (Pierce, USA) was used to determine sample protein content. The treatment was aspirated away and the biofilm was three times in deionized water. The biofilm was scrapped from the wells and placed into a glass test tube with 1 mL of deionized water. The mixture was sonicated in a water bath from 30 min then vortexed with glass beads for 2 min to completely disperse the biofilm. 25 µL of the sample was placed into a 96-well plate. 200 µL of the protein concentration determination working reagent (reagent A: reagent A. 50:1) was added to the wells and incubated for 30 min at 37 °C with constant shaking. Absorbance was measured at 572 nm. Results were compared to a calibration curve of fixed protein concentrations prepared as per manufacturer's instructions. All tests were performed in triplicates (N = 3 with ± S.D.).

***S. aureus* cellular viability after PenG/Oxa-NP treatments**

To measure *S. aureus* cellular viability after treatment with PenG/Oxa-NPs and free ABX treatments on biofilms, a filter membrane method was employed. Millipore Express PLUS membrane filter paper sections (2 cm^2) were placed on to MHA. An overnight culture was adjusted to 1×10^5 CFU mL^{-1} in saline, and 20 µL of this culture added as a droplet to the centre of the filter paper. The sample was then incubated for 24 h at 37 °C to allow a colony biofilm to form. After 24 hours, the membranes were placed onto fresh agar plates (3 mm deep) into a six-well plate and 1 mL of treatment added to each well. The plate was gently shaken in the incubator during treatment. Deionised water (1 mL) was considered the growth control. After 24 h of treatment, the membrane filter paper was peeled from the agar wells and placed into the sample tube with 1 mL of fresh MHB and 2 cm of sterile glass beads. Each sample was vortexed for 30 s at high speed to disintegrate the biofilm and inoculated the MHB with cells. 1 mL of the treatment media was retained for viability analysis. The drop plate enumeration method was used to measure cell viability in CFU mL^{-1}. To measure the viability of cells within the biofilms, 10-fold dilutions were made in fresh MHB, 10 µL droplets were placed on to MHA plates and grown for 24 h at 37 °C. CFUs were counted from the last two droplets, which contained a countable number of colonies (3−30 colonies per 10 µL drop), and averaged.

***S. aureus* fluorescent images, biofilm quantification and confocal scanning laser microscopy**

Acridine Orange (AO) was used to stain *S. aureus* biofilms, treated with PenG-Oxa-NPs and individual constituents. Biofilms were formed and treated as described above (CV staining method). The cleaned treated samples were stained with 100 µL of 0.2 wt %, AO in darkness for 5 minutes with gentle shaking. After incubation, the plate was washed three times with deionized water and allowed to air dry for 15 min. The samples were examined using an Olympus BX51 fluorescent microscope with a DP70 digital camera. The percentage of biofilm stained with OA was quantified using ImageJ v1.52d. For 3D visualisation, filter paper membrane biofilms were gently placed onto a glass slide using sterile forceps,

and stained with AO as described above for the CV biofilms. Confocal laser scanning microscopy images (CLSM) were obtained with a Carl Zeiss LSM 710 confocal microscope with Z-series images taken in 1-μm slices.

Cryostat sectioning of *S. aureus* biofilm

Membrane biofilms were prepared and treated as described above. Post treatment the membrane was carefully removed from the agar and gently washed with DPBS. A small amount of OCT embedding matrix (CellPath, UK) was placed into a plastic cassette and allowed to settle into a flat viscous liquid. Afterwards, using forceps the membrane paper with the treated biofilm on top was placed onto the OCT and then submerged in more OCT. The cassette was then snap frozen in liquid nitrogen for 5 minutes. The samples stored at -80^{o}C until sectioning. OCT embedded samples were cyrosectioned at 10 um (CM9900, Leica Biosystems) and placed onto positively charge microscope slides (CellPath, UK). Before imaging, the slides were fixed with cold acetone for 2 mins and left to evaporate at room temperature (15 mins). Finally, the slide was washed gently in PBS to remove any residual OCT and left to evaporate at room temperature (15 mins). The samples on the slides were stained with ConcanavalinA (ConA) for 30 minutes in darkness before gently washing excess dye away with deionised water. ConA was prepared to a 5 mg/mL concentration in deaonised water and centrifuged at 5000 rpm for 5 mins before used to remove any aggregated proteins in the solution, only the supernatant was used to stain the samples. Bright-field and fluorescence microscopy of the section biofilms was performed on an Olympus BX51 (Japan).

Cytotoxicity and of HaCaT and HEP G2 cells after treatment with shellac, P407, free Alcalase, free PenG/Oxa and PenG/Oxa-NPs

HaCaT and HEP G2 cells were cultured in a T75 flask using DMEM (HaCaT) or EMEM (HEP G2) supplemented with 10% FBS and 1% L-glutamine under humidified conditions at 37 °C, 5% CO_2. When a confluency of 80% was achieved, determined by visualization with an optical microscope, passaging was performed to ensure that the cells remained in the exponential phase for experimentation. Passaging was performed by removing spent media, washing in DPBS, and incubating with 1× trypsin EDTA at 37 °C 5% CO_2 for 5 min until the cells were detached in suspension. The trypsin EDTA was then neutralized with a 1:1 volumetric ratio of fresh DMEM and gently centrifuged at 400 rpm for 4 min, the supernatant was aspirated, and the pellet was resuspended in DMEM (supplemented as above) at a 1:6 ratio and transferred into a fresh T75 flask. Surplus cells used for experimentation were diluted in fresh DMEM or EMEM, supplemented with 2% FBS and 1% L-glutamine, seeded at 5×10^4 in 100 μL of media, placed into a 96-well plate, and incubated for 24 h at 37 °C 5% CO_2.

The medium was then removed, and the cells are replaced with 100 μL of treatment-infused DPBS. DPBS was used so DMEM/EMEM peptones did not interfere with the true activity of the Alcalase. The treatments with only DPBS were used as a control, 0.25 wt% shellac, 0.2 wt% P407, 0.2 wt% Alcalase,

0.1 wt% free PenG/Oxa and 0.1 wt% PenG/Oxa-NPs treatments were performed for 1 h , 6 h, and 24 h time points.

A colorimetric (MTT) cell survival and proliferation assay kit was used to measure cell viability. Treatment culture was aspirated away, and the cells washed for 2 min with fresh DPBS. Fresh DMEM/EMEM (100 µL) was added after which 100 µL of MTT reagent (50% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 50% PBS) was added. This was incubated in the same conditions for 2 h until intracellular purple formazan crystals were visible under a light microscope. After 2 h, 100 µL of the color development reagent in isopropanol with 0.04 M HCl was added for 1 h, allowing the cells to lyse and the formazan crystal to be solubilized to a homogenous blue solution. The absorbance of the blue solution was read at 570 nm on a plate reader and subtracted from a blank of media only. These data were calculated into cell count data using the Beer−Lambert extinction coefficient law using absorbance values from a fixed number of cells in media (see figure S28). The HaCaT/HEP G2 cell viability assay was repeated in three independent experiments

Statistical analysis

Data were expressed as average values ± standard deviations of the mean. P-Values of less than 0.05 were considered significant. All One-Way ANOVAs, Tukey's post-test and Student T-tests statistical analysis were performed in GraphPad v7.0.4.

Individual components and molecular structures of materials used in the preparation of shellac-P407-PenG/Oxa-Alcalase nanoparticles

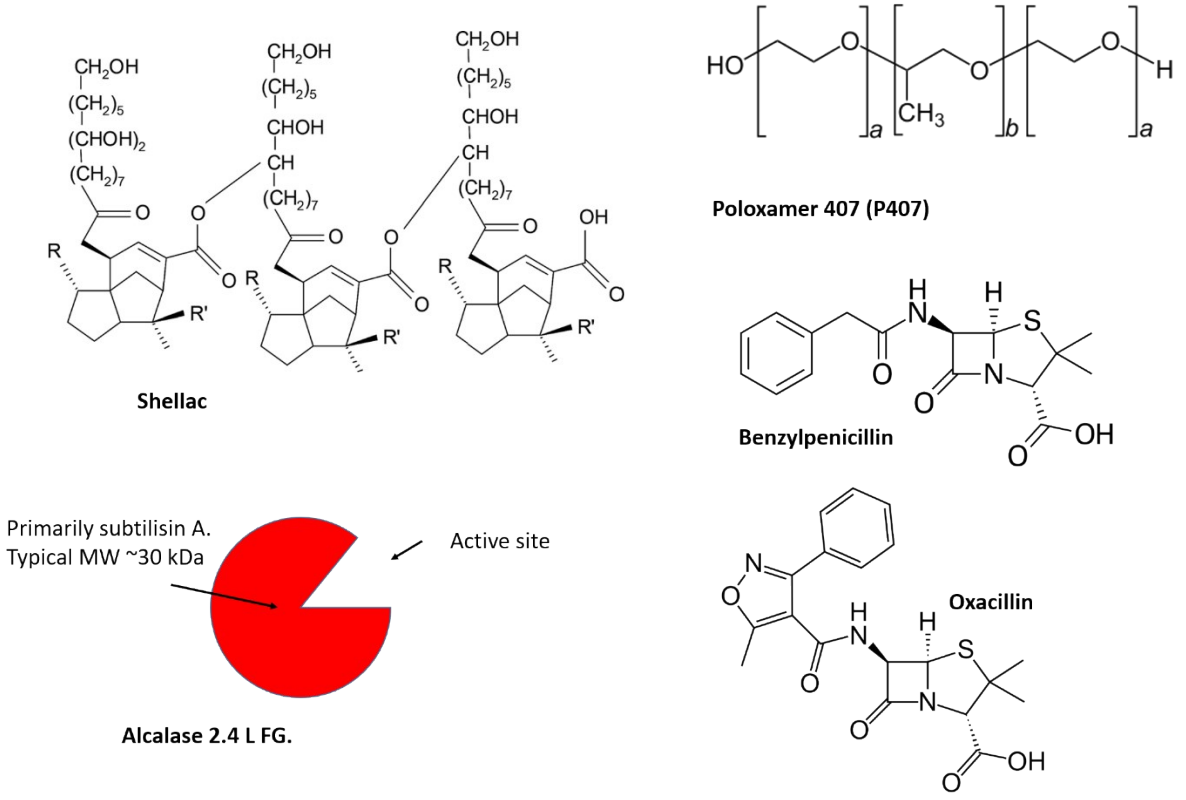

Figure S1. Individual components and molecular structures of materials used in the preparation of shellac-P407-PenG/Oxa-Alcalase nanoparticles. Shellac (SSB® Aquagold ammonia salt) R=CHO/COOH, R'=CH_2OH/CH_3, Poloxamer 407 (P407) is a triblock copolymer; consisting of a central hydrophobic block of (b) polypropylene glycol and two hydrophilic blocks of (a) polyethylene glycol. Block lengths; a = ~101 and b = ~56. Alcalase 2.4 L FG is a Serine endo-peptidase with an Isoelectric point ~pH 9 and is cationic below pH 9.

NP characterisation: Preparation, encapsulation efficiency, release kinetics and Alcalase functionalisation of PenG/Oxa-loaded shellac NPs

Figure S2A and B shows the particle size and zeta potential of 0.25 wt% shellac-0.2 wt% P407 NPs precipitated at pH 5.5 with various concentrations of PenG (0 to 0.1 wt%). The mean hydrodynamic diameter with no PenG was ~70 nm, increasing slightly up to 80 nm when synthesised with 0.1 wt% PenG. The zeta potential ranged from -19 mV to -15 mV when prepared with 0 to 0.1 wt% PenG. This is due to the residual –COOH groups within shellac which deprotonate in acid conditions yielding a negative zeta potential. Figure S2C and D shows the same hydrodynamic diameter and zeta potential of the same 0.25 wt% shellac-0.2 wt% P407 NP precipitated with oxacillin. Similar to the penicillin NP size, a range of between 70-80 nm was observed, increasing with higher concentrations of oxacillin, up to 0.1 wt%. The same is true of the zeta potential which is ranges -20 to -10 mV as the oxacillin concentration is increased. The 0.25 wt% shellac-0.2 wt% P407 was chosen as the optimum ratio for antibiotic encapsulation, as this yielded NPs with a size and zeta potential of 70 nm and -20 mV, typical of P407 sterically stabilised NPs.[33] Higher concentrations of P407 have previously shown that the size does not increase, however, the zeta potential is reduced, likely due to offsetting of the shear plane position by the accumulation of PEO chains from the P407, which is covering the COO^- groups negative surface charge. This would deleteriously affect the subsequent surface functionalisation of the NPs with the cationic protease Alcalase 2.4 L FG. Figure S3B shows a 0.25 wt% shellac-0.2 wt% P407 NP TEM image which is approximately 70 nm. Figure 3C and D show the same NP composition formulated with 0.1 wt% PenG or Oxacillin, with the size increased to 80 nm. These images are in agreement with the DLS data. The encapsulation of PenG and oxacillin was accomplished by mixing the materials at pH 10 and quickly reducing the pH to 4, allowing the rapid precipitation of the compounds. Figure S4 shows when this procedure is carried out a 4 ^oC there is an increased encapsulation efficiency of both PenG and oxacillin across a concentration range of 0.01 to 0.1 wt%, compared to preparation at 23 ^oC (room temperature). At 4 ^oC the encapsulation efficiency of PenG and oxacillin was ~75%, however, the same preparation at 23 ^oC was ~50%. This is likely due to the decreased tendency for the particles to aggregate and agglomerate at room temperate during preparation.

The encapsulation efficiency was measured by analysing the supernatant after the NPs were pelletised and comparing the absorbance vs a calibration curve created with solutions of PenG and oxacillin (Figure S5A and B respectively). Figure S6 shows the absorbance spectra of PenG and oxacillin had a λmax of ~250 nm, typical of colourless organic solutions. HPLC was used to provide further evidence of the encapsulation efficiency of PenG and oxacillin to shellac-P407 NPs. This was performed by directly measuring the concentration of antibiotic within the NP, rather than indirectly measuring the concentration not encapsulated in the supernatant (absorbance measurements). The supernatant was discarded from the antibiotic NPs and the NP re-dissolved into acetate buffer and the

antibiotic allowed to fully defuse out of the NP. Figure S7A and B show the chromatographs of 0.1 wt% PenG and 0.1 wt% oxacillin NP formulations, which had a retention time of 4 and 5.5 minutes respectively. Figure S8A and B show HPLC peak area calibration curves created using PenG and oxacillin solutions (0 to 0.1 wt%). Figure S8B shows an encapsulation efficiency of ~75% was achieved by comparing the 0.1 wt% PenG/Oxa-NP formulation against the antibiotic solutions and is in agreement with encapsulation data obtained using absorbance measurements. Table S1 shows the elemental ratios of carbon, hydrogen, nitrogen and sulphur in 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs and compared to the elemental ratios of the individual components. PenG and oxacillin show a sulphur content of 8.67 and 6.72%. Shellac showed sulphur content of 0.12%, likely from the chemical isolation of the compound from resin the product is derived from, as the chemical structure contains no sulphur. However, when analysing the sulphur content of the 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs it was shown to be 1.44 and 1.11%, with the additional sulphur coming from the antibiotics. Unfortunately, due to the sulphur content in the shellac sample alone a quantitative concentration of antibiotic encapsulation cannot be obtained, however, this data does indicate the presence of PenG and oxacillin the NP qualitatively. Figure S9 shows the FTIR spectra of shellac, P407, PenG, oxacillin and 0.25 shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs. The shellac and P407 samples show an abundance of peaks in the range between 1600-1000 cm^{-1} due to the presence of C-O bonds within the compounds. The PenG and oxacillin samples also show an abundance of peaks between the 1600-1000 cm^{-1} range. P407 has strong peak at 1100 cm^{-1} from the CH2-O-CH2 ether groups. This prominent peak is present in the shellac-P407-antibiotic NP formulation, however it is small likely due to the central hydrophobic polypropylene glycol block absorption on the shellac NP surface. The complexity of the shellac and P407 chemical structures makes unique peaks from the antibiotic encapsulated within the NPs difficult to detect. However, it does show that shellac and P407 have complexed into a NP. For additional NP characterisation we investigated the elemental composition of the antibiotic encapsulated NPs, and the individual components that the NPs are comprised of. Sulphur was chosen as the element of interest as it is present in the chemical structures of PenG and Oxa, but not the chemical structures of shellac and P407 (see figure S1 for chemical structures). CHN(S) elemental analysis of shellac showed negligible amounts of sulphur, likely derived from the manufacturing process, however, this was not detected by Energy-dispersive X-ray spectroscopy (EDS) analysis. Figure S10 shows the EDS elemental spectra for encapsulated NPs and their individual components. Shellac and P407 (figure S10A and B) shows no sulphur peak, which is consistent with their chemical structure. Samples of free PenG and Oxa (figure S10C and D) show small sulphur peaks of 11.13% and 10.55% atomic weigh, which is correct for the elemental composition of the compounds. Elemental analysis of the PenG-NPs and Oxa-NPs, formulated as 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG-NPs, and 0.25 wt% shellac-0.2 wt% P407-0.1 wt% Oxa-NPs (figure S10E and F) shows peaks of 3.82% and 3.81% for sulphur, indicating that PenG and Oxa have been successfully encapsulated into the NP in roughly the same composition.

Figure S10 shows the release kinetics of 0.1 wt% PenG and 0.1 wt% oxacillin from 0.25 wt% shellac-0.2 wt% P407 NPs was investigated as a function of pH and temperature over time. PenG and oxacillin is slowly released over 24 hours in a similar fashion. At pH 7.5 the release is more rapid, cumulating at 60% and 90% for PenG and oxacillin, compared to 40% and 50% at pH 5.5. This is due to the partial solubilisation of shellac above pH 7 allowing for a more rapid diffusion of the antibiotic from the core. The temperature was also shown to influence the release of the antibiotics from the shellac-P407 NPs. In a similar trend to the increased pH, a higher temperature of 37 ^{o}C showed that at 24 hours 65% of PenG had been release compared to 40% at 23 ^{o}C. For oxacillin 90% was release at 37 ^{o}C after 24 hours, 50% at 23 ^{o}C. The higher temperature will increase the diffusion rate in which PenG and oxacillin are released from the NP core indicating that at higher a pH and temperature the antibiotics are released more rapidly, however, in all cases it is still a gradual process. The increased rate of release of the antibiotics at pH is explained by the partial dissolving of the shellac which would increase the rate in which the antibiotics can leach from the NP interior. The 0.1 wt% PenG and 0.1 wt% oxacillin into 0.25 wt% shellac-0.2 wt% was chosen as the NP formulation to be functionalised with a coating of cationic protease 2.4 L FG. The purpose of which was to convey a positive zeta potential to the NP, increased the electrostatic attraction to the anionic bacterial cell wall, and to digest the biofilm EPS.

Figure S12A and B shows the hydrodynamic diameter and the zeta potential after coating with concentrations of Alcalase ranging from 0 to 0.6 wt%. There is an increase in size from ~80 to 90 nm which occurs up to 0.2 wt% Alcalase coating and plateaus afterwards between 0.2 – 0.6 wt%. The zeta potential is shown to flip from -15 mV to 20 mV after a coating of 0.1 wt% Alcalase with only minimal increases thereafter. Similar results are seen when the same concentrations of Alcalase are added to NPs which have encapsulated oxacillin (figure S12C and D) rather than penicillin. This is due to there being no likely difference between the NP whether encapsulating PenG or oxacillin. Figure S3A shows a TEM image of a dispersion of globular Alcalase protease, which has a diameter of ~8 to 10 nm, typical of a subtilisin A protease with a MW of ~30 kDa. A coating concentration of 0.2 wt% was chosen as this provided a good zeta potential reversal to 20 mV, increased concentrations showed no such increase indicating the surface was likely saturated with protease. Figure S3E and F shows TEM images 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG/Oxa-0.2 wt% Alcalase NPs, which have a ~10 nm larger diameter and a rough-textured surface indicating the presence of an Alcalase coating, and is consistence with data obtained with DLS. Figure S13 shows the zeta potential of 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG/Oxa-0.2 wt% Alcalase NPs change over time as a measure of the surface coating stability. The positive charge is retained in both formulations over 24 hours, dropping by ~ 10 mV. This shows the protease is retained electrostatically on the anionic shellac NP with only a minimal release over 24 hours.

For short-term storage the NP suspension was pelletised and the supernatant removed. The semi-wet NP was then stored at 5^{o}C and individual aliquots removed and left to return to room

temperature afterwards. The size and zeta potential were measured to evaluate the NP after storage. We additionally measured the encapsulation efficiency of the NPs after storage to determine if any antibiotic had leached out. Figure S14 shows that after resuspending the NP, after 24 hours of storage, the size is approximately 87 nm and 88 nm for 0.1 wt% PenG-NPs and 0.1 wt% Oxa-NPs respectively. There is only a negligible difference with an additional 24 hours of storage for each NP sample. This indicates that storage at 5^{o}C does reduce the size of the NP. The same is true of the zeta potential which was measured after the same storage time and conditions. Upon redispersion and sonication the NP retained their positive zeta potential with no reduction depending on the length of time storage. Crucially, the concentration of either PenG or Oxa is required to remain high after storage, otherwise storing the NP is not viable. We investigated the encapsulation efficiency of the 0.1 wt% PenG-NPs and 0.1 wt% Oxa-NPs by supernatant absorbance. After the NP pellets where redispersed into the deionised water (buffered to pH 5 using acetate buffer), the NP suspension was again pelletised on the supernatant examined to determine the antibiotic concentration within. Figure S15 shows that after 1 day storage in a semi-wet pellet that approximately 1% of the encapsulated antibiotic is lost out of the NP, 69% for PenG and 68% for Oxa, compared to 0.1 wt PenG/Oxa encapsulation for fresh NPs (figure S4). This very small amount is due to the very low amount of water on the pellet. The antibiotic can only diffuse out of the NPs if the concentration in the surrounding solution goes below its equilibrium concentration of partitioning between particle and solution. The small amount of antibiotic that leaches out of the pelletised NPs will quickly saturate the small amount of liquid surrounding the pellet.

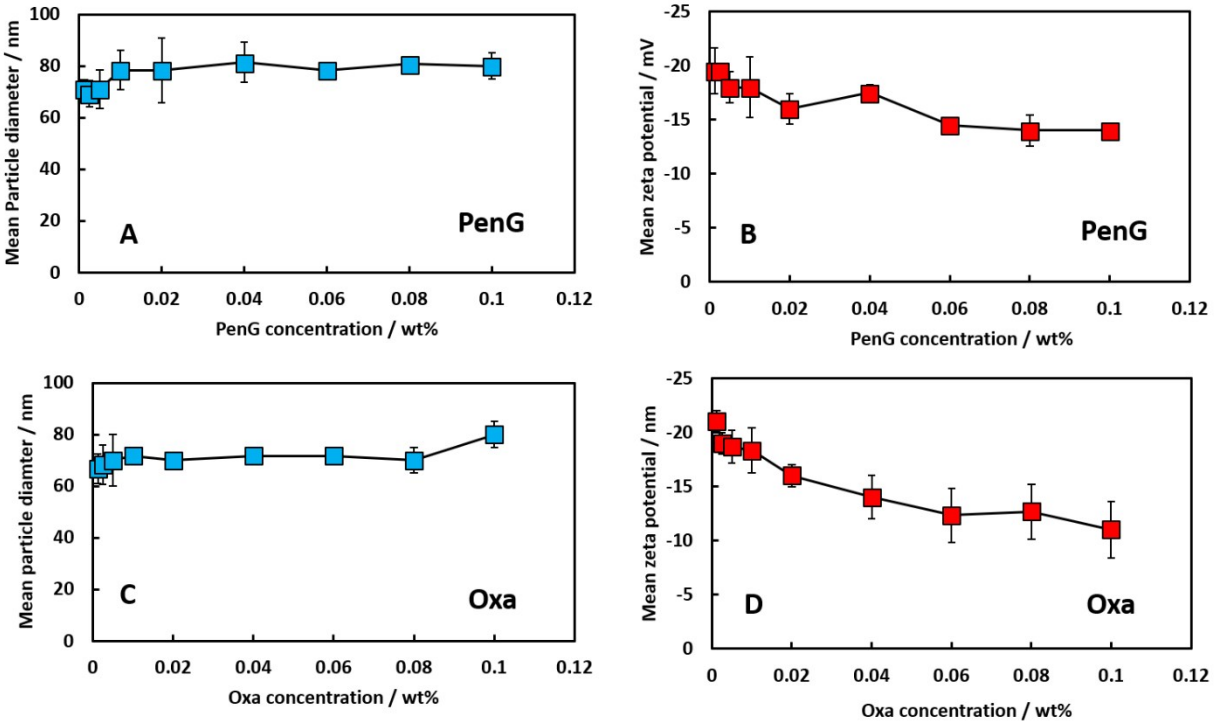

Figure S2. Size and zeta potential of different concentrations of penicillin G or oxacillin into 0.25 wt% shellac-0.2 wt% P407 NPs. (A) Shows size and (B) zeta potential of different concentrations of Penicillin G, (C) shows size and (D) zeta potential of different concentrations of Oxacillin. Measurements were taken at pH 5.5, 25 ^{o}C, RI 1.516, Abs 1.000 using a Malvern Zetasizer Nano ZS. Each value represents a triple replicate with ± S.D.

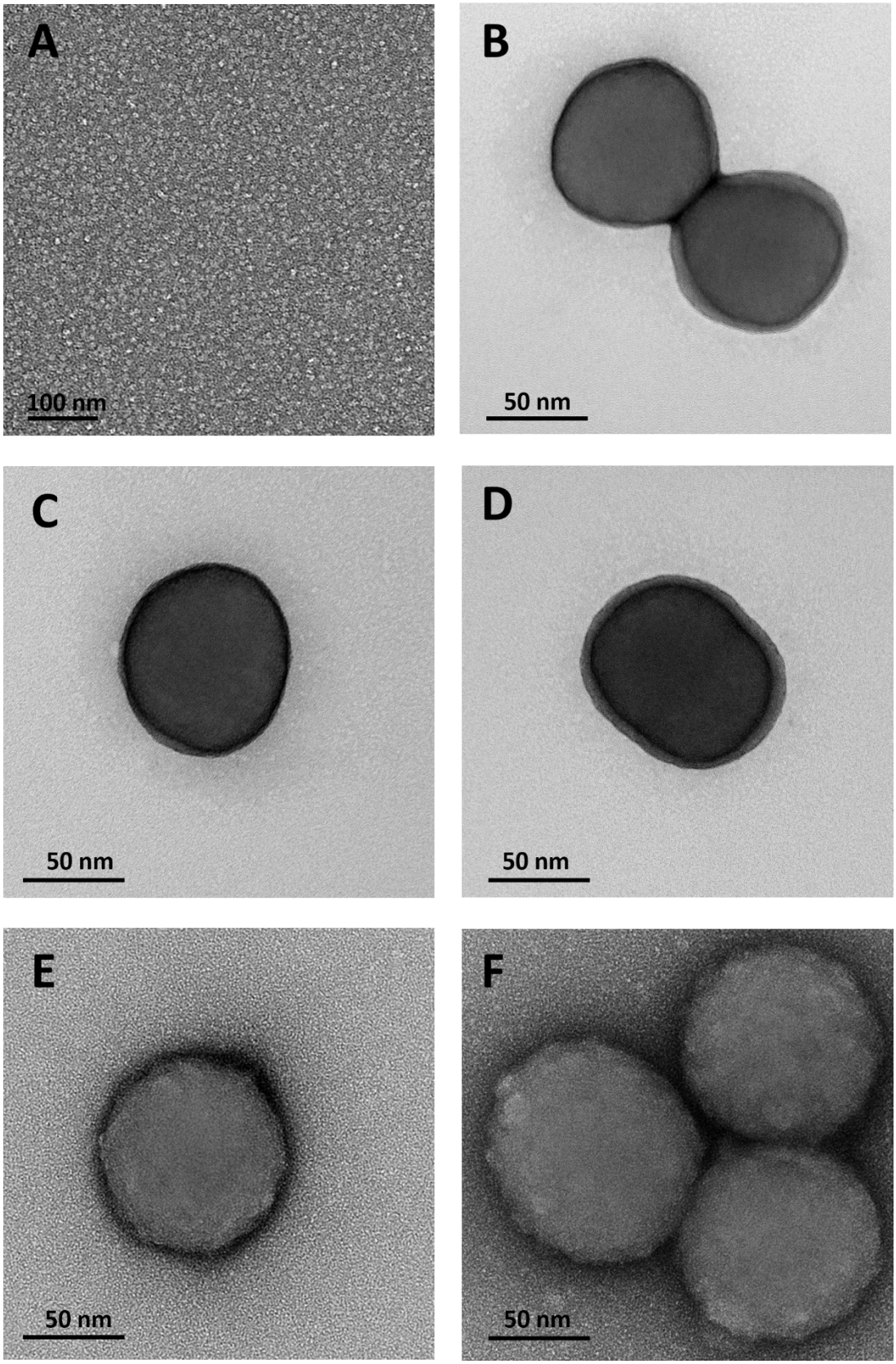

Figure S3. TEM photographs of (A) Alcalase 2.4 L FG, (B) 0.25 wt% Shellac-0.2 wt% P407 NPs, (C) 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% PenG NPs, (D) 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% Oxa NPs, (E) 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% PenG-0.2 wt% Alcalase NPs and (F) 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% Oxa-0.2 wt% Alcalase NPs.

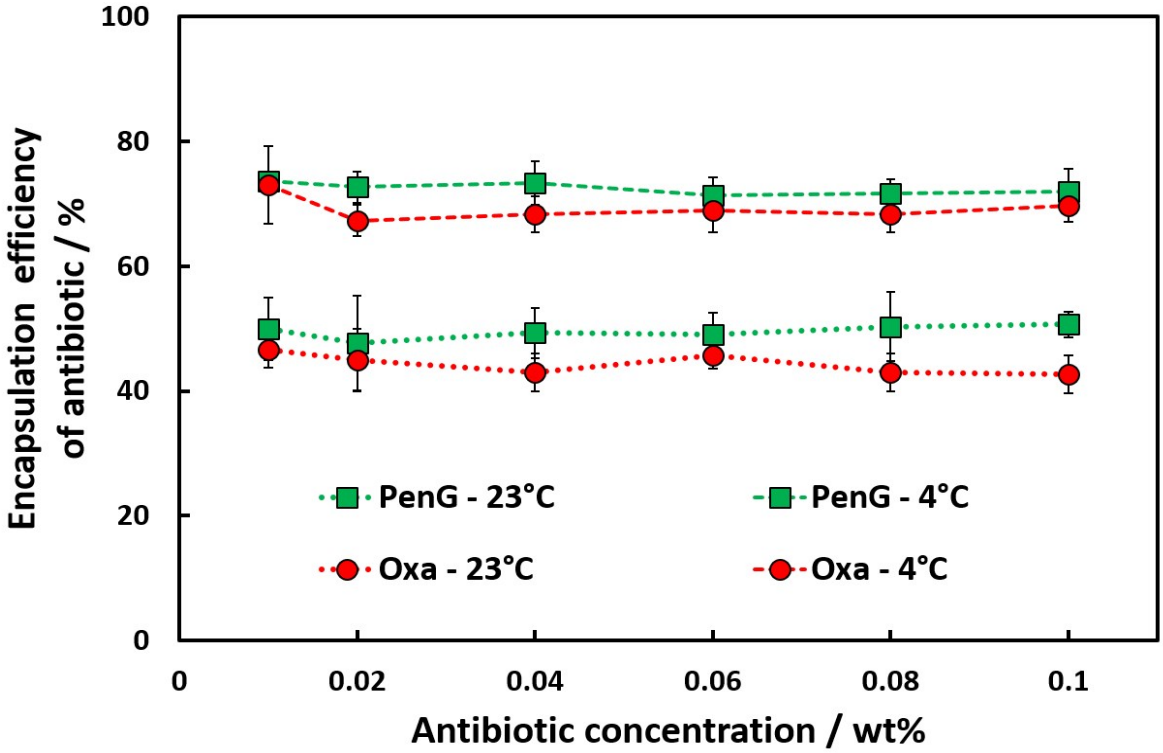

Figure S4. Encapsulation efficiency of varied concentrations of penicillin G and oxacillin into 0.25 wt% shellac-0.2 wt% P407 NPs. The shellac solution was adjusted to pH 10 and incubated at 23 °C and 4 °C for 1 hour to reach temperature. The P407 and antibiotic was then added, and the solution quickly reduced to pH 4. The mixture was then incubated at either 23 oC or 4 oC for 5 mins. Afterwards, the precipitated NP dispersion was increased to pH 5.5. pH adjustments were made using drops of 0.25 M HCl or 0.25 M NaOH. The nanoparticles were centrifuged for 30 minutes at 8500 rpm to pelletise leaving the supernatant with unencapsaulted AmpB. Absorbance (250 nm) of the supernatant was measured using a spectrophotometer to determine encapsulation efficiency. The follow equation was used to calculate the encapsulation efficiency;

$$Encapsulation\ efficency\ (\%) = \frac{total\ antibiotic\ - uncapsulated\ antibiotic}{total\ antibiotic} \text{ x } 100$$

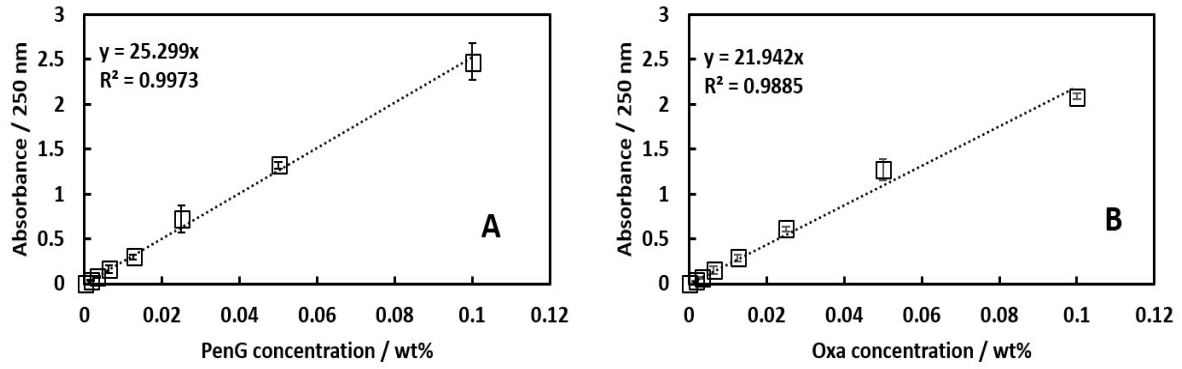

Figure S5. Standard calibration curves of difference concentrations of (A) Penicillin G and (B) Oxacillin. Absorbance measurements were taken 250 nm, pH 5.5 and room temperature.

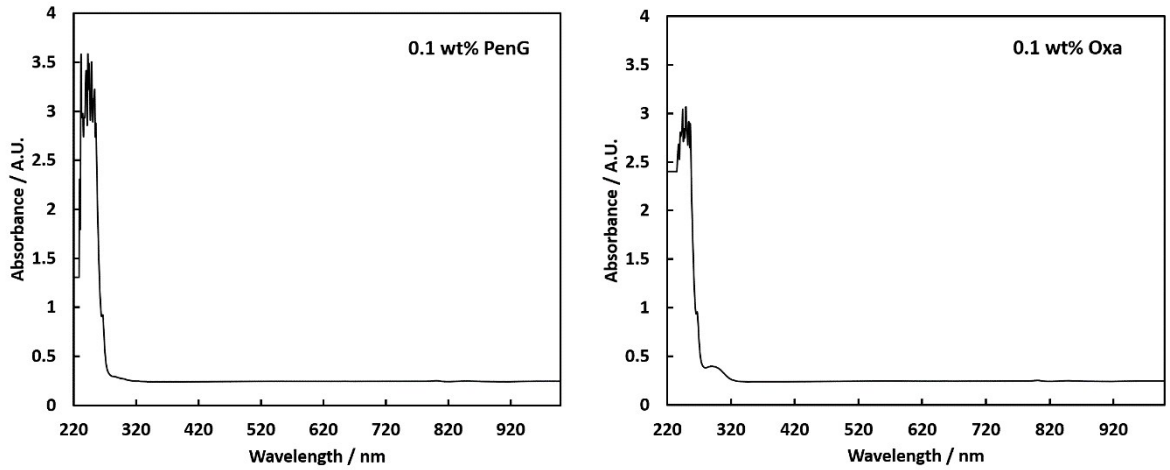

Figure S6. Absorbance spectra of PenG and Oxa measured between 220 and 1000 nm. Absorbance measurements were taken 250 nm, pH 5.5 and room temperature using a quartz cuvette. Measurements were taken using a PerkinElmer lambda 25 UV-Vis spectrophotometer.

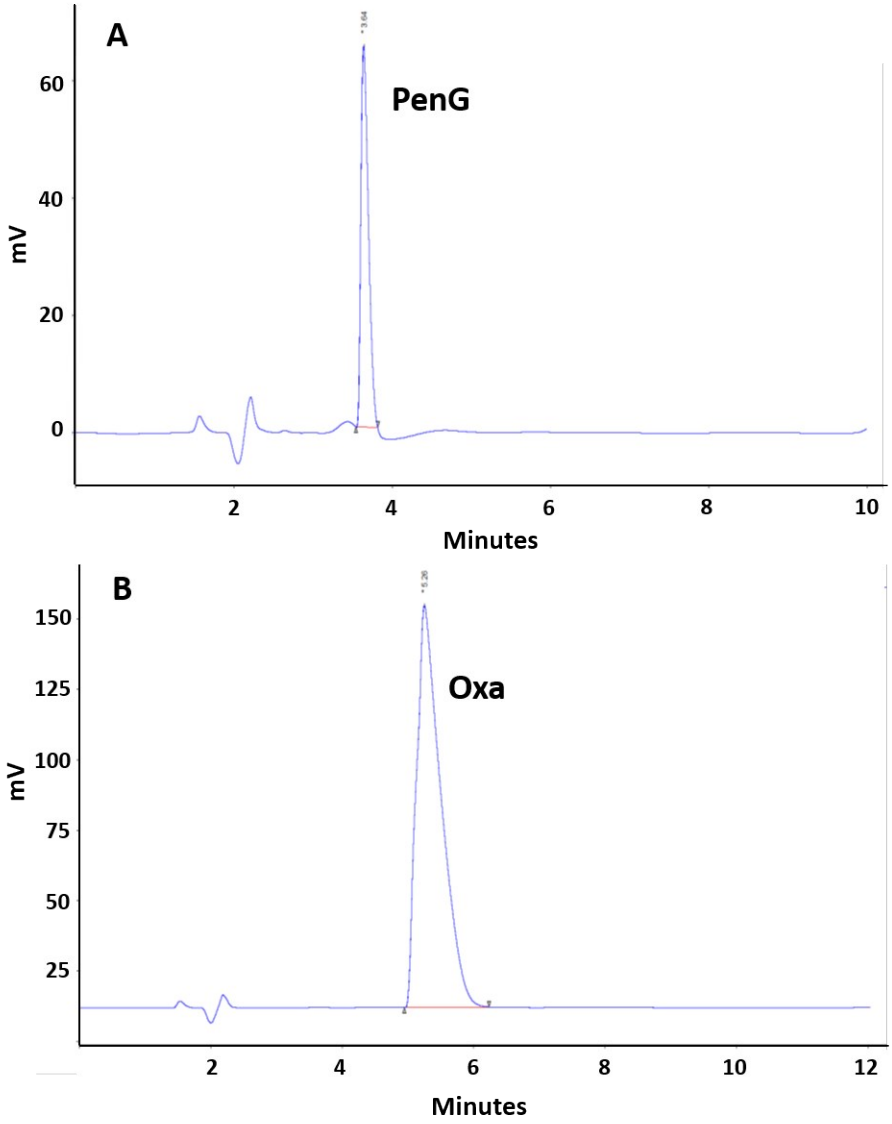

Figure S7. HPLC chromographs of (A) PenG - 1mg/mL (0.1 wt%) and (B) oxacillin - 1mg/mL (0.1 wt%) shown for illustrative purpose.

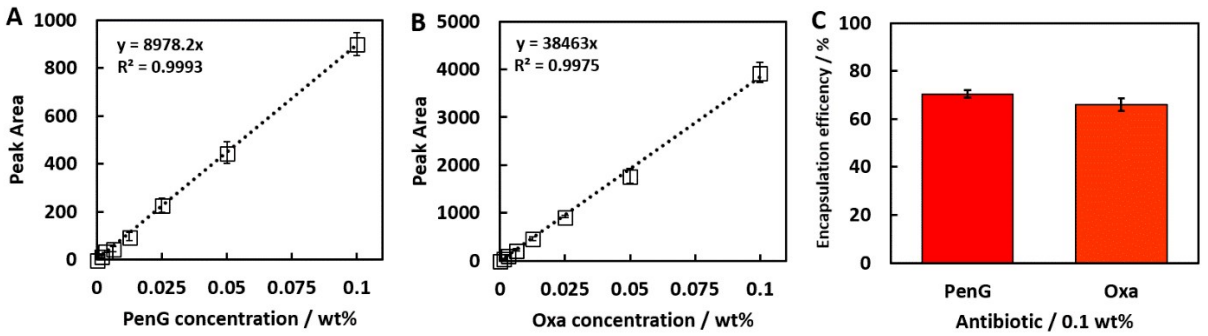

Figure S8. PenG (A) and (B) Oxacillin (B) HPLC calibration curves. Calibration curves were obtained from 0 to 0.1 wt% PenG/Oxa dissolved into acetate buffer. (C) Shows the encapsulation efficiency of 0.25 Shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs. Data was interpolated from PenG/Oxa peaks in the NP samples compared to PenG/Oxa calibration curves. 20 mL of 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs were pelletized and dissolved into 20 mL of acetate buffer (0.1 M, pH 5.5). The supernatant was discarded. 2-fold serial dilutions were made into acetate buffer (0.1 – 0 wt% PenG/Oxa). The samples were sonicated for 5 mins. 20 µL of each sample was injected into a 5 µm C18 column (Phenomenex, UK) and pumped at 1300 psi, 1 mL/min. The mobile phase was ammonium acetate (10 mM, pH 4.5) and acetonitrile in the ratio 75:25 (V:V) under isocratic elution. UV-absorbance at 220 nm was used for sample detection (Perkin-Elmer 785 A UV/VIS Detector, UK). The peak area was measured using Azur software. The same process was used to in PenG/Oxa antibiotic calibration curves.

Table S1. Elemental analysis of 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs, and the individual components. The PenG/Oxa content in the nanoparticle is calculated based on the sulphur (32.065 m_a) content of PenG/Oxa. NP samples were prepared and pellitised, and dried overnight under vacuum at room temperature to leave the dry mass of the encapsulated NP.

Element	**Shellac / %**	**P407 / %**	**PenG* / %**	**Oxa** / %**	**0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG / %**	**0.25 wt% shellac-0.2 wt% P407-0.1 wt% Oxa / %**
C	27.42	56.01	53.62	51.61	45.11	42.66
H	9.09	10.57	4.95	4.74	8.56	8.79
N	0.97	0.22	7.95	9.54	6.55	7.06
S	0.12	0	8.67	6.72	1.44	1.11
					% PenG content in shellac-P407 NPs	15.04 %
					% Oxa content in shellac-P407 NPs	13.92 %

*Penicillin G – 334.4 g/mol, $C_{16}H_{18}N_2O_4S$, **Oxacillin – 401.4 g/mol, $C_{19}H_{19}N_4O_5S$
PenG calculation; (1.44/32)*334.4 = 15.048
Oxa calculation; (1.11/32)*401.4 = 13.923

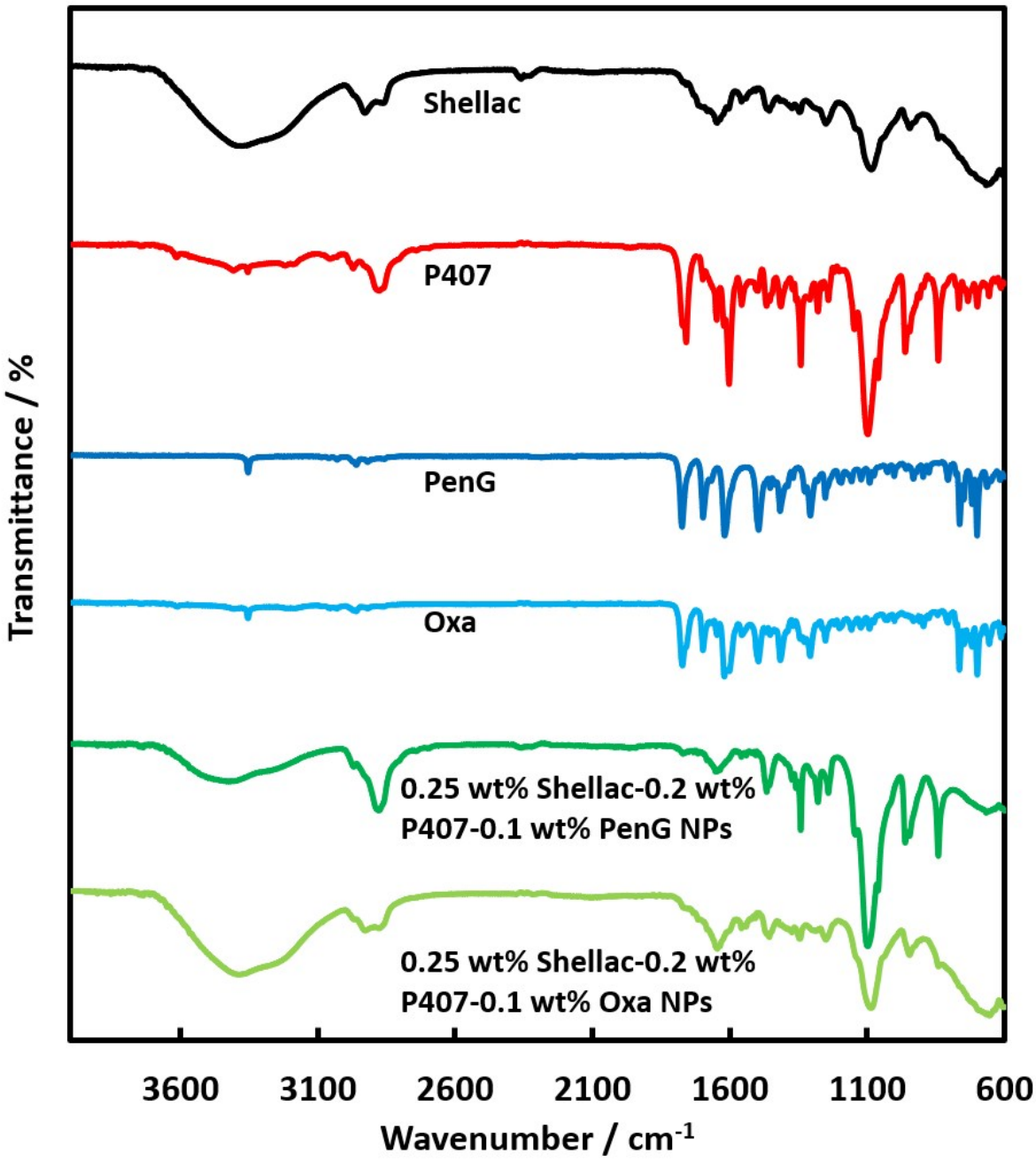

Figure S9. Fourier transform infra-red (FTIR) spectra of Shellac, P407, 0.25 wt%, free PenG, free Oxa, 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% PenG NPs and 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% Oxa NPs. Samples were prepared by removing water using a Silica gel desiccant by in vacuum chamber for 1 day, then dried at 60^{o}C for 2 days. The spectra were measured between 4000-600 cm-1 using PIKE ATR diamond settings. A spectrum was obtained with a blank of just air to reduce transmittance interference with the samples.

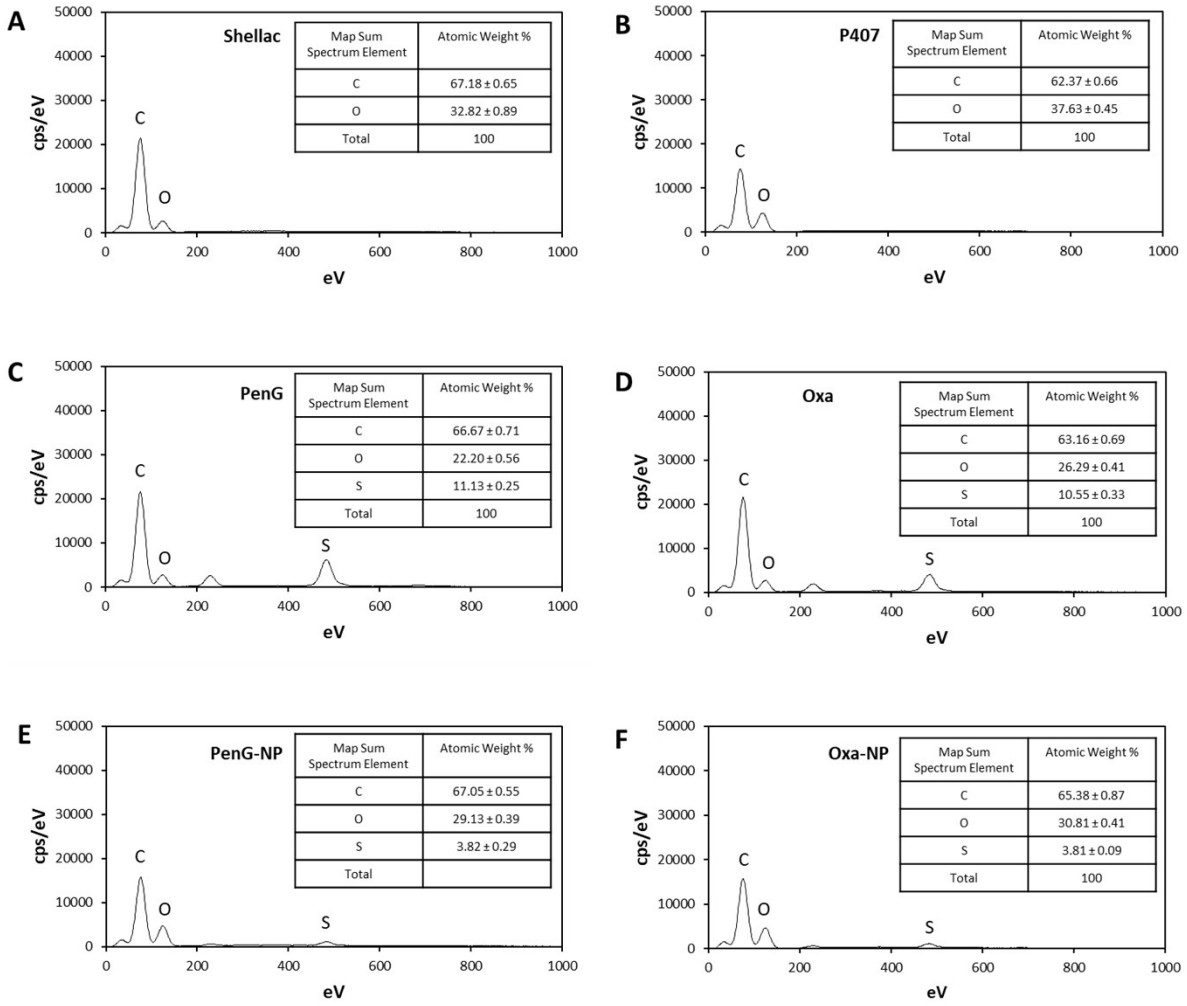

Figure S10. EDX spectra of (A) shellac, (B) P407, (C) PenG, (D) Oxa, (E) 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG-NPs, and (F) 0.25 wt% shellac-0.2 wt% P407-0.1 wt% Oxa-NPs. The measurement for shellac, P407, PenG and Oxacillin was taken three separate locations and the data averaged. The measurement for the NP was taken in the centres of three separate NPs and averaged. A Nanoanalysis X-Max 65-T detector and the INCA software were used to produce the elemental analysis spectra.

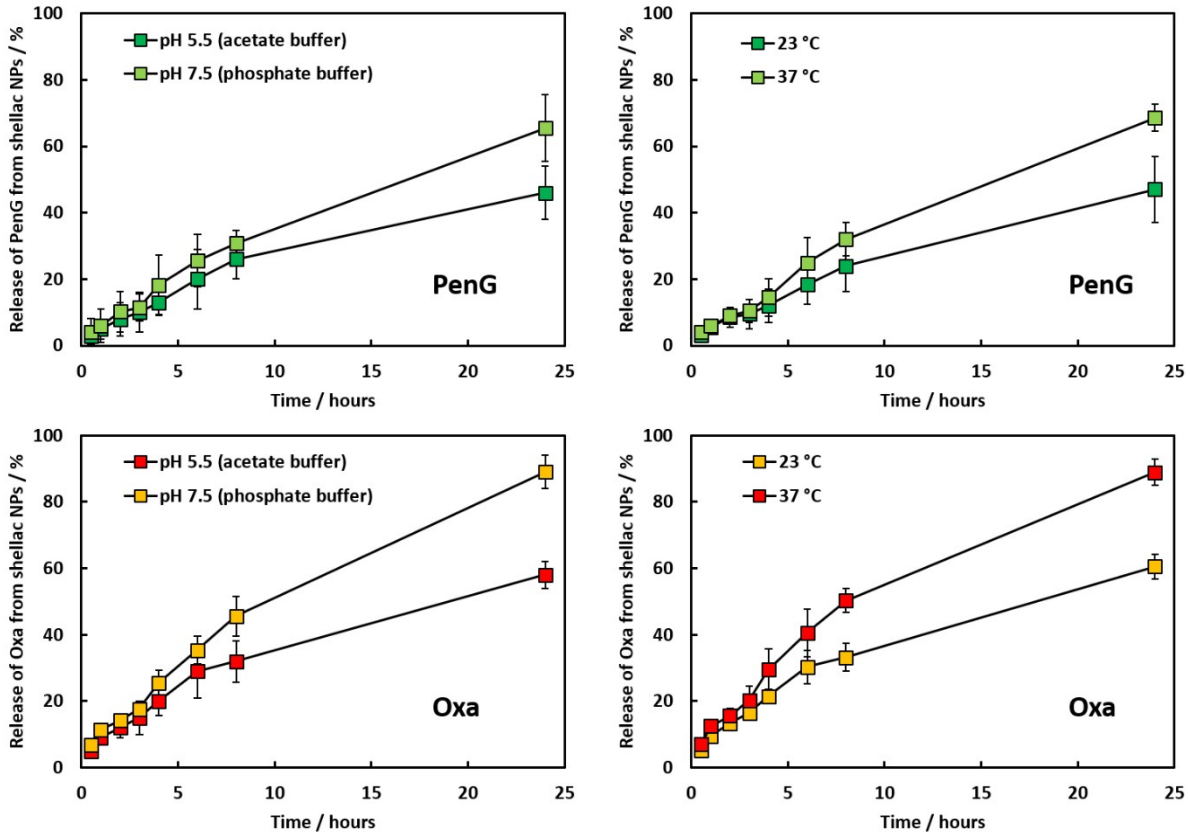

Figure S11. Release percentage of PenG and Oxa from 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs as a function of pH and temperature. The release percentage of the temperature experiments were performed at pH 5.5, buffered with acetate buffer. For both experiments 50 mL of the 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% ABX NP formulation was placed in to a 10-12 kDa MWKO dialysis bag. The dialysis bag was placed into 500 mL of the specific buffered and at a specific temperature. 1 mL aliquots were taken at specific time points on the absorbance of the aliquot equated into the concentration of PenG/Oxa in the buffer using a standard calibration curve. Absorption was measured at 250 nm. The follow equation was used to calculate the release kinetics;

$$Antibiotic\ release\ (\%) = \frac{amount\ of\ antibiotic\ in\ the\ buffer\ solution\ at\ a\ specific\ time}{amount\ of\ antibiotic\ encapsulated\ into\ shellac\ NP} \text{ x } 100$$

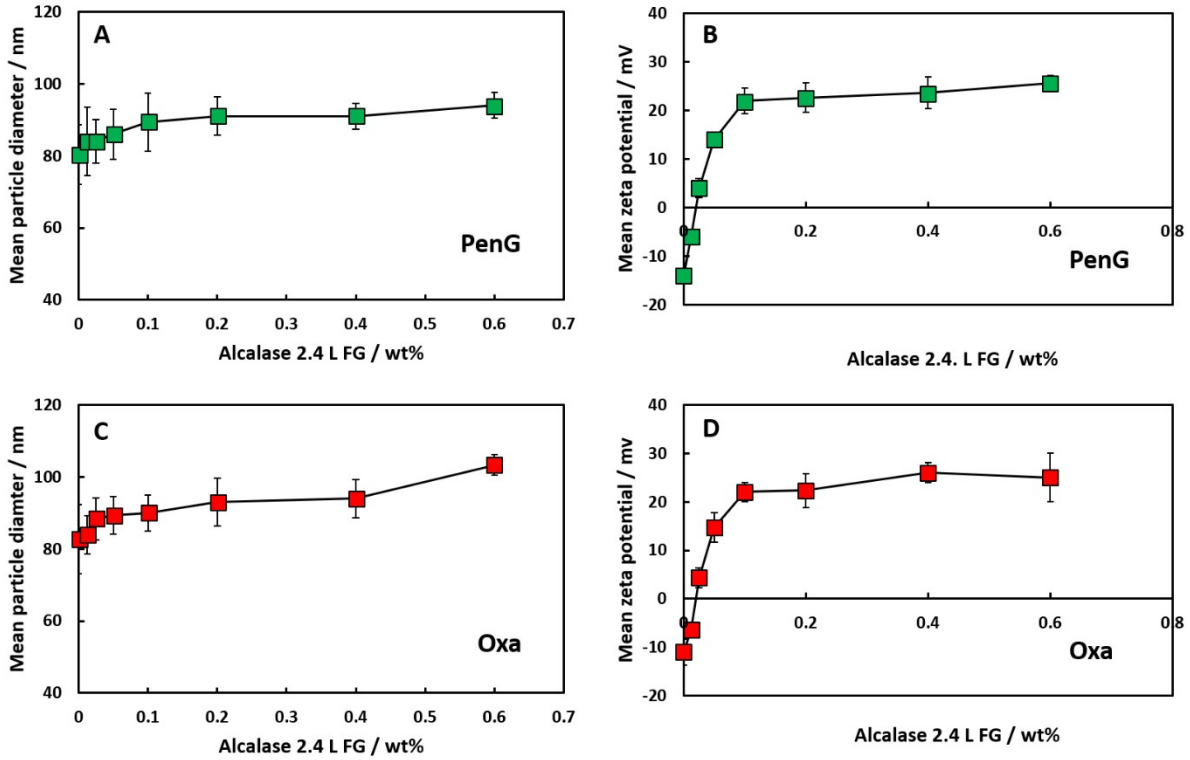

Figure S12. The (A) mean particle diameter and (B) zeta potential of 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG NPs coated with various concentrations of Alcalase 2.4 L FG. The (C) mean particle diameter and (D) zeta potential of 0.25 wt% shellac-0.2 wt% P407-0.1 wt% Oxa NPs coated with various concentrations of Alcalase 2.4 L FG. Various concentrations of Alcalase were prepared in 25 mL solution and mixed for 30 min at room temperature with an 25 mL of 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs suspensions at pH 5.5. Afterwards the mixture was centrifuged at 8000 rpm for 30 mins to pelletize the NPs. The pellet was redispersed into 25 mL of deionised water buffered to pH 5.5 (acetate buffer) yielding a stock solution of 0.25 wt% Shellac-0.20 wt% P407-0.1 wt% PenG/Oxa-0.2 wt% Alcalase NPs. Measurements were taken at pH 5.5, 25 ^{o}C, RI 1.516, Abs 1.000 using a Malvern Zetasizer Nano ZS. Each value represents a triple replicate with ± S.D.

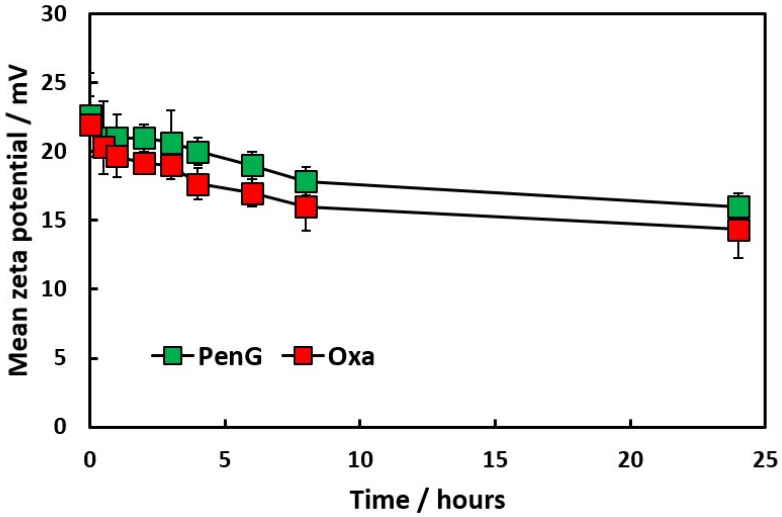

Figure S13. The zeta potential of 0.25 wt% Shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs coated with 0.2 wt% Alcalase 2.4 L FG over time. After coating with Alcalase 2.4 L FG the NPs were centrifuged at 8000 rpm and the pellet washed three times in deionised water. The pellet was re-dispersed into deionised water adjusted to pH 5.5 using acetate buffer. Zeta potential measurements were taken at different time points up to 24 hours to investigate the stability of the Alcalase coating.

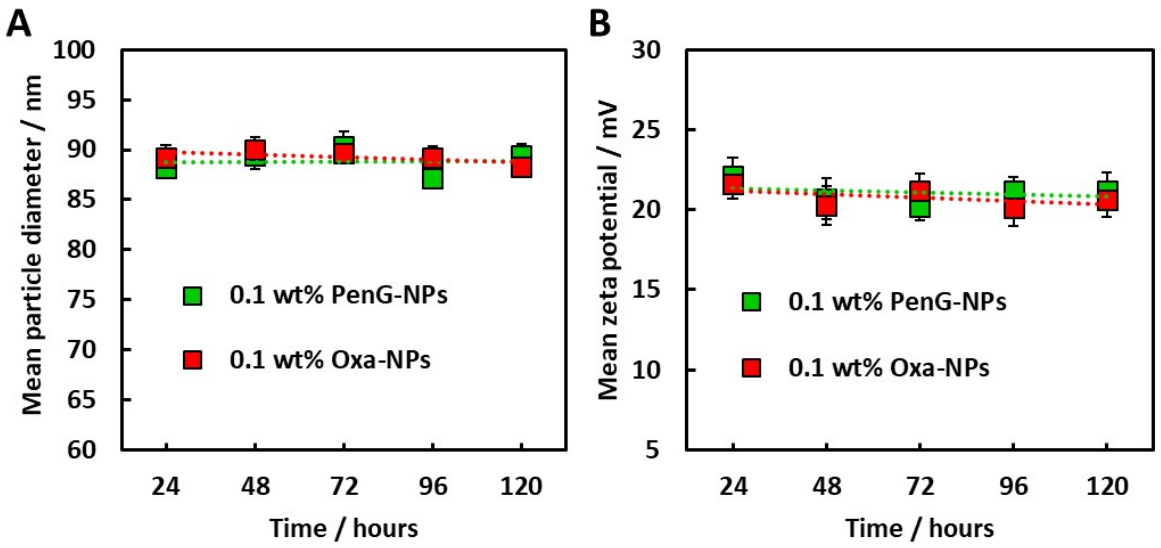

Figure S14. The **(A)** mean particle diameter and **(B)** zeta potential of 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG/Oxa NPs coated with 0.2 wt% Alcalase 2.4 L FG. After NP synthesis the NP suspension was pelletised by centrifugation at 8000 rpm for 30 mins. Afterwards the supernatant was discarded and the semi-wet pellet frozen and stored at -20oC. After storage, the pellet was resuspended into deionised water buffered to pH 5.5 (acetate buffer) to the same volume as synthesised with constant shaking. The samples were then sonicated for 5 minutes in a water bath. Measurements were taken at pH 5.5, 25 oC, RI 1.516, Abs 1.000 using a Malvern Zetasizer Nano ZS. Each value represents a triple replicate with ± S.D.

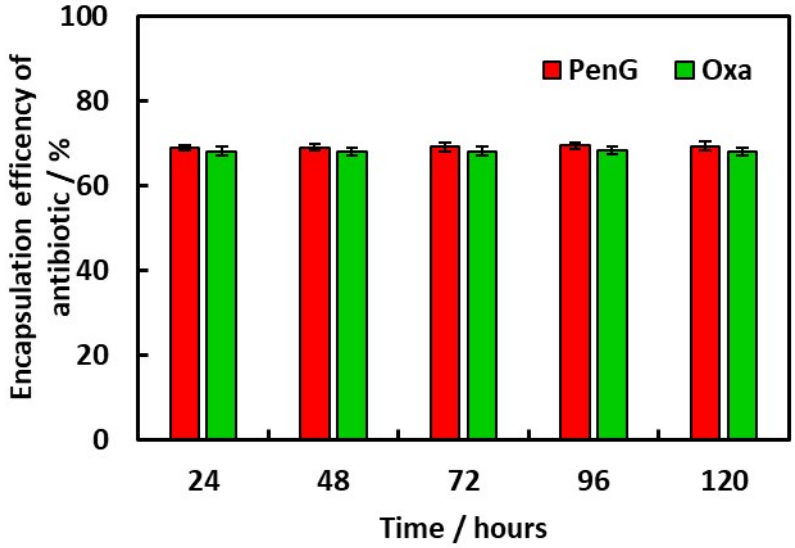

Figure S15. Encapsulation efficiency of varied concentrations of penicillin G and oxacillin into 0.25 wt% shellac-0.2 wt% P407-0.2 wt% Alcalase NPs. Absorbance (250 nm) of the supernatant was measured using a spectrophotometer to determine encapsulation efficiency. The follow equation was used to calculate the encapsulation efficiency:

$$Encapsulation\ efficency\ (\%) = \frac{total\ antibiotic\ -uncapsulated\ antibiotic}{total\ antibiotic} \text{ x } 100$$

Additional Results

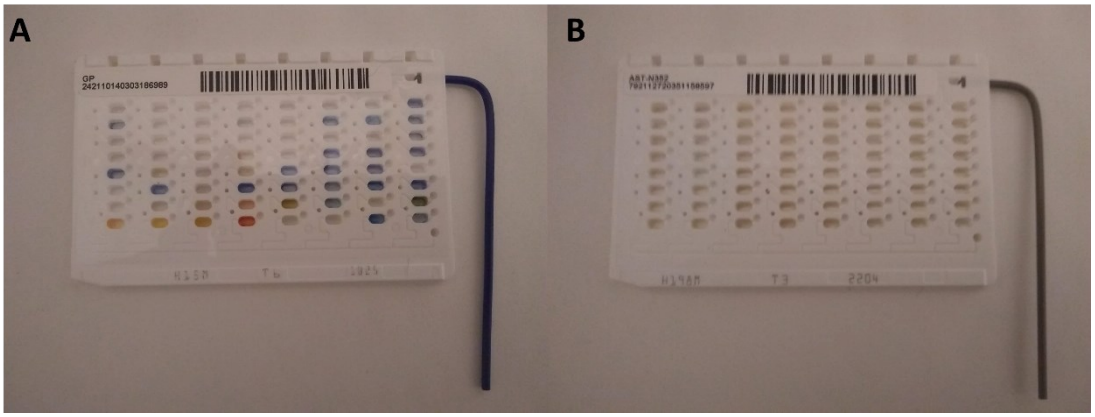

Figure S16. Photographs of (A) VITEK 2 GP (21342) and (B) VITEK 2 AST-P635 (416911) Gram-positive identification and AST cards

Table S2. VITEK2 *Staphylococcus aureus* Antimicrobial Susceptibility Screen

Selected Organism: Staphylococcus aureus					
Comments: Rule 5: Chloramphenicol not used for food animals. (Source: CLSI 2015, VET01S, 3rd edition). Rule 6: tetracycline tested as the class representative for susceptibility to chlortetracycline, doxycycline, minocycline and oxytetracycline. Organisms that are susceptibility to tetracycline are also considered susceptible to doxycycline and minocycline. However, some organisms that are intermediate or resistant to tetracycline may be susceptible to doxycycline or minocycline or both. (Source: CLSI 2015, VET01S, 3rd edition). Rule 8: Cefoxitin is used as a surrogate for oxacillin, report oxacillin susceptible or resistant based on the cefoxitin result. (Source: CLSI 2015, VET01S, 3rd edition). Rule 11: Clindamycin is also used to test for susceptibly to lincomycin. Clindamycin is more active than lincomycin against most staphylococcal strains. (Source: CLSI 2015, VET01S, 3rd edition).					
Identification Information	**Card**: GP		**Lot Number**: 2420469403		**Analysis time:** 7.85 hours
Organism Origin	VITEK 2				
Selected Organism	99% Probability **Staphylococcus aureus**				
	Bionumber: 030412067763271		**Confidence**: Low discrimination		
Susceptibility Information	**Card**: AST-P635		**Lot Number**: 7350480103		**Analysis time:** 8.41 hours
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Cefoxitin Screen	POS	+	Teicoplanin	<= 0.5	S
Benzylpenicillin	>= 0.5	R	Vancomycin	1	S
Oxacillin	>= 4	S	Tetracycline	<= 1	S
Gentamicin	<= 0.5	S	Tigecycline	<= 0.12	S
Inducible Clindamycin Resistance	NEG	-	Fusidic Acid	<= 0.5	S
Erythromycin			Mupirocin	<= 2	
Clindamycin	0.25	S	Chloramphenicol	8	S
Linezolid	2	S	Rifampicin	<= 0.03	S
Daptomycin	0.25	S	Trimethoprim	1	S

*+= Deduced drug *= AES modified ** User modified. Parameter Set: Global CLSI-based+Natural Resistance V2 17 NOV2017*

Figure S17. Zones of growth inhibition for right oxacillin (1 µg concentration) and left penicillin G (10iu). Inoculum was at 0.5 McFarland incubated at 35 ± 1 ^{0}C for 18 hours.

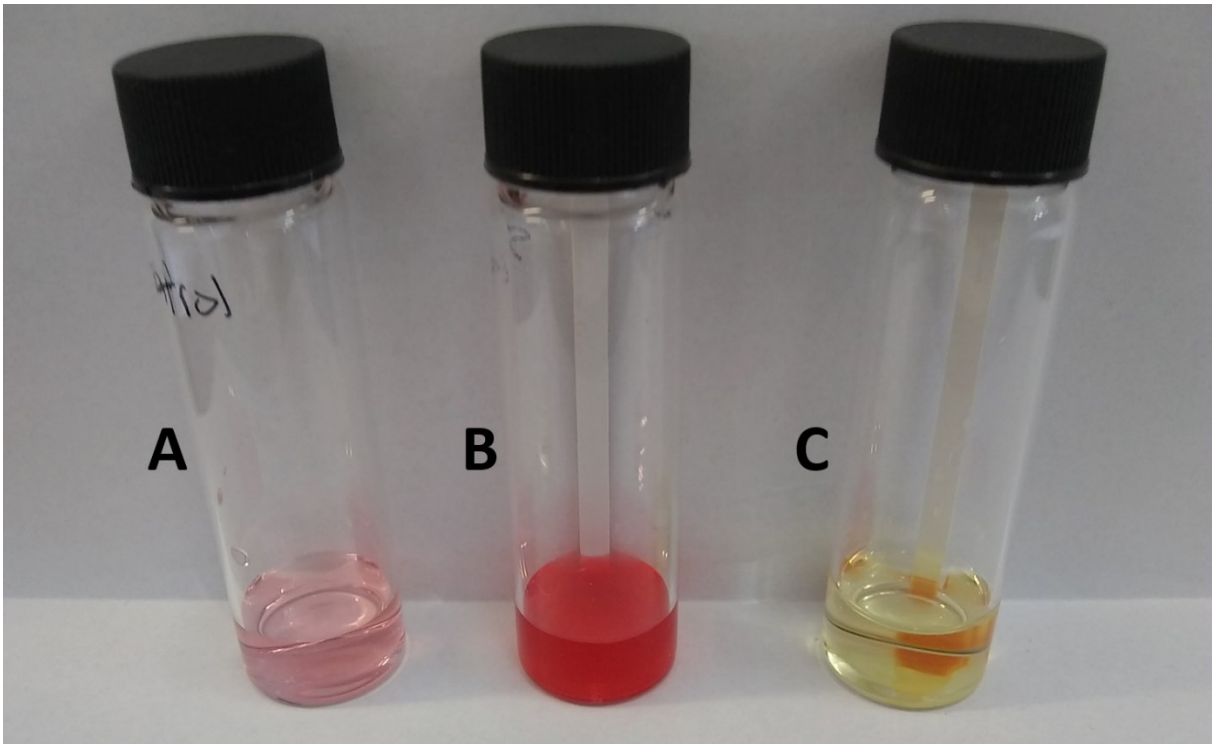

Figure S18. Beta-lactamase detection. The plastic strip contains an active zone saturated with penicillin G and an acid-basic indicator. The process is based on the hydrolysis of the beta-lactam ring in penicillin G producing penicillioc acid. This caused acidification of the bacterial suspension (yellow indicated the presence of penicillioc acid and thus beta-lactamase. 3 colonies of *S. aureus* and *S. epidermidis* (positive control) were placed into 1 mL of moistening solution and a test strip was added. The bacterial suspension was incubated at room temperature for 10 minutes with shaking. (A) Shows the pink coloured moistening solution with no bacteria, (B) shows the *S. epidermidis* suspension and (C) shows the *S. aureus* suspension. Red indicates negative and yellow positive for beta-lactamase.

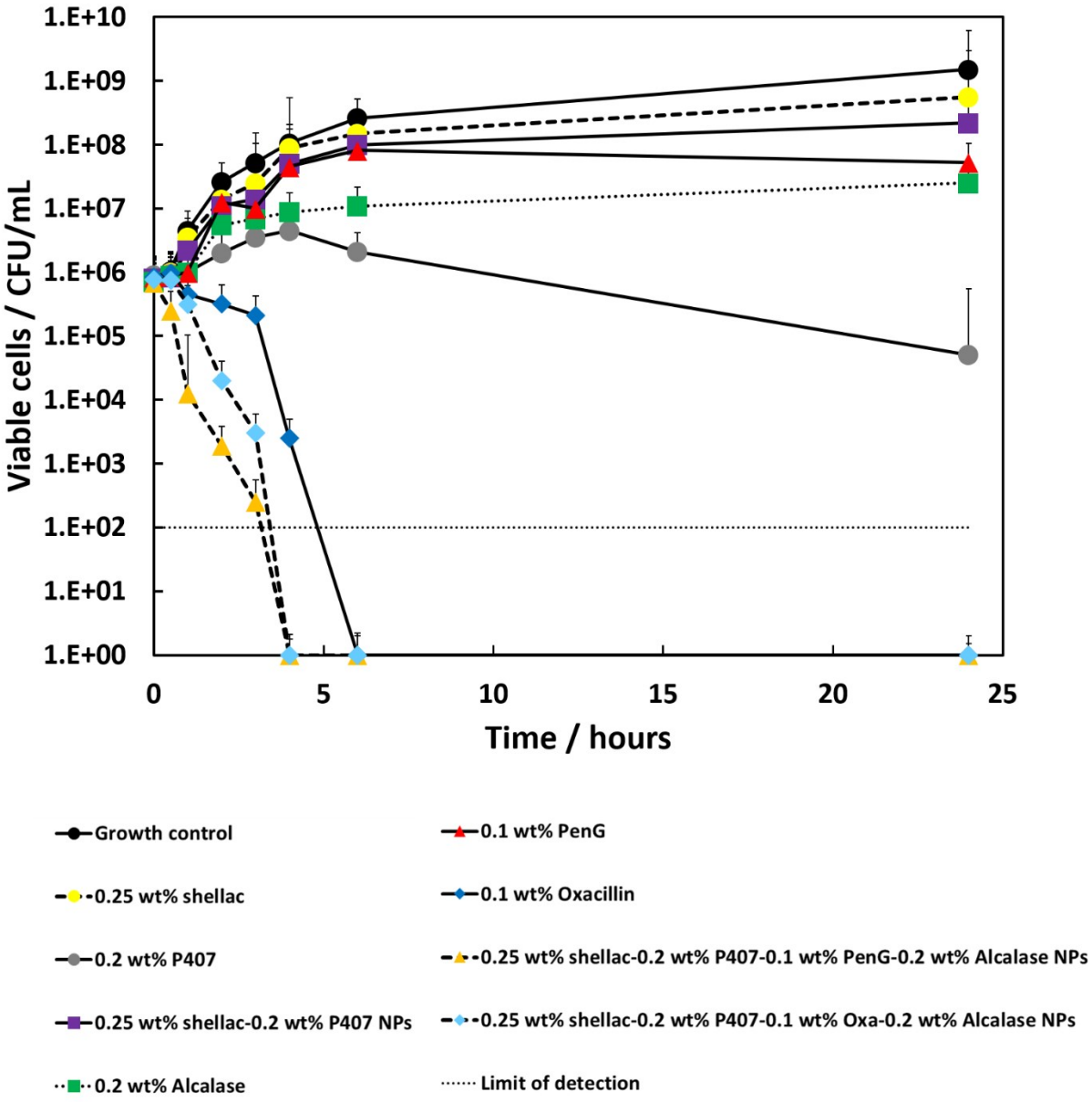

Figure S19. Time kill assays against *S. aureus*. Growth control (untreated). 0.25 wt% shellac, 0.2 wt% P407, 0.25 wt% shellac-0.2 wt% P407 NPs and 0.2 wt% Alcalase are considered controls. 0.1 wt% free PenG and 0.1 wt% Oxacillin were compared to the same antibiotic concentration encapsulated into Alcalase-coated shellac NPs. The results are presented as means (n=3).

Table S3. *S. aureus* MIC and MBC tabulated data.

	MIC	MBC
Shellac	> 0.25 wt% (2500 µg/mL)	> 0.25 wt% (2500 µg/mL)
P407	> 0.2 wt% (2000 µg/mL)	> 0.2 wt% (2000 µg/mL)
Shellac-P407 NPs	> 0.25 wt% (2500 µg/mL)	> 0.25 wt% (2500 µg/mL)
Alcalase	0.1 wt% (1000 µg/mL)	> 0.2 wt% (2000 µg/mL)
PenG	0.05 wt% (500 µg/mL)	> 0.1 wt% (1000 µg/mL)
Encapsulated PenG-NPs	0.0015 wt% (15 µg/mL)	< 0.0003 wt% (03 µg/mL)
Oxa	< 0.0003 wt% (3 µg/mL)	< 0.0003 wt% (3 µg/mL)
Encapsulated Oxa-NPs	< 0.0003 wt% (3 µg/mL)	< 0.0003 wt% (3 µg/mL)

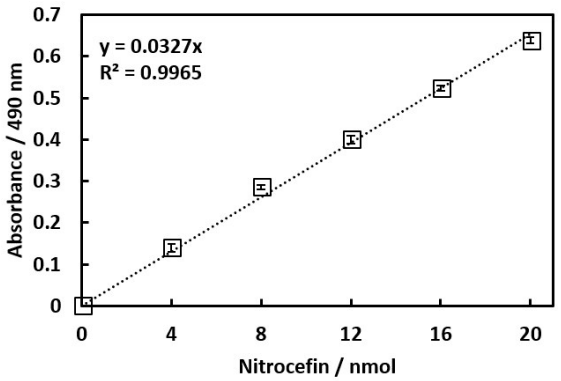

Figure S20. Nitrocefin standard calibration curve. The nitocefin stock solution was hydrolysed by adding 8 µL of nitrocefin to 8 µL of Beta Lactamase Hydrolysis Buffer and 28 µL of DMSO (ration 1:2:7) into an Eppendorf tube. The mixture was reacted at 60 ^{o}C for 10 minutes before cooling to room temperature (23 ^{o}C) and centrifuged at 1000 rpm for 1 minute. 0, 2, 4, 6, 8 & 10 µL of the hydrolyzed Nitrocefin Standard (2 mM) was added into a series of wells in a 96-well plate to generate 0, 4, 8, 12, 16 & 20 nmol/well of Nitrocefin Standard. The final volume of each well was adjusted to 100 µL/well using Beta Lactamase Buffer solution. Absorbance was read at 490 nm, 0 nmol nitrocefin was used as a blank.

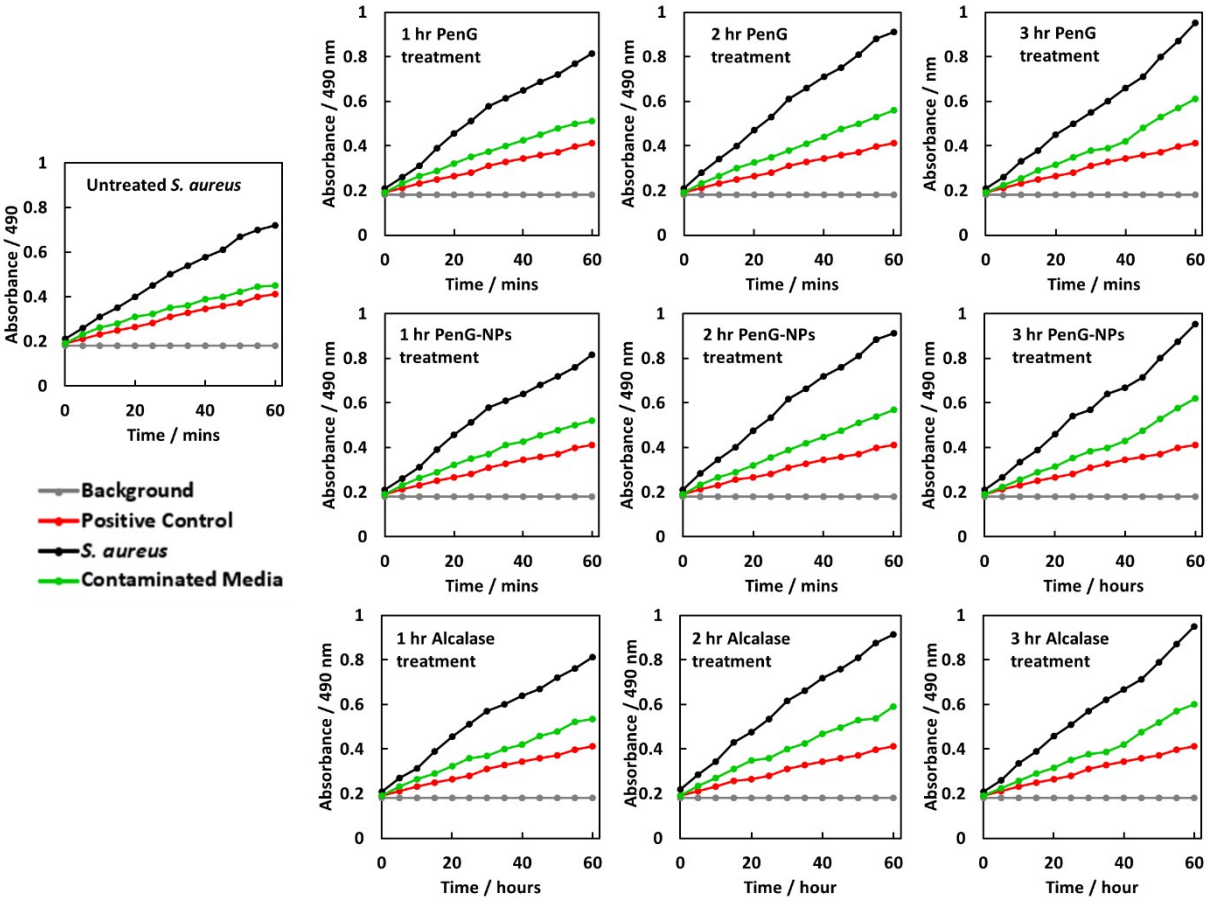

Figure S21. Comparison of beta lactamase activity of *S. aureus* after treatment with free PenG vs PenG-NPs over time. *S. aureus* samples were obtained after no treatment, or after treatment with either 0.1 wt% PenG or 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG-0.2 wt% Alcalase (referred to as PenG-NPs) or 0.2 wt% Alcalase. *S. aureus* cells, contaminated (culture) media, a positive control and a background reading for the reaction mix were analysed. Note; there is no discernible difference in beta lactamase activity of *S. aureus* when treated with either free PenG, PenG-NPs (both 0.1 wt%) or 0.2 wt% Alcalase between 1 and 3 hours.

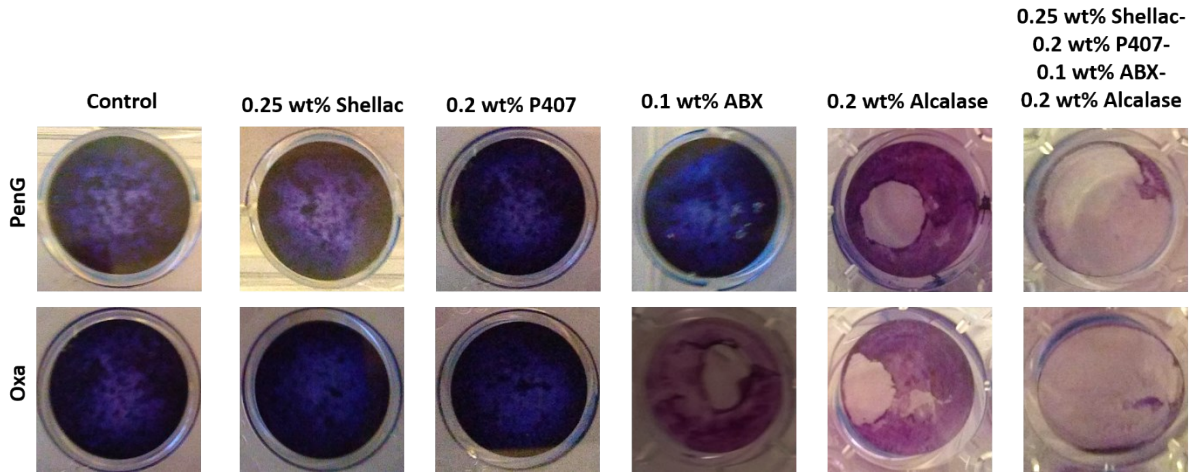

Figure S22. Photographs of *S. aureus* biofilms after CV staining and washing (before solubilization in acetic acid).

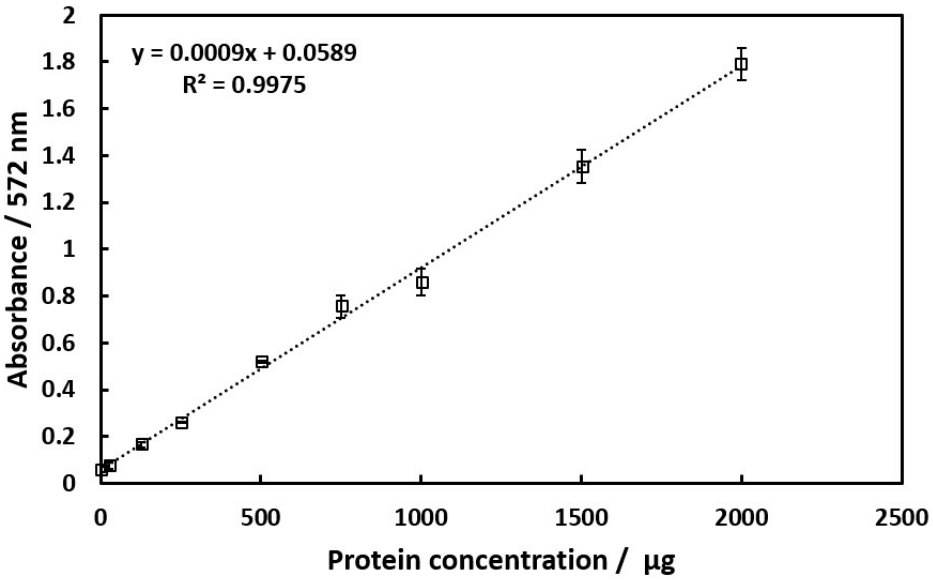

Figure S23. BCA (bicinchoninic acid assay) standard curve. N=3 with ± S.D.

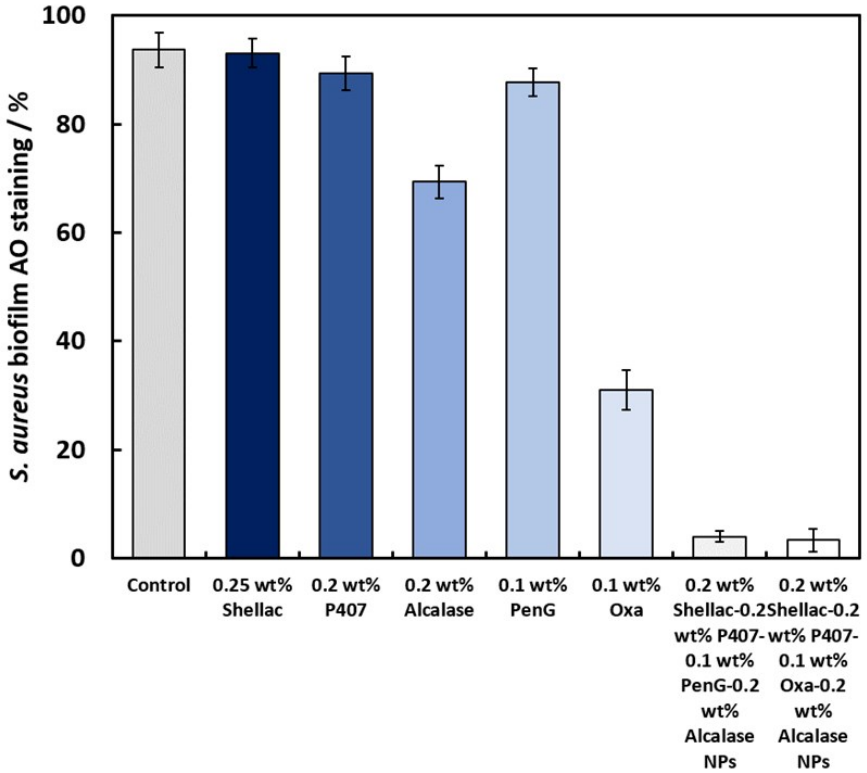

Figure S24. Quantification of AO staining of *S. aureus* biofilms treated for 24 hours. The % of biofilm stained with OA was quantified using ImageJ v1.52d.

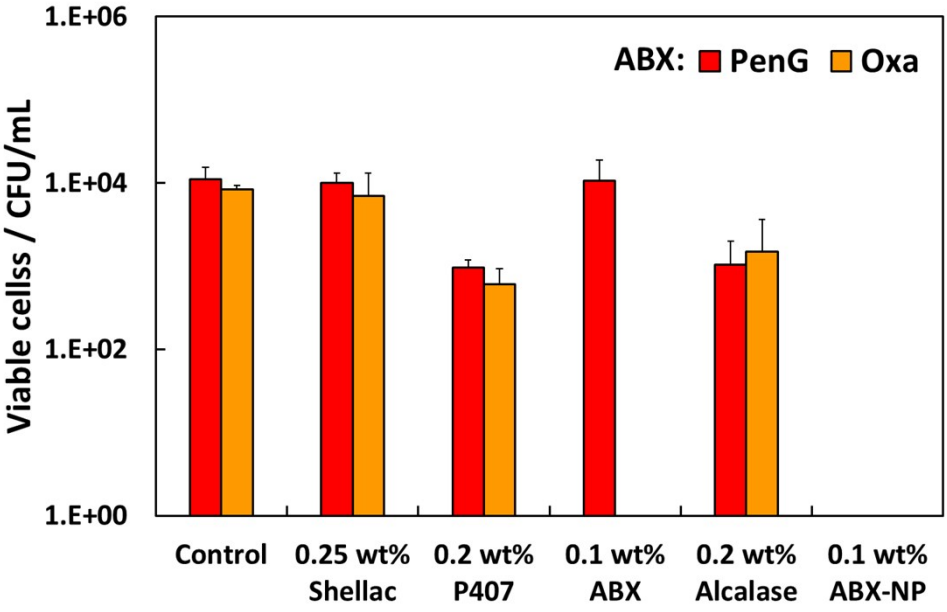

Figure S25. 24 hour grown *S. aureus* biofilm cellular viability after anti-biofilm treatments. CFU/mL of treatment media after 24 hours. Post-treatment 1 mL of the treatment media was serial diluted. The agar drop plate method was used to elucidate the CFU/mL. N=3 with ± S.D.

Cytotoxicity and of HaCaT and HEP G2 cells after treatment with shellac, P407, free Alcalase, free PenG/Oxa and PenG/Oxa-NPs

A preliminary human cell cytotoxicity experiment was performed on HaCaT and HEP G2 cells to investigated if the shellac, P407, free Alcalase. Free antibiotic, and PenG/Oxa-NPs were toxic to human cells. HaCaT cells were chosen as they a good proxy for studying human keratinocytes, a cell type which would be exposed to treatment in topical applications. HEP G2 were chosen as they are good proxy for studying the effects of xenobiotics. Figure S26A and B shows that 0.25 wt% shellac had only a marginal reduction in cell viability over 1, 6- and 24-hour time points. 0.2 wt% P407 showed a slightly decreased viability, particularly after 24-hour treatment. This is to be expected of a surfactant which are known to be cytotoxic due to their ability to destroy the plasma membrane of cells. Interestingly, 0.25 wt% shellac-0.2 wt% P407 showed only a marginal reduction in viability in both the HaCaT and HEP G2 cells. This is likely due to the absorption of the P407 onto the shellac NP reducing the ability of the P407 to damage the cells. 0.2 wt% Alcalase showed again only a small reduction in viability over 1, 6- and 24-hour time points. 0.1 wt% free PenG/Oxa demonstrated only a small cytotoxic effect. This is to be expected as antibiotic supplementation of cell culture media is routinely used, particularly PenG, and is generally considered safe to use. A similar result was observed with the 0.1 wt% PenG/Oxa-NP treatments indicating the concentrating of antibiotic into a NP centre had little effect on cell toxicity. The data shows that the PenG/Oxa-NPs had very little impact on cell viability in both HaCaT and HEP G2 cells, providing evidence they may be suitable for *in vivo* treatment topically on infected chronic wounds. Figure S27A and B showed the cytotoxicity of PenG-NPs and Oxa-NPs on on HaCaT and HEP G2 cells as a function of concentration dependence after 24 hours of treatment. Dilutions of the NPs were made into serialised PBS. The results of the 0.1 wt% PenG/Oxa-NPs for both the HaCaT and HEP G2 cells are the same as revealed in figure S26A and B. As the concentration is reduced the viability of the cells is shown to increase slightly, gradually improving towards the control of no treatment. This provides further evidence that the 0.1 wt% PenG/Oxa-NPs are only slightly toxic to mammalian cells over 24 hours.

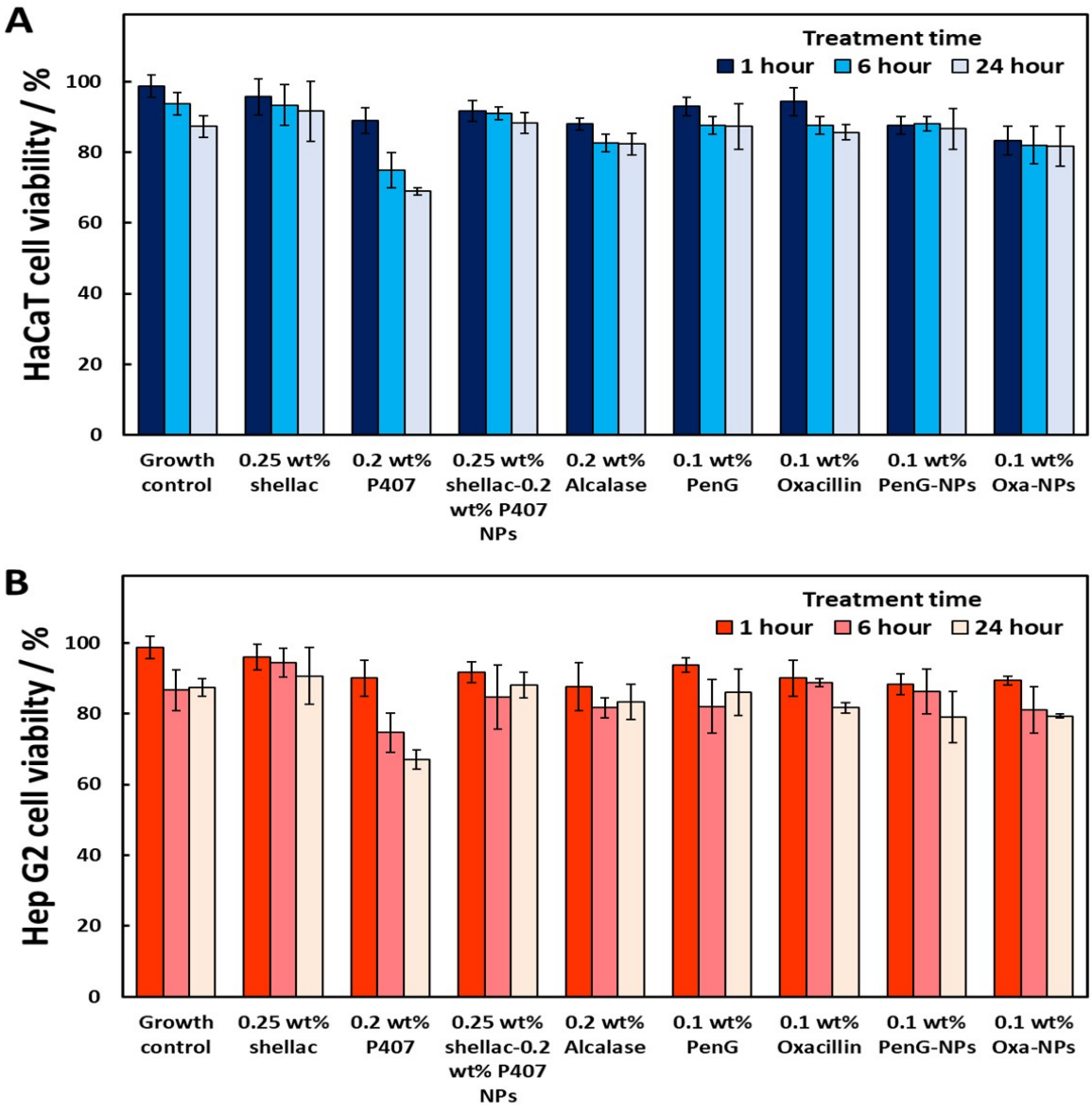

Figure S26. (A) HaCaT and **(B)** Hep 2G viability after treatment with 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG/Oxa-0.2 wt% Alcalase NPs compared to 0.1 wt% free PenG/Oxa. NP components treated at the same concentration as the NP formulation were also tested. Cells were seeded at 1×10^4 and incubated for 24 hours at 37 °C in 5% CO_2 atmosphere (2 wt% FBS). The medium was then removed and replaced with treatment infused media under the same conditions. Viability counts were performed at 1, 6, and 24 hours. 0.1 wt% PenG-NP and 0.1 wt% Oxa-NP refers to 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG-0.2 wt% Alcalase NPs and 0.25 wt% shellac-0.2 wt% P407-0.1 wt% Oxa-0.2 wt% Alcalase NPs. N=3 with ± S.D.

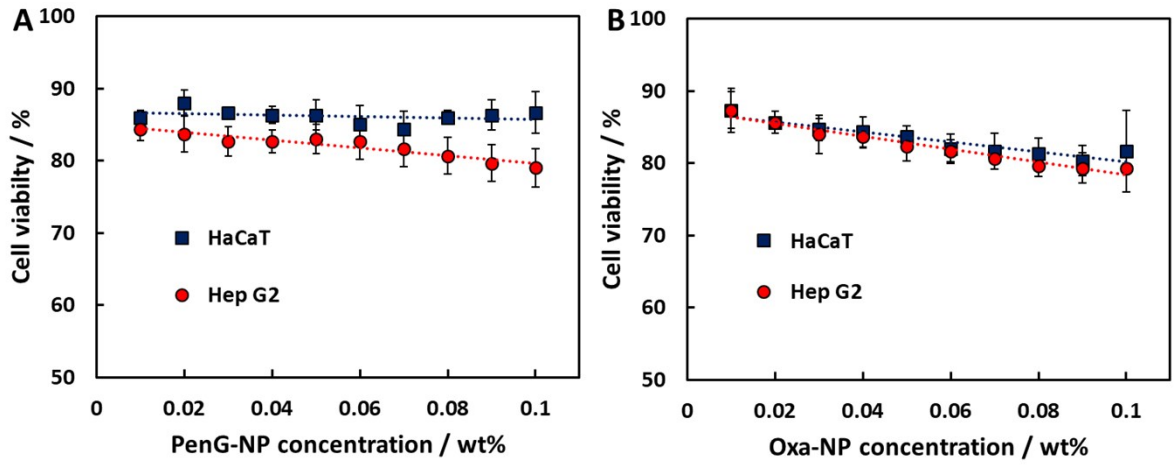

Figure S27. HaCaT and Hep 2G viability after treatment with decreasing concentrations of free **(A)** PenG-NPs and **(B)** Oxa-NPs. Cells were seeded at 1×10^4 and incubated for 24 hours at 37 °C in 5% CO_2 atmosphere (2 wt% FBS). The medium was then removed and replaced with treatment infused media under the same conditions. Viability counts were performed at 24 hours. 0.1 wt% PenG-NP and 0.1 wt% Oxa-NP refers to 0.25 wt% shellac-0.2 wt% P407-0.1 wt% PenG-0.2 wt% Alcalase NPs and 0.25 wt% shellac-0.2 wt% P407-0.1 wt% Oxa-0.2 wt% Alcalase NPs. N=3 with ± S.D.

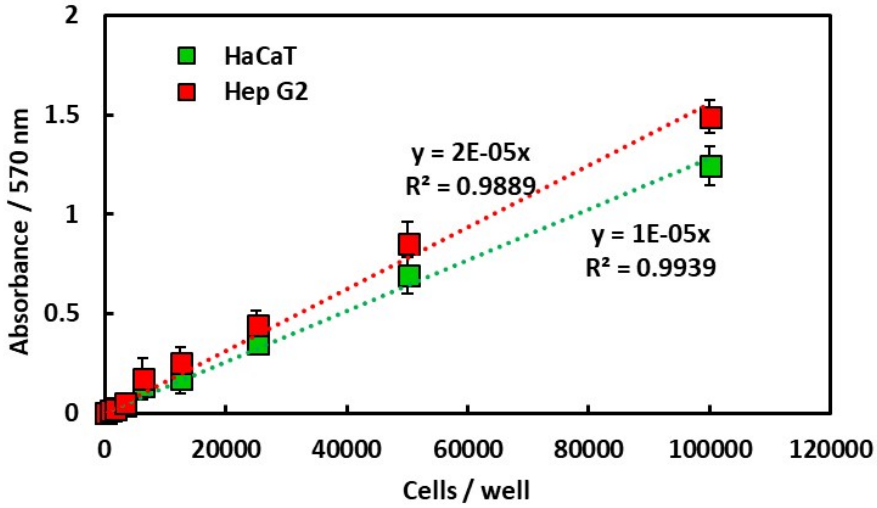

Figure S28. Calibration curve of HaCaT and Hep G2 cells. Various cell concentrations were seeded in DMEM (HaCaT) and EMEM (Hep 2G) medium and absorption values obtained at 570 nm. These data were used to calculate the number of cells in treatment / untreated wells. N=3 with ± S.D.

Table S4. Time-Kill assay statistical analysis between free PenG and encapsulated PenG at 24-hour time point. Data were expressed as average values ± standard deviations of the mean. P-values of less than 0.05 were considered significant. All One-Way ANOVAs and Tukey's post-test statistical analysis were performed in GraphPad v7.0.4.

Multiple Comparison	**P-value**	**Significance**
Free PenG vs PenG NPs	0.00000306	***

Table S5. Crystal Violet, dry mass weight and protein concentration statistical analysis between free PenG/Oxa and PenG/Oxa NPs. Data were expressed as average values ± standard deviations of the mean. P-values of less than 0.05 were considered significant. All Student's T-tests were performed in GraphPad v7.0.4.

Multiple Comparisons	**P-value**	**Significance**
PenG vs PenG-NPs (CV Staining)	0.00001824	***
Oxa vs Oxa NPs (CV Staining)	0.00326552	***
PenG vs PenG-NPs (Dry Mass Weight)	0.00113258	**
Oxa vs Oxa NPs (Dry Mass Weight)	0.00572886	**
PenG vs PenG-NPs (Protein Concentration)	0.00000477	***
Oxa vs Oxa NPs (Protein Concentration)	0.00120271	**

Table S6. *S. aureus* biofilm viability statistical analysis after 1, 6 and 24 hour treatments, comparing free PenG/Oxa and PenG/Oxa NPs. Data were expressed as average values ± standard deviations of the mean. P-values of less than 0.05 were considered significant. All Student's T-tests were performed in GraphPad v7.0.4.

Multiple Comparisons	**P-value**	**Significance**
PenG vs PenG-NPs (1 hour)	0.00108547	**
Oxa vs Oxa NPs (1 hour)	0.00120226	**
PenG vs PenG-NPs (6 hours)	0.00096656	***
Oxa vs Oxa NPs (6 hours)	0.00263979	***
PenG vs PenG-NPs (24 hours)	0.00037384	***
Oxa vs Oxa NPs (24 hours)	0.00044592	***

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