SUPPLEMENTARY DATA

Methods

Materials and Reagents

Poly(D,L-lactide-co-glycolide) (PLGA) 50:50 (Resomer® RG 503 H) was obtained from Evonik (Germany), polysorbate 80 from Caelo (Hilden, Germany), medium-chain triglycerides, clarithromycin and phosphotungstic acid hydrate from Sigma-Aldrich (St. Louis, USA), ultrapure chitosan chloride ProtasanTM UP CL113 with a molecular weight of 50–150 kDa and a degree of deacetylation between 75% and 90% from NovaMatrix (FMC Bio-Polymer, Drammen, Norway). Soy lecithin (Lipoid® S75) was kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Calu-3 (HTB-55), A549 (CCL-185), THP-1 and RAW 246.7 cells were bought from the DSMZ GmbH (Braunschweig, Germany). Minimum essential medium (MEM) containing Earl's salts and L-glutamine was obtained from Gibco (Life Technologies, Paisley, UK). RPMI 1640 medium containing L-glutamine was acquired from PAA Laboratories GmbH (Pasching, Austria). Fetal calf serum (FCS) and nonessential amino acid (NEAA) were bought from Lonza (Vervieers, Belgium) and GE Healthcare (PAA Laboratories, Pasching, Austria), respectively. Acetone, ethanol, methanol and all other chemicals and solvents used were analytical grades. Water was purified with a Milli-Q water purification system (Merk Millipore, Billerica, USA).

Fluorescent nanocapsules preparation

The fluorescent polymer was prepared using 5-fluoresceinamine (FA) chemically bound to PLGA as described by Horisawa et al. (39). Briefly, PLGA (3.07 g), FA (0.0583 g) and DMAP (0.0408 g) were completely dissolved in 30 mL of acetonitrile

at room temperature under light protection and gentle stirring. The FA-PLGA was purified by precipitation with purified water and separated by centrifugation. After separation of excessive reagents with repeated dissolvation in acetone and precipitation with ethanol, FA-PLGA was lyophilized (Alpha 2-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany).

Physicochemical characterization

The average particle size and polydispersity index (PdI) of formulations were determined by dynamic light scattering using ZetaSizer ZS (Malvern Instruments, UK) after their dilution (500x) in water. The zeta potential was measured by electrophoretic mobility technique using a ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, UK) after their dilution (500x) in 10 mM/lL NaCl aqueous solution to a conductivity around 1.4 ± 0.1 mS/cm. The pH values of the suspensions were measured using a potentiometer Fiveeasy FE20 (Mettler Toledo, Schwerzenbach, Switzerland) at room temperature (25 °C). The stability of CS-CLARI-NC was evaluated during a week in different pHs (7.4, 5,8 and 3,8) using 100mM Phosphate buffer pH.

Total clarithromycin content in the nanocapsules was determined after the dissolution of 0.1 mL of the formulation in 10 mL of methanol:water/50:50 to ensure complete dissolution of all material and further dilutions was used to achieve the linear range of the analytical method. Besides, CLARI not entrapped in the nanocapsules was determined after separation from the nanocarrier in the ultrafiltrate from the aqueous medium by ultrafiltration-centrifugation technique at 15,000 rpm for 30 min using the Ultracel YM-100 (Amicon® Millipore Corporation, United States). The encapsulation efficiency (EE, %) was calculated by the quotient of CLARI encapsulated (the

difference between the total and the free CLARI concentrations) and the total drug content multiplied by 100. All the analyses were carried out at least in triplicate of different batches and are expressed as mean ± standard deviation (SD).

The *in vitro* release profiles of CLARI from nanocapsules were evaluated by the dispersion method. Briefly, 10 µL of the formulation was added in several Eppendorf flasks with 500 µL of the medium. We tested the release at 100 rpm and 37 ºC in different mediums: Phosphate buffer (PBS) pH = 7.2, Mueller-Hinton broth (MHB) and tryptone soya broth (TSB). At appropriate time intervals (0.5, 1, 1.5, 2, 4, 8, 24 h), three tubes of each formulation were removed and centrifuged at 15,000 rpm and 4^{O}C for 10 minutes. The amount of drug released in the supernatant was diluted with the proper amount of mobile phase and quantified by LC/MS-MS method. The amount of drug release was calculated as [(amount of drug release)/(amount of drug total content) X 100].

LC-MS/MS method

Concentrations of CLARI were quantified by LC-MS/MS system with the method previously validated (20). It was used a TSQ Quantum® Access MAX Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with Accela 1250 pump and Accela Autosampler. The drug was separated with an Accucore RP-MS column (150 mm x 2.1 mm, 2.6 µm, Thermo Fisher Scientific, San Jose, CA, USA) kept at 30 ºC. The injected samples were eluted isocratically with a mobile phase consisted of 70% of methanol containing 0.1 % of formic acid (v/v) and 30% of ammonium acetate buffer (5 mM) containing 0.1 % of formic acid (v/v) at a flow rate of 0.25 mL/min during 4 min. The compound ionization was carried out with a heated electrospray ionization source (HESI-II)

operating in positive mode (ESI+). The following MS conditions were used: sheath gas using nitrogen at a flow rate of 35 arbitrary units; collision gas with argon; vaporizer temperature at 300 °C; ion transfer capillary temperature at 250 °C; skimmer offset at 0 V; spray voltage at 4000 V. MS analysis was preformed in selected reaction monitoring (SRM) mode. The transition of the precursor ion of CLARI 748.3 m/z to the production 158.1 m/z was measured at collision energy of 28 V, scan time of 0.1 s, scan width of 0.02 m/z, and tube lens offset of 111 V. The following parameter values were set for amitriptyline, used as internal standard (IS): transition 278.5 m/z to 233.1 m/z, collision energy 14 V, scan time 0.1 s, scan width 0.02 m/z, and tube lens offset 91 V. The peak areas were automatically integrated using the software Thermo Xcalibur (v. 2.2, San Jose, CA, USA). The software parameters were as follows: peak detection algorithm, ICIS; smoothing points, 5; baseline window, 80; area noise factor, 5; peak noise factor, 10.

Sample extraction was carried out with 0.1 mL of samples and 0.9 mL of methanol:water/1:1 (v/v) containing the IS (500 ng/mL) under ultrasonication for 10 minutes. After centrifugation at 10.000*g* for 10 min, the supernatant (5 µL) was injected using partial loop injection mode in the LC-MS/MS system. The developed method was linear in the concentration range of 5–500 ng/mL ($R^2 > 0.99$), limit of detection of 1 ng/mL and limit of quantification of 5 ng/mL.

Bacterial cultivation and antimicrobial susceptibility assay

The following culture conditions were used to grow the bacteria:

- *Staphylococcus* spp.: were grown in Mueller-Hinton broth (MHB; 0.2 % beef infusion solids, 1.75 % casein hydrolysate, 0.15 % starch, pH 7.4; 37 °C, 230 rpm, for 24h).

- *Streptococcus* spp.: were grown in tryptic soy broth (TSB; 1.7 % peptone casein, 0.3 % peptone soymeal, 0.25 % glucose, 0.5 % NaCl, 0.25 % K_2HPO_4, pH 7.3, 37 °C, microaerophilic conditions without shaking for 24h).
- *Mycobacterium abscessus:* were grown on 7H9 medium supplemented with 10% OADC, 37 °C, 230 rpm for 96h).

The cultures were diluted to ca. $5x10^6$ colony-forming units (CFU)/mL in the respective growth medium. Serial dilutions of free CLARI and formulations at 1 mg/ mL were prepared as duplicates in sterile 96-well plates. Bacteria suspensions were added, and plates were incubated overnight (16-18 h, 37 °C). Minimum inhibitory concentrations (MIC) values were determined in at least two independent experiments by visual inspection of the plates and are defined as the lowest sample concentration used were no visible growth of the indicator strain was observed. For *M. abscessus*, Clinical and Laboratory Standards Institute (CLSI) recommendation was followed, in which free bacteria solution was adjusted to an OD (600) of 0.0005 to reach 5 x 10^5 CFU/mL, and incubated at 37°C for 14 days to check bacteria growth and exclude CLARI resistance (38).

Colony Forming Unity (CFU) assay

Infected cells were lysed with 1 mL ice-cold sterilized Milli-Q water to release intracellular bacteria. The lysates containing released *S. aureus* were serially diluted (0.9% NaCl), plated on brain heart infusion (BHI) agar plates, and cultured overnight at 37°C. The limit of detection for this plate counting method was 400 CFU/mL. *M. abscessus*-infected cells were washed, and bacteria were grown on Luria-Bertani (LB) agar as described before (39).

Murine S. aureus wound infection model

For the murine *S. aureus* wound infection model, PBS-washed bacterial cells obtained from exponential growth phase cultures (i.e., after 2 h of growth in tryptic soy broth [TSB; Becton Dickinson, Heidelberg, Germany] at 37°C and 150 rpm) were used as inocula. Seven weeks old female hairless mice (SKH1-Hr^{hr}; Charles River, Sulzfeld, Germany) were anesthetized by intraperitoneal injection of 100 mg/kg body weight ketamine hydrochloride (Zoetis, Berlin, Germany) and 10 mg/kg of xylazine hydrochloride (Bayer, Leverkusen, Germany) and treated with a dose of carprofen (5 mg/kg, Zoetis, Berlin, Germany). After disinfection of the skin with ethanol (70%) full-thickness excisional punch wounds (Ø 5 mm) were created on both flanks through the skin down to the panniculus carnosus. These wounds were stabilized using silicone rings and subsequently infected with 10 µL of a PBS suspension containing 10^5 cells of *S. aureus* strain Newman. Infected wounds were allowed to dry for 2 min and afterward covered with Tegaderm (3M, Neuss, Germany). 10 µL aliquots of a CLARI-NC 10 µg/mL suspension or an equal amount of BLANK-NC were spotted onto the infected wounds at 3, 48 and 96 hours post-infection (pi). After six days pi, mice were sacrificed, and full-thickness tissues harvested for microbial analyses. Excised wounds were homogenized in 1 mL PBS with a hand disperser (POLYTRON® PT 1200 E, Kinematica, Luzern, Switzerland), and serial dilutions of the homogenates were plated on sheep blood agar plates. CFU rates were determined after 24h of cultivation at 35ºC.

Zebrafish infection model and determination of colony-forming units (CFU)

S. aureus Newman was grown overnight in TSB medium (17 g/L tryptone, 3 g/L soy, 5 g/L NaCl, 2.5 g/L K2HPO4, 2.5 g/L glucose) at 37°C. The next day, the bacterial

culture was centrifuged at 15 000 rpm for 5 min. The supernatant was discarded and the cell pellet was washed twice with PBS buffer. Finally, the pellet was resuspended in PBS and OD600 was adjusted to 4 (corresponding to ~$2x10^9$ CFU/mL). Embryos at 30 hours post-fertilization (hpf) were dechorionated using Pronase (final concentration: 50 µg/mL) and anaesthetized through immersion in tricaine (final concentration: 170 µg/mL). Embryos were placed on 1.5 % agar plates and 4.2 nL the bacterial suspension was microinjected into the yolk circulation valley (YCV) or into fourth hindbrain ventricles (4V). Alternatively *S. aureus* was also heat-inactivated for 1h at 100°C and used as a negative control. After injections, embryos were left to recover for 2 h in 0.3x Danieau´s solution. After the recovery phase, embryos were placed in 6-well plates with 20-30 embryos per condition per well. Treatment was done through addition of test solutions directly in 0.3x Danieau´s solution. Embryos/larvae were observed twice daily by microscopy for survival until 120 hpf (90 hpi). Embryos/larvae were considered dead when no heartbeat was observed. To confirm the injected CFU load, we injected equal volume of bacterial cells into 1 mL of PBS, we performed decimal dilutions and plated them on CASO agar plates (15 g/ L agar, 15 g/L casein peptone, 5 g/L NaCL, 5 g/L soy peptone, pH 7.3±0.2). At the end of the experiment, the remaining alive larvae per condition were pooled and homogenized in 1mL PBS using a micropestle. Homogenates were decimally diluted and plated on CASO agar plates.

Zebrafish embryos/larvae microscopy

Embryos/larvae were anesthetized through immersion in tricaine at a final concentration of 170 µg/mL. Images were acquired using fluorescence stereomicroscope Leica M205 FA and LasX software.

Cell Culture

Calu-3 cells (passage number from 35 to 44) were cultivated in MEM containing Earl's salts and L-glutamine supplemented with 10% FCS, 1% NEAA and 50 mM sodium pyruvate. For deposition studies with Calu-3 cells, 100 units/mL penicillin and 100 µg/mL streptomycin were added to avoid cell contamination. A549 (passage number from 10 to 16) and THP-1 (passage number from 55 to 60) cells were grown in RPMI-1640 medium with *L*-glutamine supplemented with 10% FCS. The cells were propagated in 75 cm^2 flasks at 37 ºC, 5% CO_2 and 95% humidity and regularly tested for mycoplasma infection.

After detaching of cells from 75 cm^2 flasks with Trypsin, 1.5 X 10^5 cells/cm^2 cells were determined by a CASY® Model TT (Innovatis AG, Reutlingen, Germany) and were seeded in 12 well Transwells polyester inserts (1.12 cm^2, 0.4 µm pore size, Corning Costar, Lowell, MA, USA) with 1.5 mL and 0.5 mL of medium in basolateral and apical compartment, respectively. After 2 days, the cells were cultivated at ALI with apical medium completely removed and the cells were fed only by basolateral. The experiments started after cell differentiation and ideal epithelial barrier have been formed (12 -14 days) (40).

In Vitro Deposition with the Air-Liquid Interface Cell Exposure (ALICE) system

After Calu-3 cell differentiation and ideal epithelial barrier were formed (12 – 14 days), the formulations were deposited on Calu-3 monolayers using the ALICE® (VITROCELL Systems GmbH, Waldkirch, Germany). The ALICE system generates a dense cloud of droplets, which is gravitationally settled into the exposure chamber

containing cells cultured at the air-liquid interface. This system was presented and validated by Lenz et al.(39). Briefly, the ALICE is composed of a droplet generator (nebulizer), an exposure chamber, an incubation chamber providing temperature (37°C) and humidity (nearly saturated) suitable for maintenance of cells. The nebulizer (Aeroneb® Lab, Aerogen, Galway, Ireland) generated a dense cloud of droplets from 1 mL CLARI, CLARI-NC or CS-CLARI-NC. These droplets were generated by the Aerogen's OnQ technology using the Standard Volume Median Diameter Nebulizer Unit with a particle size between 4.0 µm and 6.0 µm. The dense cloud of droplets generated by the Aeroneb® Lab nebulizer is emitted into the exposure chamber where they carefully homogenize as a single cloud to be deposited onto cells cultured at the air-liquid interface by gravitational settling of single droplets, with a better in vivo correlation. After 15 min of exposition, the cells were returned to the culture plate filled with 1.0 mL of prewarmed medium in the basolateral compartment and kept on an incubator set to 37 ºC, 5% CO_2 and 95% humidity with a rotational shaker at 150 rpm for 24 h.

Cell Viability Study

The in vitro cytotoxicity in A549, Calu-3, THP-1 and RAW 246.7 cells of drug-loaded nanocapsules (CLARI-NC and CS-CLARI-NC), blank nanocapsules (CS-BLANK-NC) and free drug (CLARI) were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase activity (LDH) assays. 200 µL of cell suspension at 50,000 cells/mL (A549) or 100,000 cells/mL (Calu-3, THP-1 and Raw 246.7) were seeded in central wells of 96-well plates and placed in an incubator at 37 °C supplemented with 5% CO_2 for at least 90% of confluence (2-3 days). THP-1 cells were additionally

pretreated with 10 nM phorbol 12-myristate 13-acetate (PMA) for 24 h in 5% CO_2 at 37°C to induce maturation of the monocytes into macrophage-like adherent cells.(44) After removing medium, 200 µL of freshly prepared formulations in the medium was added at various concentrations (1, 10 and 100 µg/mL) (n=3) with Triton X-100 1% or only medium as a positive or negative control, respectively. Following 24 h incubation, 100 µL of cell supernatants were withdrawn to another 96-well plate for the LDH assay. Additionally, it was added 100 µL of LDH reaction mixture prepared as recommended by the manufacturer (Cytotoxicity Detection Kit PLUS, Roche, Mannheim, Germany), homogenized for 5 min at 15 – 25 ºC protected from light and measured at 490 nm using a plate reader Infinite M200 PRO (Tecan, Grödig, Austria). Simultaneously each well of the rest of the initial 96 well plate received 10 µL of a 5 mg/ml MTT solution in PBS. After 4 h of incubation at 37 °C, the medium was removed and replaced with DMSO (100 µL) to dissolve the formazan crystals in 10 minutes. The absorbance was measured at 550 nm using a plate reader Infinite M200 PRO (Tecan, Grödig, Austria). The percentage of viable cells in each well was calculated as the absorbance ratio between group tests cells and untreated control cells.

MTT [(3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to determine the cell viability of Calu-3 monolayers after 24h of exposition of CLARI, CLARI-NC and CS-CLARI-NC with the ALICE system. Firstly, 50 µL of 5 mg/ml MTT solution in PBS pH 7.4 were added to each well. After 4h of incubation, 500 µL of DMSO were added to each well and the cells were lysed on an orbital shaker for 10 minutes. Three samples of 0.1 mL from each well were transferred to a 96 well plate. The absorbance was read at 550 nm with the plate reader Infinite M200 PRO (Tecan, Grödig, Austria). The obtained results were compared to cell

monolayers without the exposition process at the ALICE device and with the addition of Triton X-100 1% as negative and positive controls, respectively.

Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM)

Raw 246.7 cells were visualized with SEM and CLSM after 2 h, 18h or 2h of incubation and more than 16h after changing the medium of treatment of *S. aureus* and fluorescent CLARI-NC or CS-CLARI-NC. Also, samples without any treatment were used as a control. For SEM, samples were fixed with 1.0% glutaraldehyde in 0.2 M HEPES buffer (pH 7.4) for 1 h, dehydrated in a graded ethanol series (from 30% to 100%), finally dried with hexamethyldisilazane and mounted on stubs. Finally, they were sputtered with a 10 nm layer of gold (Quorum Q150R ES, Ashford, UK) and observed at 5 kV under high vacuum conditions on a Zeiss EVO HD15 SEM (Zeiss, Oberkochen, Germany). While the CLSM samples were fixed with 3% paraformaldehyde solution for 1 h and then quenched for 15 min in PBS containing 50mM glycin, followed by dehydration in a graded ethanol series (from 30% to 100%), finally dried with hexamethyldisilazane, added on a glass slide and coverslipped. The samples were kept at 5 ± 3 ºC until imaging with a confocal laser scanning microscope (Zeiss LSM 800, Carl Zeiss GmbH, Göttingen, Germany).

Immunocytochemistry

Fluorescent nanocapsules were incubated in Calu-3 or RAW cells for 4h or 24h. The cells were washed with PBS and fixed with 3% paraformaldehyde solution (PFA; Stock 16%; 15710-S, Electron Microscopy Sciences, USA) for 30 min, followed by 15 min quenching in PBS containing 50 mM glycine and permeabilization for 20 min with 0.05% (w/v) saponin and BSA 1.0 % (w/v) in PBS. *S. aureus* was detected by

anti-Staphylococcus aureus antibody ab20920 (Abcam), anti-rabbit (1:200); tight epithelial junctions was detected with anti-ZO-1 (rabbit anti-ZO-1, Catalog No 61-7300, Invitrogen). Both antibodies were incubated for 2h at room temperature. The secondary antibodies for *S. aureus* and ZO-1 (polyclonal Alexa-Fluor 633, conjugated goat anti-rabbit, Catalog No. A21070, Invitrogen) were diluted in PBS (1:400) and incubated for 1 hour at room temperature, protected from the light. When Actin staining was carried-out, the cells were incubated with 0.1 mL of fluorophore phalloidin in PBS 1:500 for 30 min at room temperature and washed three times with PBS. Nuclear staining was performed with 0.2 mL of DAPI (1:500) for 20 min and washed once with PBS. The Snapwells filters were removed from the holder, mounted on a glass slide with DAKO medium (Product No. S302380-2, DAKO) and coverslipped. Images were obtained with a confocal laser scanning microscope (Zeiss LSM 800, Carl Zeiss GmbH or Leica TCS SP2 AOBS, Leica Microsystems) maintaining the same laser power, gain and offset settings.

Lysotracker red staining and image analysis

In brief, infected and non-infected macrophage were washed with PBS and treated with 50 μg/mL gentamicin for 90 min. After repeated washing with PBS, 10 μg/mL of NC-CLARI, NC-CS-CLARI or free drug was incubated for 18 h at 37°C. Lysosomes of RAW246.7 cells were stained for 2 h using LysoTracker Red DND-99 (200 nM). Samples were fixed and stained as described befopre, and further analyzed with fluorescent confocal laser scanning microscope (Zeiss LSM 710 Carl Zeiss GmbH, Göttingen, Germany). Zeiss files (.lif) were opened with the plug-in BioFormats of ImageJ (NHI). The following parameters were measured, 1) intracellular nanocapsules (measuring the FITC signal per cell), 2) bacterial growth (measuring the signal from anti-*S. aureus* antibody), 3) co-localization of

LysoTracker red with NCs or *S. aureus*, and 4) co-localization of *S. aureus* with NCs. For all measurements, the channels were splitted and the interested channel subjected to a pixel threshold.