Electronic Supplementary Information (ESI)

Flame-made silica-coated phosphorescent nanoprobes for selective cell targeting and dynamic bioimaging of pathogen-host cell interactions

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Materials and Methods

Particle synthesis

SiO_2-coated YVO_4:Eu^{3+}, Bi^{3+} (10 at Eu, 20 at% Bi) nanoparticles were produced by an enclosed flame spray pyrolysis (FSP) reactor as described in detail elsewhere [1]. In brief, the core material metal precursors [yttrium(III) nitrate hexahydrate (99.8% trace metals basis), ammonium metavanadate (99%), europium(III) nitrate pentahydrate (99.9% trace metals basis), bismuth(III) nitrate pentahydrate (ACS reagent, >98.0%), and solvents 2-ethylhexanoic acid (2-EHA) and acetic anhydride were obtained from Sigma Aldrich] were dissolved in 2-EHA and acetic anhydride at 2:1 volume ratio mixture at 0.4 M (total metal concentration). The precursor solution was fed to the FSP nozzle and atomized by O_2 (1.5 bar pressure drop) and further ignited by a premixed methane/oxygen (1.5 L min^{-1} and 3.2 L min^{-1}) flame. The FSP reactor was enclosed with quartz glass tubes and sheathed by 40 L min^{-1} O_2. The core nanoparticles were encapsulated by the swirl injection of hexamethyldisiloxane

(HMDSO, Sigma Aldrich, purity ≥99%) vapor carried by nitrogen (15 L min^{-1}, PanGas, purity >99.9%) at room temperature through a metallic ring with 16 equidistant openings (Figure 1a in main manuscript). The ring was placed on top of a quartz glass tube (20 cm long). The reactor was terminated by a quartz glass tube (30 cm). The HMDSO vapor was supplied by bubbling nitrogen through liquid HMDSO (350 mL) in a glass flask (max. 500 mL).

Particle characterization

The as-prepared nanoparticles were characterized by X-ray diffraction (Rigaku MiniFlex 600, CuKa radiation). Crystal phases were identified by comparison with reference structural models for tetragonal YVO_4 (COD: 9011137) and cubic Y_2O_3 (COD: 7205917). Average crystal size was determined with the software PDXL2 (Rigaku) using the (200) crystal plane of tetragonal YVO_4. Scanning transmission electron microscopy and energy-dispersive X-ray spectroscopy studies were performed with a FEI Talos F200X system, after dispersing the powders in ethanol and depositing them onto a perforated carbon foil supported by a copper grid. Photoluminscence spectra were obtained with a PerkinElmer LS55 spectrophotometer with an emission/excitation slit 2.5 nm, and voltage 800 V. Dynamic light scattering and zeta potential was performed with a Zetasizer Ultra (Malvern Panalytical). Fourier transform infrared spectroscopy with attenuated total reflection (FTIR-ATR) module was performed on an Agilent Cary 630 instrument.

Protein biofunctionalization

Protein functionalization was performed in phosphate buffered saline (PBS, Substrate Unit, Karolinska University Hospital) in the presence of antiCK8 antibody (Novus Biologicals) or bovine serum albumin (BSA, Thermo Fisher Scientific). Nanoparticles were dispersed in water by bath ultrasonication at 1 mg mL^{-1} and diluted in PBS for their biofunctionalization with proteins by incubating the nanoparticle suspensions (250 μg mL^{-1}) with proteins at room temperature for 30 min at a mass ratio 1:1 and washed

by centrifugation (10000 rpm). Protein loading quantification was performed using the Pierce BCA protein assay (Thermo Fisher Scientific).

Stability studies

Nanoparticle suspensions were prepared at 1mg/mL using a cup-horn sonicator (Vibra-Cell VCX 750) for 10 minutes at 80% amplitude with a pulsing of 5s on 1s off and diluted to 0.5 mg/mL. For UV-Vis a cuvette had the lower ~1 cm covered with black pen, a reference measurement was taken using this cuvette filled with PBS. The cuvette was then emptied and the nanoparticle suspension was added and the absorbance at 550 nm monitored. For the DLS measurements: 1 mL of the sample was inserted and particle concentration measurements were run every 5 minutes with a total of 50 repetitions.

A549 cells, S. pneumoniae and growth conditions

A549 cells were cultivated in accordance to the American Type Culture Collection (ATCC) guidelines as previously described [2]. For maintenance of the cell culture, A549 cells were cultivated in 25 cm^2 flasks, while for microscopy imaging experiments cells were cultivated in 12-well-plates in DMEM cell culture medium (Gibco) supplemented with 10% fetal calf serum (Gibco). The GFP-expressing *S. pneumoniae* serotype 2 R6 strain was obtained from Prof. Jan Willem Veening [3] and cultivated in Todd-Hewitt broth medium as previously described [4]. Briefly, bacteria were grown until a 600 nm optical density of 0.25-0.30, aliquots of 1 mL bacterial cultures were prepared and stored at -80°C.

Incubation of A549 with nanoparticles and Phalloidin, and infection with S. pneumoniae

Plastic coverslips (diameter 13 mm, Thermo Fisher Scientific) were placed inside each well of 12-well-plates and A549 cell monolayers (volume 1 mL) were grown in each well until confluence was reached. Coverslips with adherent A549 cell monolayers were washed twice with sterile PBS and were either left at the bottom of the wells (upright orientation) or removed from the bottom to be placed

gently up-side-down on the surface of the medium containing the nanoparticles (inverted orientation). For dynamic bioimaging experiments, cells were incubated for 1 h with either nanoparticles or Phalloidin (Thermo Fisher Scientific) diluted in A549 culture medium to concentration of respectively 30 μg mL^{-1} and 2 units mL^{-1}. Cells were then washed three times with sterile PBS to remove the excess of NP and Phalloidin. Prior to the infection experiment, 1 mL bacterial aliquot (see "A549 cells, *S. pneumoniae* and growth conditions" section) was thawed and centrifuged at 10000 rpm for 3 minutes and re-suspended with 1 mL sterile A549 culture medium. Serial dilutions were made in sterile PBS and plated on blood-agar plates for CFU count. 900 μL of cell culture medium was added to each well and 100 μL of approximately 10^6 Colony Forming Unites (CFU) of GFP-expressing *S. pneumoniae* was added in each well. After 1 hour, the medium was removed and cells were washed three times with PBS to remove the unattached bacteria. Cells and bacteria were then imaged real-time.

Cytotoxicity

A549 cells were grown until 90% confluent and 10,000 cells/well were seeded into a 96 well plate and allowed to attach overnight. Once reached confluence, the cells were washed and the medium was exchanged for medium containing the desired concentration of nanoparticles. The cells were allowed to incubate at 37°C for 4 hours at which point resazurin was added and allowed to incubated for a further 4 hours. The cells and particles were then centrifuged and the supernatant was measured in the LS55B luminescence spectrometer with an excitation wavelength of 540 nm and an emission wavelength at 590 nm.

Live-cell imaging

Microscopy was performed using a DV Elite microscope (Applied Precision) using a scientific complementary metal-oxide-semiconductor (sCMOS) camera with a 60X immersion oil objective. Images were acquired using the imaging program Softworx (Applied Precision). To visualize the red

fluorescence of nanoparticles, a DAPI filter set with 390 to 418 nm excitation and a TRITC filter set with 593 nm to 645 nm emission were used; to image the red fluorescence of Phalloidin, a TRITC filter set with 542 to 627 nm excitation and 593 to 645 nm emission; to image fluorescent pneumococci a FITC filter set with 475 to 528 nm excitation and 525 to 545 nm emission. All filters were used with a quad polychroic mirror, with exposure time 0.2 s. Coverslips with infected A549 cells were removed from the wells of the 12-well-plate and placed up-side-down on microscope glass slides with Vectashield (Vector Laboratories). Slides were maintained at 37°C in a temperature-controlled chamber during the experiment. For live-cell imaging videos, phase contrast and fluorescent images were taken every 2 seconds for 10 minutes in total. For z-stack images (step 0.5 □m) phase contrast (DIC) and fluorescence images were taken after 10 seconds, 2, 5, 10, 30 minutes exposure to the laser.

Red fluorescence quantification

Red fluorescence signal was quantified using ImageJ. An RGB Profile Plot was generated for each image taken and the average intensity of red color in each image was calculated using all the signal intensity values measured in every square pixel of each image.

Lysates of A549 cells for SDS-page gel electrophoresis

Lysates of A549 cells were prepared as previously described [4]. Briefly, 250 µL of RIPA lysis buffer was added to confluent A549 cells grown in T25 flasks. Cells were scraped, harvested and centrifuged 13000 rpm for 10 min at 4°C; afterwards, the cell lysate in the supernatant was collected. The quality of each cell lysate was assessed by SDS-PAGE following Coomassie staining.

Western blot analysis for Cytokeratin 8 detection

A549 cell lysates were loaded onto a 10% NuPage Novex Bis-Tris Gel (Thermo Fisher Scientific) and electroblotting was performed using the Biorad Trans-Blot Turbo Transfer System. To detect

Cytokeratin 8 protein, a rabbit anti Cytokeratin 8 antibody (1:1000 diluted, Abcam) was used as positive control combined with the secondary antibody HRP-conjugated goat anti rabbit (1:5000 diluted, GE Healthcare). Antibodies used for Western blot experiments were diluted in PBS supplemented with 0.1% Tween (PBS-T) and 1% dry milk.

Statistical analysis

For multiple comparisons the nonparametric ANOVA test was used to assess the presence of the differences between the groups; the Dunn´s test was then used to make pairwise comparisons (software GraphPad).

Results

Table S1. Average zeta-potential and hydrodynamic diameters of the nanoprobes alone and functionalized with BSA and antiCK8 measured in water (and cup-horn ultrasonication)* and PBS (in bath ultrasonication). Approximate protein content in the nanoprobes measured by the BCA assay. The change in the zeta-potential before/after biofunctionalization is attributed to the presence of proteins [5].

	Zeta-potential (mV)	***Hydrodynamic diameter (nm)***	***Protein content (wt%)***
NP only*	-20.9 ± 2.0	174 ± 2	-
NP only	-19.1 ± 2.0	796 ± 101	-
NP-antiCK8	-15.8 ± 2.1	339 ± 43	47.6
NP-BSA	-18.5 ± 1.48	432 ± 42	4.1

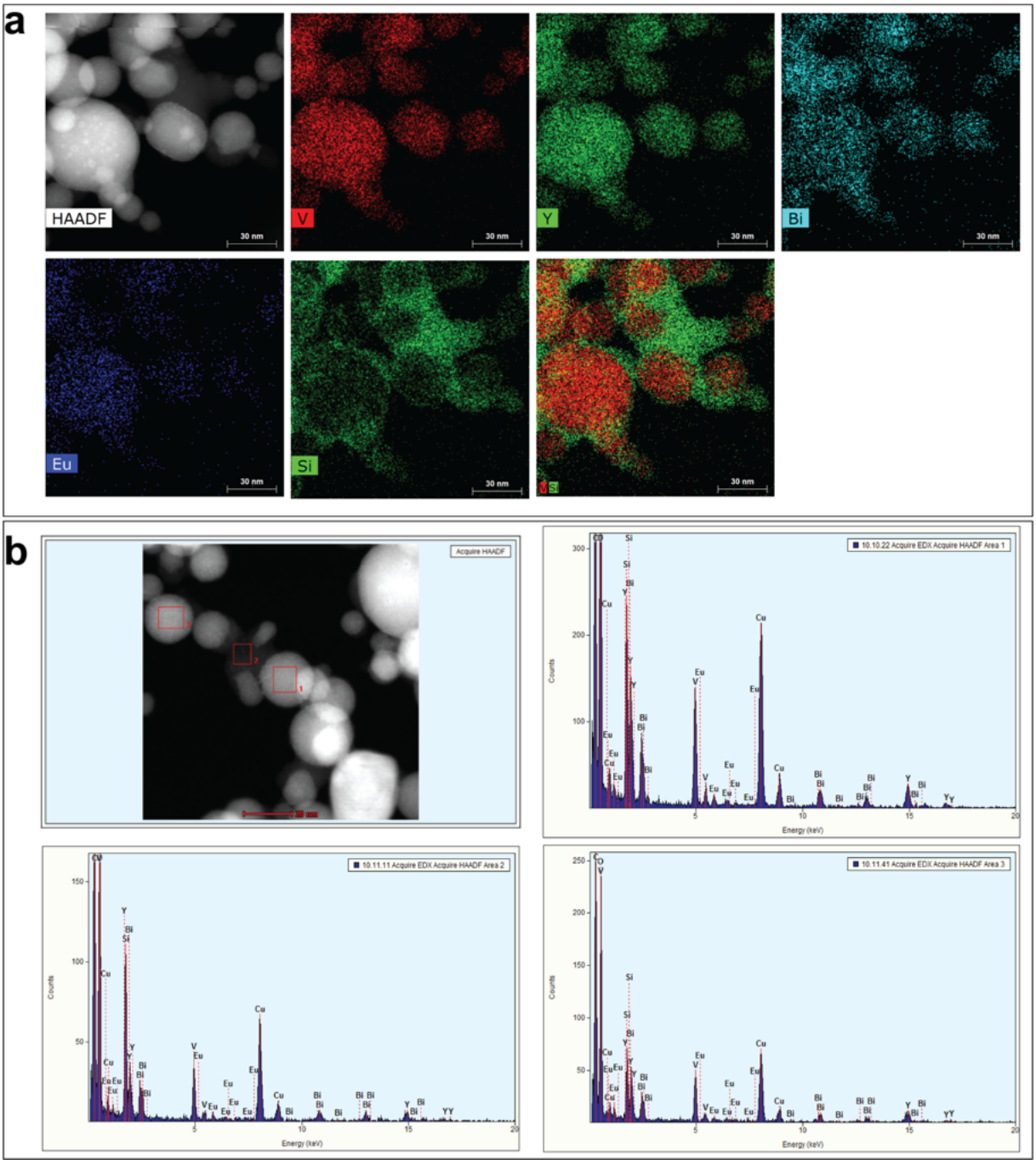

Figure S1. (a) Additional STEM-EDX images monitoring the signal from all elements here, verifying the homogeneous incorporation of all core elements and the presence of the SiO_2 shell encapsulating that core. (b) EDX spectra of three areas in the STEM image again validating the composition of the developed nanoparticles as well as highlighting the high purity of them. The C and Cu signals originate from the carbon coated copper grid that was used for imaging.

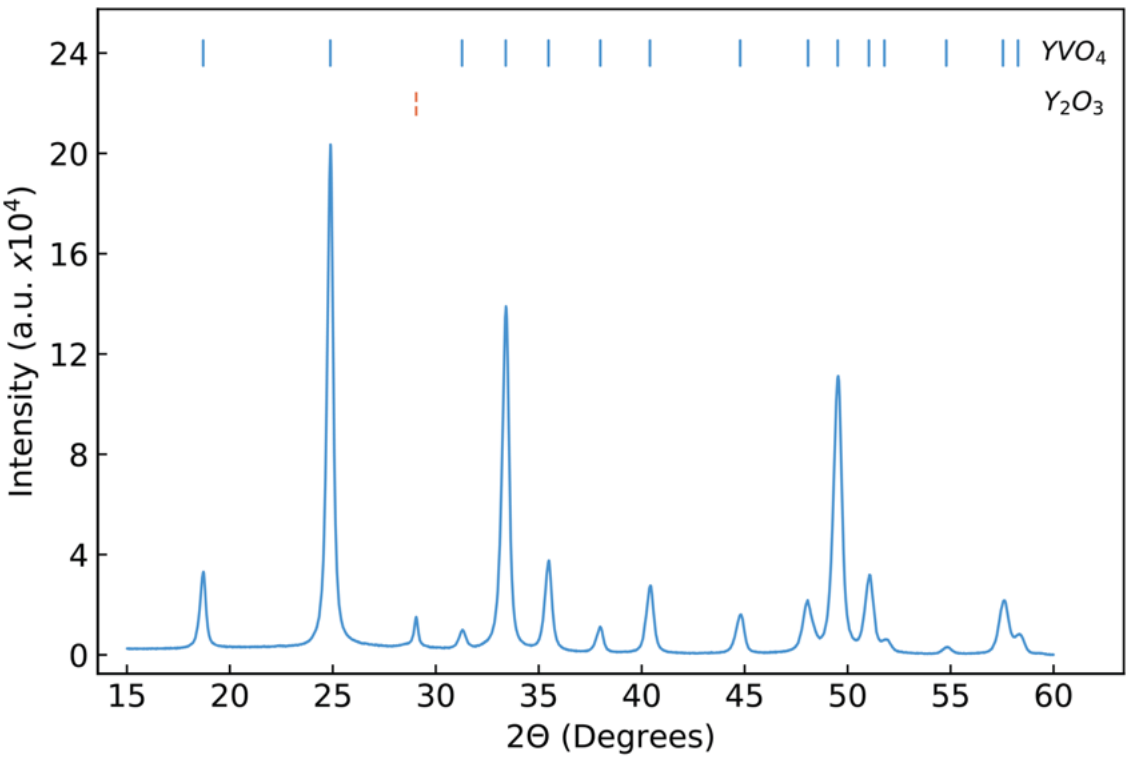

Figure S2. X-ray diffraction pattern of the SiO_2-coated YVO_4:Eu^{3+},Bi^{3+} nanoparticles along with the peak positions attributed to the YVO_4 and the Y_2O_3 crystal phases. From Rietveld refinement analysis, the mass fractions of YVO_4 and Y_2O_3 are 97% and 3%, respectively. The XRD pattern of the SiO_2-coated YVO_4:Eu^{3+},Bi^{3+} nanoparticles exhibit the same crystallinity as the pattern from the uncoated ones [1].

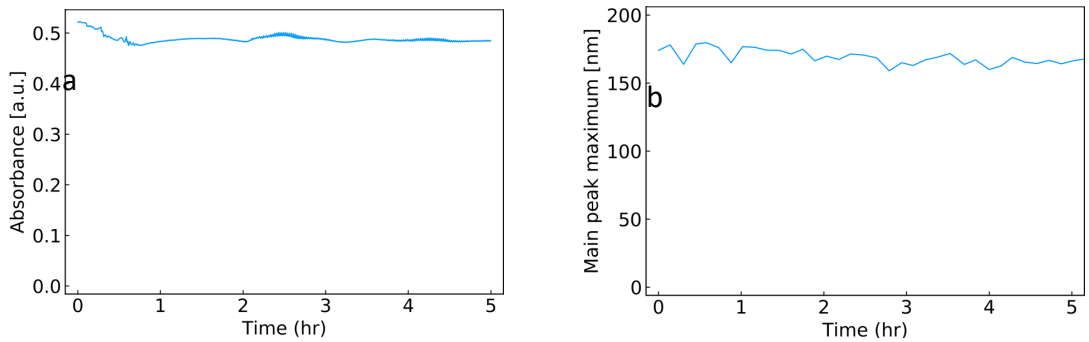

Figure S3. The sedimentation profile (a) and average DLS size (b) of the as-prepared nanoprobes in water monitored over time (5 hours).

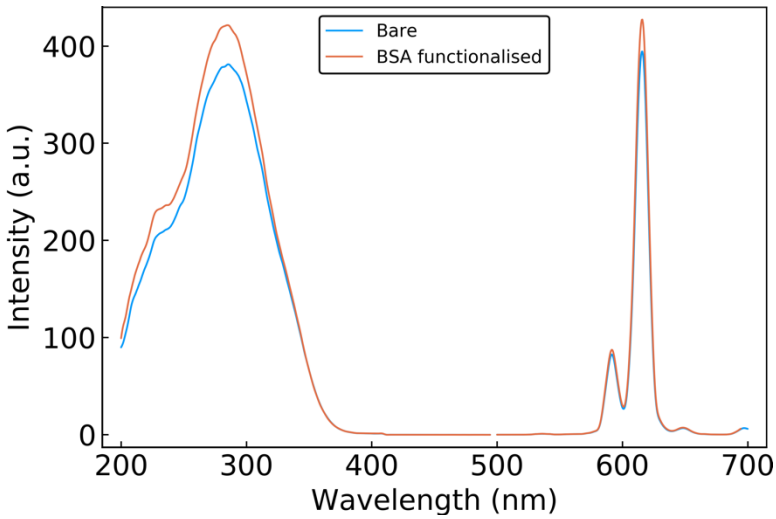

Figure S4. The excitation/emission spectra of the bare NPs and the BSA-functionalized NPs exhibit no differences.

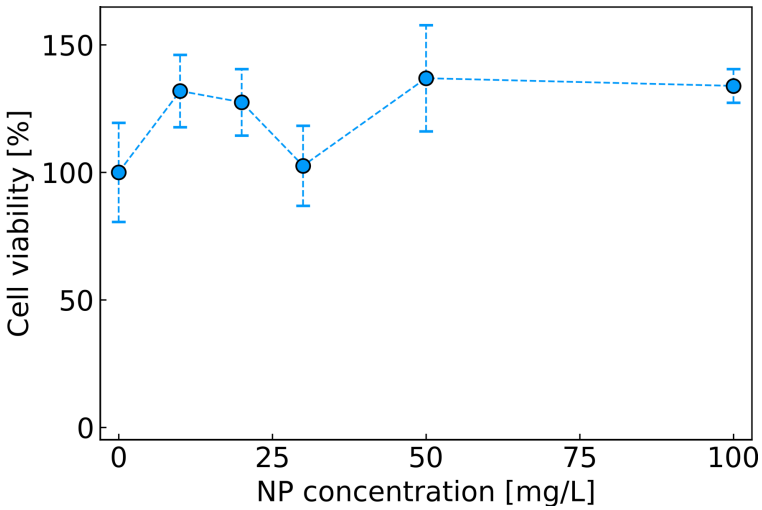

Figure S5. The cell viability of A549 cells (measured as metabolic activity from the resazurin assay) after 4 hour incubation with different concentrations of the as-prepared nanoprobes.

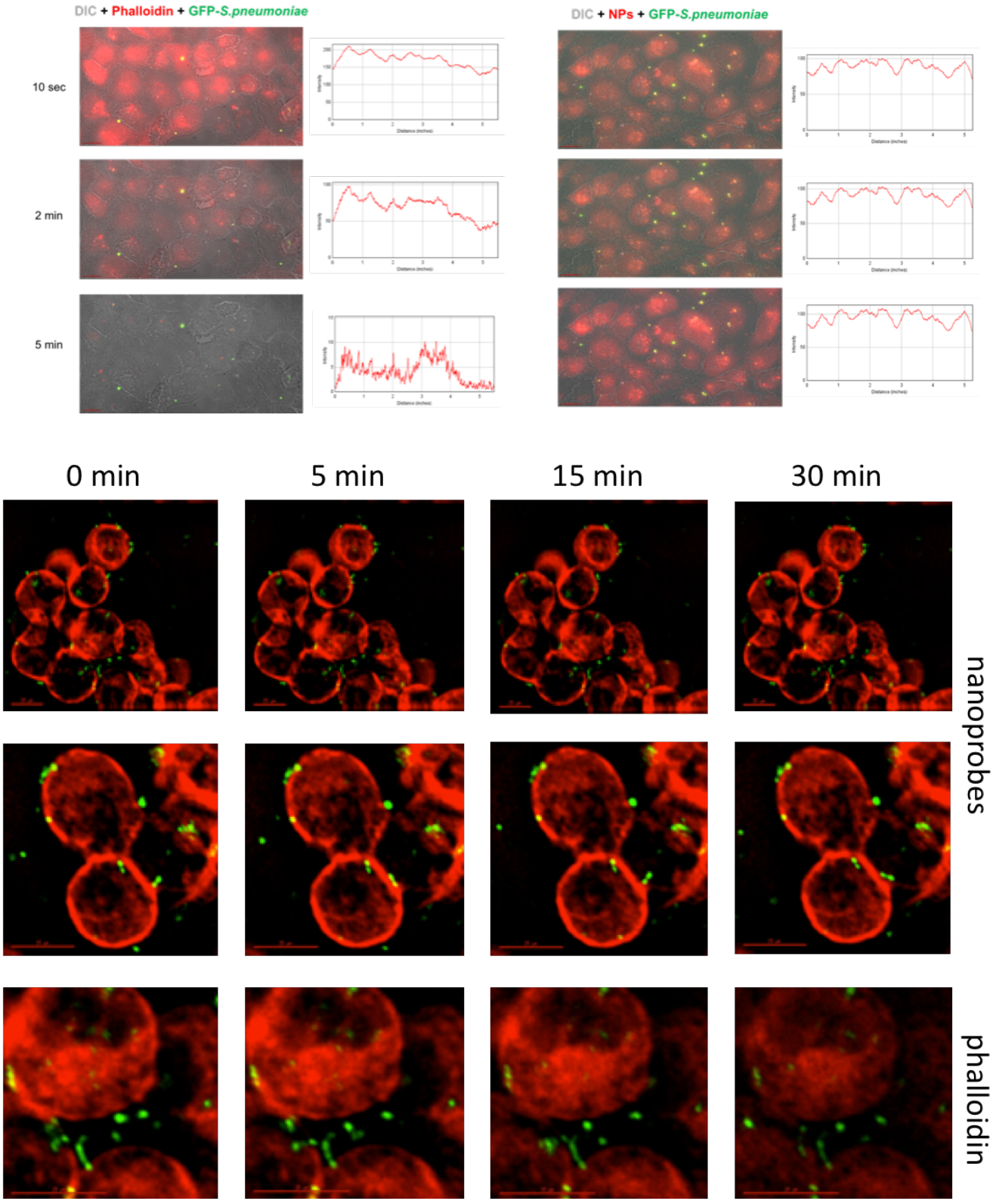

Figure S6. A549 human lung cancer epithelial cells were challenged with *S. pneumoniae* bacteria expressing GFP. The cells were stained with either phalloidin or with the here developed nanoprobes and images were taken continuously for up to 30 min, monitoring the red signal intensity.

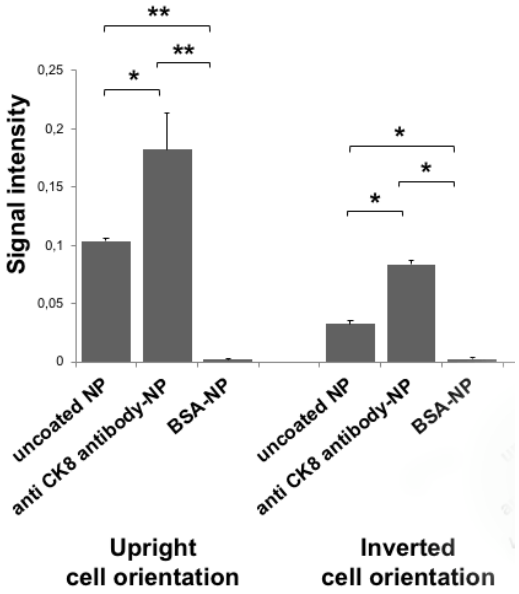

Figure S7. Quantitative analysis of the nanoparticle uptake from fluorescence microscopy images monitoring the red fluorescence for NP concentration of C = 100 mg/L, N = 3.

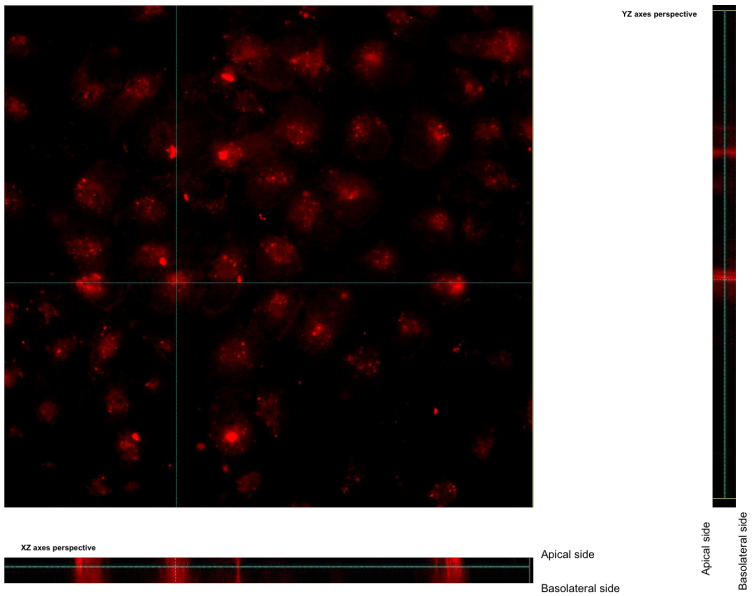

Figure S8. Z-stacks images of A549 cells incubated with the developed nanoprobes, showing their presence inside the cells.

References

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