Electronic Supplementary Material (ESI) for Biomaterials Science.

Antimicrobial and pro-angiogenic properties of soluble and nanoparticle-immobilized LL37 peptides

Michela Comune[1], Akhilesh Rai[1,2*], Paulo Palma[3,4], Chiara TondaTuro[5], Lino Ferreira[1,2*]

[1]CNC- Centro de Neurociências e Biologia Celular, CIBB - Centro de Inovação em Biomedicina e Biotecnologia, Universidade de Coimbra, Coimbra, Portugal
[2]Faculdade de Medicina, Universidade de Coimbra, Portugal
[3] Institute of Endodontics, Faculdade de Medicina, Universidade de Coimbra, Portugal
[4]Center for innovation and Research in Oral Sciences (CIROS), Faculdade de Medicina, Universidade de Coimbra, Portugal
[5]Department of Mechanical and Aerospace Engineering, Politecnico di Torino, Italy

MATERIALS and METHODS

Materials. $HAuCl_4.3H_2O$, $Na_3C_6H_5O_7$ and HEPES acquired from Sigma-Aldrich were used as received. Lyophilized LL37 peptide modified with a C-terminal cysteine (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTESC) was purchased from Caslo Laboratory, Denmark. The peptide was synthesized by conventional solid-phase synthesis, purified by high performance liquid chromatography, and characterized by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy. The purity of the peptide was 96% (**Fig. S5**). Rhodamine B isothiocyanate and HEPES were purchased from Sigma.

NP preparation. LL37 peptides (0.5 mM) were dissolved initially in DMF (100 μL) followed by addition of HEPES (900 μL, 100 mM, pH 5). $HAuCl_4.3H_2O$ (10^{-2} M, 50 μL) was added to a peptide solution (0.25 mM, 950 mL) to make the final concentration of $HAuCl_4$ to 0.5 mM and the NP synthesis was carried out at 25°C. The synthesized LL37-Au NPs were centrifuged at 11,000 rpm for 20 min at 4 °C followed by washing with Milli-Q water to remove unreacted peptides and HEPES. Spherical Au NPs were also synthesized via citrate reduction method [1]. An aqueous $HAuCl_4$ solution (0.5 mM, 90 mL of water) was boiled in a 250 mL round bottom flask while being stirred after which an aqueous sodium citrate solution (120 mg in 10 mL Milli-Q water) was added. The reaction was allowed to run until the solution reached a wine-red color, indicating the reaction was completed. Fluorescent Au NPs and LL37-Au NPs were prepared by addition of DMSO solution of rhodamine-B isothiocyanate (0.5 mM) to achieve a final concentration of 25 μM for flow cytometry and confocal microscopy studies. Free rhodamine molecules in the colloidal NP solution were removed by centrifugation at 11,000 rpm for 15 min at 4°C followed by one washing with Milli-Q water. The pellet obtained after centrifugation was redispersed in Milli-Q water and then dialyzed. For the fluorescent labeling of LL37 peptides, they were dissolved in PBS buffer (pH 7.5; 1 mg/mL) followed by the reaction with rhodamine B-isothiocyanate (dissolved in 25%

methanol; 0.5 mM) for 24 h in dark, at room temperature. The labeled peptides were then purified by dialysis (3 kDa dialysis membrane).

CD measurement. CD spectra of LL37 peptides, LL37-Au NPs and Au NPs were collected over a wavelength range of 200 to 300 nm with a data interval of 0.5 nm, scanning speed of 200 nm/min, path-length of 5 nm and response time of 2 s. K2D3 algorithm was used to estimate the secondary structures.

Antimicrobial activity. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442) and *Staphylococcus epidermidis* (ATCC 12228) were grown at 37 °C and maintained on TSY-agar plates. Bacteria were also grown in TSY media at 37 °C for overnight and cell counts were done at OD 600 nm. Different amounts of LL37-Au NPs (40-80 μg/mL) and soluble LL37 peptides (10-30 μg/mL) were added into bacterial suspensions (10^5 CFU bacteria/mL) in the presence of 10 % (v/v) human serum (HS), and incubated for 20 h at 37 °C. 100 μL aliquots were taken out from the respective suspensions at 20 h and diluted in PBS buffer 100 times and plated on TSY-agar plates followed by incubation at 37 °C. Visible colonies were counted after 24 h of incubation.

Antimicrobial mechanism. The genetic engineered *E. coli* ML-35p was used to investigate the effect of LL37 peptides and LL37-Au NPs in the permeability of the outer membrane (OM) and inner membrane (IM) of the bacteria [2]. Nitrocefin and *ortho*-nitrophenyl-β-galactoside (ONPG) were used as the chromogenic molecules to monitor the permeabilization of OM and IM respectively of *E. coli* ML-35p. It is known that nitrocefin cannot permeate the OM and reach to the periplasmic space. However, the OM permeabilization facilitates the crossing of nitrocefin to the periplasm. Nitrocefin is cleaved by β-lactamase to produce color change that can be evaluated using an UV-vis spectrophotometer at 486 nm. There is no lactose permease in this bacteria therefore ONPG cannot traverse IM. When IM is permeable to ONPG, it is cleaved by β-galactosidase to *o*-nitrophenol in cytoplasm that can be monitored using an UV-vis spectrophotometer at 420 nm.

E. coli ML-35p bacteria were grown in TSB at 37 ºC for 14 h. The grown bacteria were washed in PBS (pH 7.2) three times, followed by dilution of the culture to 10^6 cfu/mL in 10% HS. LL37-Au NPs (80 μg/mL), and LL37 peptides (30 μg/mL) with 30 μM nitrocefin or 2.5 mM ONPG were incubated with 10^6 cfu/mL of bacteria. Time dependent absorbance was measured simultaneously at 420 and 486 nm to monitor color changes with ONPG and nitrocefin respectively for 20 h, using a BioTek synergy MX microplate reader at 37 ºC.

Cell culture. Human umbilical vein endothelial cells (HUVECs, Lonza, Maryland, USA) were cultured in endothelial growth medium (EGM-2, Lonza). Cells with 4-6 passages were used in all experiments. The cells were sub-cultured at a ratio of 1:3 until achieving the number of cells required for the experiment.

Cytotoxicity studies. HUVECs were seeded at a density of 1×10^4 cells per well and cultured in a 96-well plate for 24 h and then incubated with LL37 peptides, LL37-Au NPs or Au NPs in culture medium at different concentrations. After 5 and 48 h, CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was used to assess the ATP production of the cells according to the supplier's instructions. For ROS quantification, cells were seeded at a density of 4×10^4 cells/cm^2 in 24-well plates and cultured for 24 h. Either LL37-Au NPs or Au NPs suspended in culture medium at different concentrations were added to cells and left for 5 h and 48 h. Before the treatment ended, cells were exposed to 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, 100 μM), a dye commonly used to measure intracellular changes in ROS, for 1 h. After washing the cells twice, they were trypsinized, centrifuged and resuspended in PBS to proceed with flow cytometry analysis (FACS Canto II, BD Biosciences). Hydrogen peroxide (1 mM) was used as a positive control for ROS production. In addition, propidium iodide (PI) (1 μg/mL final concentration) was used to quantify cell viability and to exclude the dead cells (i.e. PI positive cells) from the DCFH analysis.

NP internalization studies. HUVECs were seeded at a density of 300,000 cells per well in 6-well plates and cultured for 24 h. Then, LL37-Au NPs or Au NPs suspended in serum-containing medium were incubated with cells for 5 h or 48 h. After incubation, cells were washed three times with PBS to remove non-internalized NPs. Cells were then detached using 0.2% (v/v) trypsin in PBS, centrifuged, counted and resuspended in nitric acid solution (1 mL, 69%, v/v). After acidic digestion, samples were diluted to 4 mL in Milli-Q water and gold was quantified by ICP-MS, using a Bruckner 820-MS instrument (Fremont, CA, USA). Elemental analysis detection of Au^{197} was performed after a calibration of the apparatus using gold (Panreac) as standard at 5, 10, 50, 100, 250, 500 and 1000 μg/L. Iridium (Panreac) was used as internal standard at 20 μg/L. Considering the total cell number in each conditions, total Au^{197} per cell and NPs per cell were determined.

Membrane potential. HUVECs were seeded at a density of 4×10^4 cells/cm^2 in 24-well plates and cultured for 24 h. Then, LL37-Au NPs, Au NPs or LL37 peptides in culture medium at different concentrations were added to cells. After 5 and 48 h, cells were harvested, resuspended in medium with 3,3′-dipentyloxacarbocyanine iodide $DiOC_5(3)$ (5 nM, Sigma-Aldrich) and incubated for 5 min at room temperature in the dark, prior to the analysis with the flow cytometer (FACS Calibur, BD Biosciences). $DiOC_5(3)$ is a charged lipophilic dye that emits a fluorescent signal proportional to the membrane potential [3]. Cells upon hyperpolarization (i.e., cell interior becomes more electronegative with respect to the exterior) internalize more dye and therefore have more fluorescence. Conversely, cells upon depolarization internalize less dye and have less fluorescence. Cells treated with gramicidin (10 μM) or valinomycin (10 μM) was used as controls for cell depolarization and hyperpolarization, respectively.

FACS analyses. Cells were dissociated from the culture plate by exposure to cell dissociation buffer (Life Technologies) for 3-5 min and gentle pipetting, centrifuged and finally resuspended in PBS supplemented with 0.1% (v/v) BSA. The single cell suspensions were aliquoted ($1.5x10^5$ cells per condition) and stained with either isotype controls and antigen-specific primary antibodies Anti-

EGFR antibody (ab30, Abcam, dilution 1:200) for 30 min at 4°C. After the incubation with primary antibodies, cells were washed 3 times and incubated with anti-mouse Alexa fluor 488 (Life technologies 1:1000 dilution) for 30 min. Once the incubations were terminated, the cells were centrifuged at 1000 rpm, 20°C for 5 min, washed 3 times with cold PBS and then resuspended in PBS containing 0.1% BSA (400 µL) for FACS analysis. Differently for FPRL1 quantification, cells were fixed with 0.1% (v/v) paraformaldehyde (PFA) for 10 min, washed, permeabilized with saponin 0.02% (v/v) for 20 min and then stained with either isotype controls and antigen-specific primary antibodies anti-FPRL1 (ab101702, Abcam, dilution 1:200) for 30 min at 4°C. After the incubation with primary antibodies, cells were washed 3 times and incubated with anti-rabbit Alexa fluor 488 (Life technologies 1:1000 dilution) for 30 min. All conditions were performed in triplicate. FACS Calibur (BD Biosciences, San Diego, CA) and Cyflogic software were used for the acquisition and analysis of the data.

Confocal microscopy analyses. HUVECs were plated on a µ-slide 8 well, ibiTreat (Ibidi, Germany) (30.000/well) and left to adhere overnight before adding rhodamine LL37-Au NPs (at 200 µg/mL) in EGM-2. After 30 min, 4 h or 24 h of NPs incubation, cells were washed three times with PBS and were fixed with paraformaldehyde (4%, v/v) for 10 min, at room temperature, and washed three times with PBS. After blocking with BSA (1%, w/v, in PBS) for 1 h, fixed HUVECs were incubated with mouse anti-EGFR antibody (Abcam, dilution 1:1000) for 2 h, washed three times with PBS and permeabilized with 0.3% Triton X-100/PBS for 1 h. Cells were incubated with primary antibodies early endosomal marker EEA1 (Cell Signaling C45B10, 1:100 dilution) and the late endosome/lysosome marker protein RAB7 (Cell Signaling D95F2, 1:100 dilution) diluted in BSA solution according to the manufacturer's instructions and incubated for 2 h at room temperature. Secondary antibodies were anti-mouse Alexa fluor 647 and anti-rabbit Alexa fluor 488 (both from Life technologies 1:1000 dilution). Un-bound antibody was removed by washing three times with PBS before staining the nucleus of cells with Hoechst (Life technologies, dilution

1:1000) for 10 min. Confocal images (40x objective) were taken, using the optimal pinhole for better discrimination between foci and assuring no overexposure or bleed-through between channels. Images were exported to ImageJ, and colocalization analysis was performed using the automated co-localization tool named JACOP.

Evaluation of Ca^{2+} intracellular release. HUVECs were seeded at a density of 1.5×10^4 cells/well and cultured for 48 h in black opaque 96-well plates. After a period of starvation (7 h, in EBM-2), cells were loaded with Fura-2 calcium fluorescent indicator by incubation with 5 μM of the membrane permeable acetoxymethyl (AM) derivative Fura-2/AM (Molecular Probes), using basal medium as vehicle (35 μL/well) for 1 h at 37 °C in 5% CO_2. The medium was replaced and cells were incubated in the same conditions for more 30 min to allow the hydrolysis of the acetoxymethyl (AM) esters by cellular esterases, resulting in intracellular capture of the probe. Cells were then washed twice with 100 μL sodium salt solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose, 10 mM HEPES-Na^+ pH 7.4) and again immediately before the incubation or not with test compounds. Fluorescence reading was performed at 25 °C (Spectramax Gemini EM, Molecular Devices, with SoftMax Pro Software) by measuring emission at 510 nm, using two alternating excitation wavelengths (340 and 380 nm). Cells were then exposed to different concentrations of LL37 peptides, LL37-Au NPs and Au NPs. VEGF (100 ng/ml) was used as positive control for intracellular calcium release. For measurements in the presence of FPRL-1 receptor inhibitor, cells were pre-treated with FPRL-1 antagonist WRW4 (Calbiochem, 10 μM), for 1 h before Fura-2/AM probe incubation.

Matrigel assay. HUVECs were seeded at a density of 2×10^5 per well in 6-well plates and cultured for 24 h in EGM-2. Cells were exposed to LL37-Au NPs (200 μg/mL) or Au NPs (200 μg/mL) for 4 h and then washed, trypsinized, counted and seeded again at a concentration of 1×10^4 cells per 50 μL of M199 medium on 15-well slide (IBIDI, Germany) previously coated with 10 μL of Matrigel (BD Biosciences) per well and incubated for 30 min at 37 °C. For measurements in the

presence of FPRL-1 receptor inhibitor, cells were pretreated with FPRL1 antagonist WRW4 (Calbiochem, 10 μM) for 1 h before adding NPs. In addition, 10^4 cells per 50 μL of M199 medium were seeded on top of the polymerized Matrigel in the presence of LL37 (5 μg/mL) with and without 10 μM of FPRL1 Antagonist WRW4, VEGF (50 ng/mL). After 4 h, images were taken by a In Cell Microscope 2000 (GE Healthcare) (objective 2×) and then ImageJ was used to calculate the number of tube-enclosed regions for each conditions relative to control.

Measurement of VEGF by ELISA. HUVECs were seeded at a density of 4×10^4 cells/cm^2 in 24-well plates and cultured for 24 h in EGM-2 without VEGF. Then cells were exposed to LL37 (5 μg/mL), LL37-Au NPs (200 μg/mL) or Au NPs (200 μg/mL) suspended in culture medium without VEGF, at different concentrations. LL37 peptides (5 μg/mL) and LL37-Au NPs (200 μg/mL) were also incubated in the presence of 10 μM of FPRL-1 antagonist WRW4. After 24 h of incubation, cell medium from all the conditions was collected and used to quantify VEGF secretion. Supernatant content was measured by a human VEGF ELISA development Kit (PeproTech) according to the manufacture's instruction. A calibration curve was established for the calculation.

CAM assay. CAM assay was performed in chicken eggs as described previously [4]. Water (5 μL, vehicle, per egg), LL37 peptides (70 μg per egg), LL37-Au NPs (200 μg per egg), Au NPs (200 μg per egg) or bFGF (400 ng per egg) were mixed with 1% methylcellulose (5 μL) and placed onto the CAMs and incubated for 3 days. After injection of 20% Luconyl black 0600 (BASF, Ludwigshafen, Germany), photographs were taken. CAMs were fixed, embedded in paraffin, sectioned, stained with a Masson-Goldner solution, and vessels were counted in hot spots as described previously [5].

Statistical analyses. Statistical analyses were performed by a t-test or one-way ANOVA test with a Newman-Keuls test applied post hoc for paired comparisons of means (GraphPad Prism 5.0 software).

References:

1. J., P.C. Turkevich et al., Discuss. Faraday Soc. 1951, 11, 55-75.
2. R.F. Epand et al., Antimicrob. Agents Chemother. 2010, 54, 3708-3713.
3. N.Y. Yount et al., Proc. Nat. Acad. Sci. 2009, 106, 14972-14977.
4. R. Koczulla, J. Clin. Invest. 2003, 111, 1665-16672.
5. D.K. Nambiar, Methods Mol. Biol. 2016, 1379, 107-115.

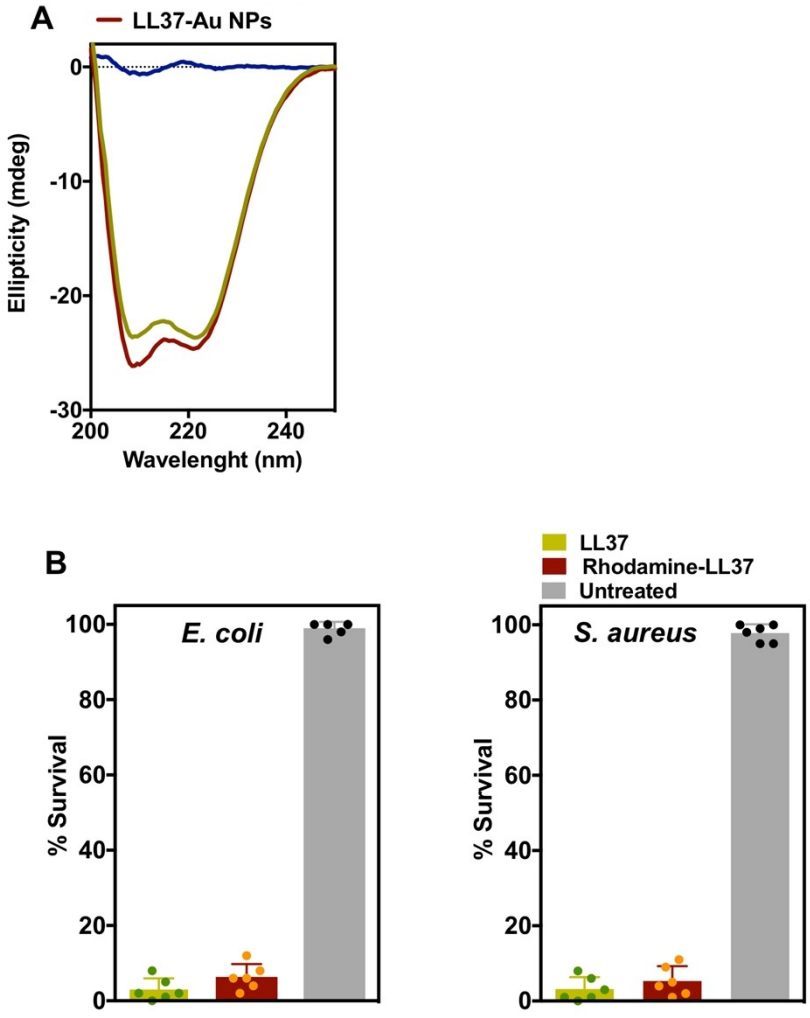


Figure S1- CD and antimicrobial activity measurements. (A) CD spectra of bare Au NPs, LL37 peptides and LL37-Au NPs. (B) Antimicrobial activity of LL37 peptides (30 □g/mL) labeled with or not with rhodamine against *E. coli* and *S. aureus*.

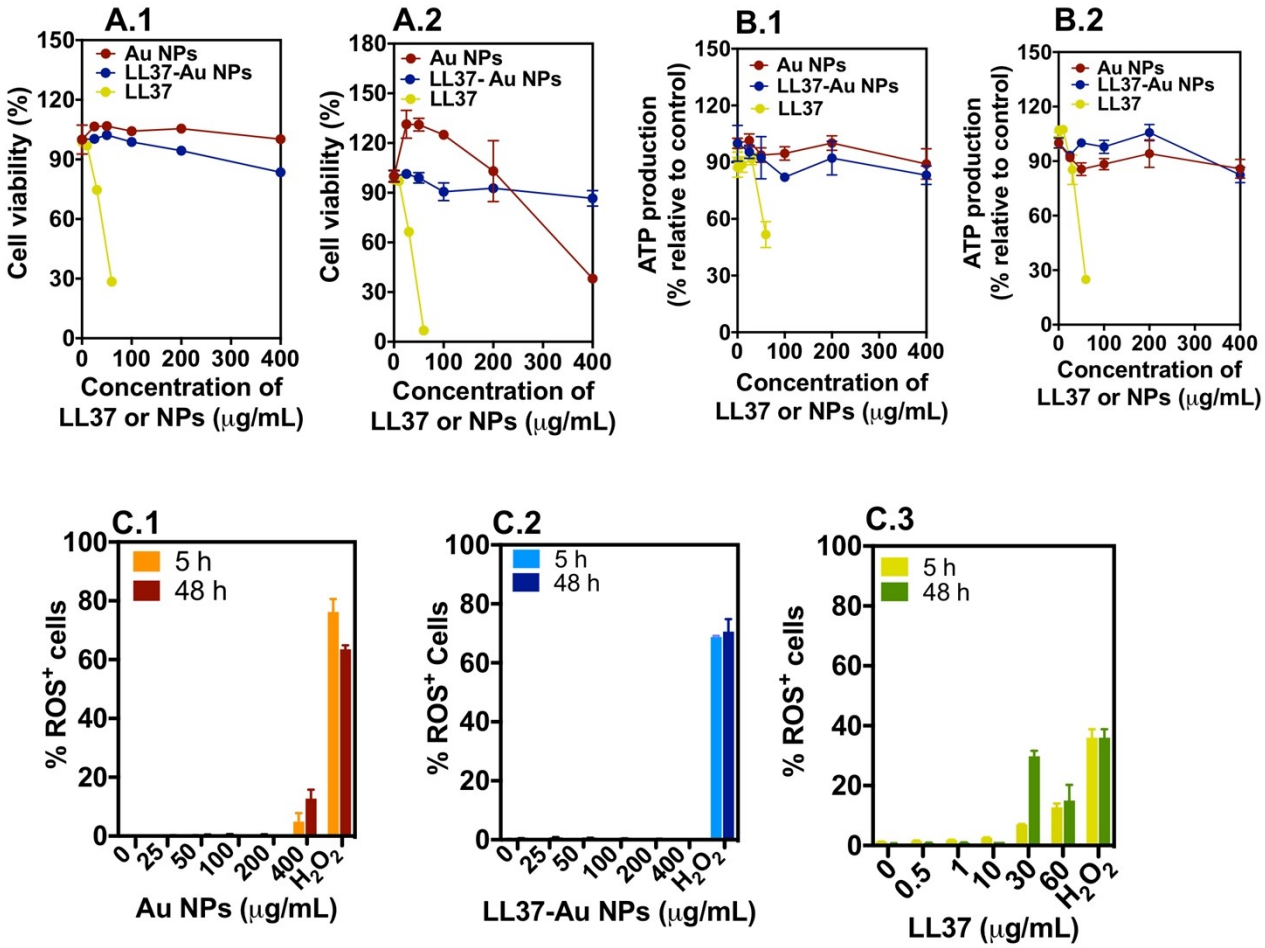


Figure S2- Cytotoxicity of LL37 and LL37-Au NPs. (A,B) HUVECs were exposed for 5 h (A.1 and B.1) or 48 h (A.2 and B.2) to different concentrations of soluble LL37, Au NPs and LL37-Au NPs, followed by the quantification of the viability (PI staining followed by flow cytometry evaluation) (A.1, A.2) or ATP kit (B.1, B.2). Results are average ± SEM, n=3. (C) Percentage of ECs expressing ROS. Non-treated cells and hydrogen peroxide-treated cells were used as negative and positive controls, respectively. Results are average ± SEM, n=2-3. Our results show that ECs treated with LL37 or LL37-Au NPs up to a concentration of 30 μg/mL and 400 μg/mL (ca. 100 μg/mL of conjugated LL37), respectively, have no significant decrease in cell viability or increase in ROS production. Interestingly, a decrease in cell viability and an increase in ROS production were observed for LL37 peptides above 30 μg/mL. Moreover, 400 μg/mL Au NPs were also cytotoxic to ECs.

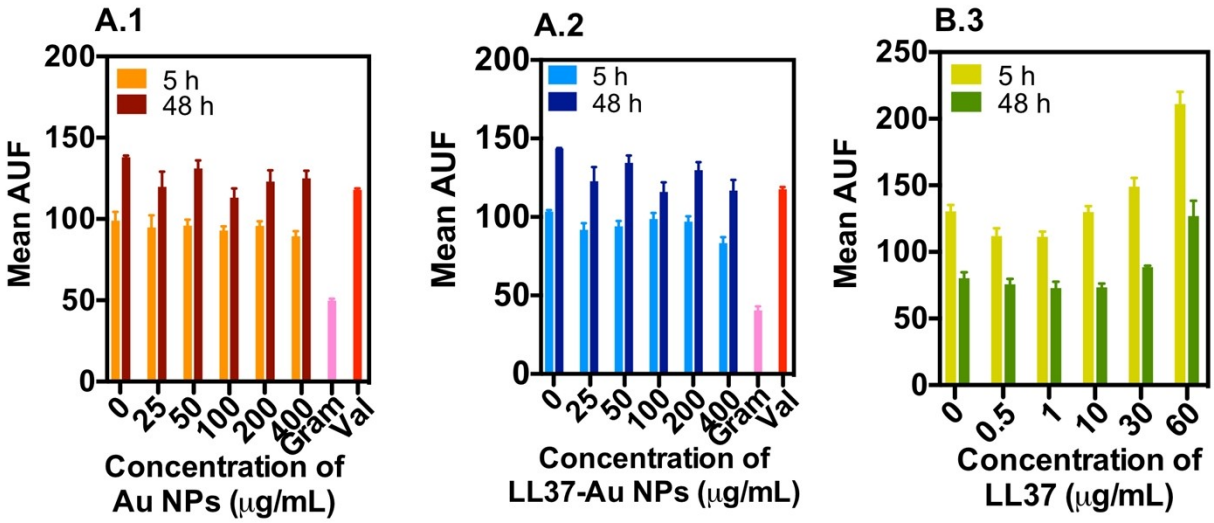


Figure S3- Effect of LL37 and LL37-Au NPs on the potential of HUVEC membrane. Gramicidin (10 μM), a non-selective ionophore that causes cell depolarization, and valinomycin (10 μM), a K^+ ionophore that causes cell hyperpolarization, were used as controls. Measurement of the membrane potential. Results are average ± SEM, n=3. Results are average ± SEM, n=3. Our results show that the membrane potential of HUVECs was not altered upon incubation either with LL37-Au NPs or Au NPs up to 400 □g/mL. On the other hand, HUVECs hyperpolarized after 5 h of exposure to soluble LL37 peptide below 30 μg/mL, while depolarized above 30 μg/mL.

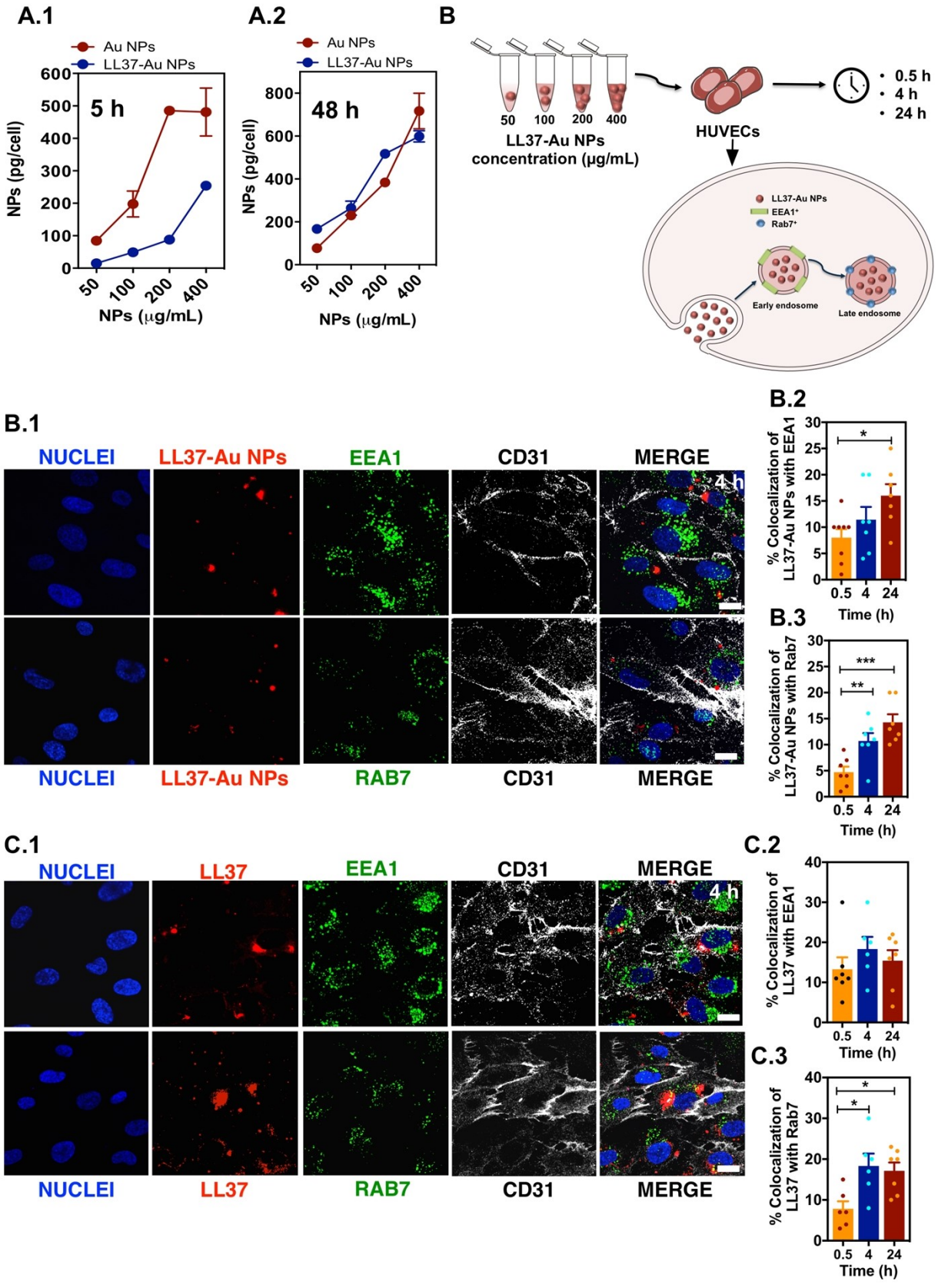
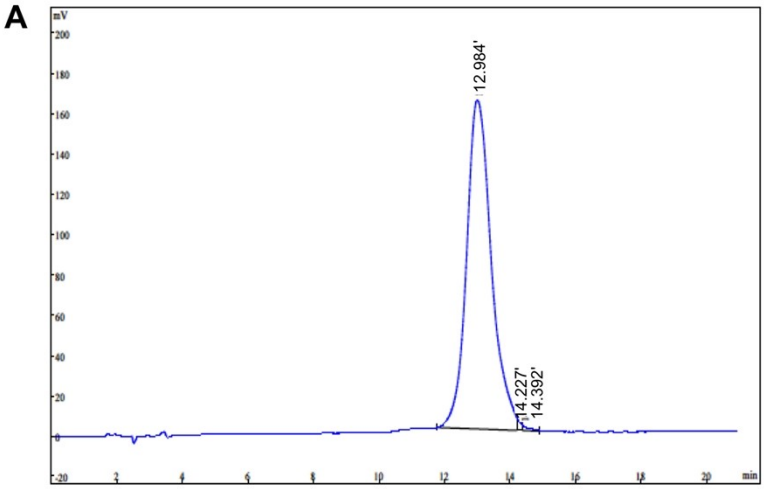


Figure S4- Internalization study of LL37 peptides and LL37-Au NPs by ECs. (A) ICP-MS study of internalization of Au NPs and LL37-Au NPs by HUVEC at 5 h (A.1) and 48 h (A.2). The results

are presented in mass of NPs and not mass of gold. Results are Mean ± SEM (n=3). Intracellular trafficking of LL37-Au NPs. (B) The representative schematic diagram shows the different concentrations of LL37-Au NPs and exposure time evaluated for the internalization study along with the colocalization of LL37-Au NPs in early and late endosomes. Cells were exposed for 30 min, 4 h and 24 h to 100 µg/mL of fluorescently-labeled LL37-Au NPs (B.1) and 1 µg/mL fluorescently-labeled LL37 (C.1). At the end of each time, cells were washed, fixed and stained for endolysomal compartment markers as early endosome/lysosome marker protein EEA1 (cell membrane was stained for CD31). Confocal images of fluorescently-labeled LL37-Au NPs (B.1) or LL37 peptide (C.1) intracellular trafficking in ECs. Bars correspond to 15 µm. Co-localization of LL37-Au NPs (B.2 and B.3) or LL37 peptide (C.2 and C.3) with EEA1 or RAB7 compartments. Results are Mean ± SEM (n=6-8). Statistical analysis was done using Turkey's multiple comparisons 1 way ANOVA test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



Peak Results

Rank	Time	Conc.	Area	Height
1	12.984	99.32	8425025	162932
2	14.227	0.3927	33309	4782
3	14.392	0.2862	24280	2099
Total		100	8482614	169813

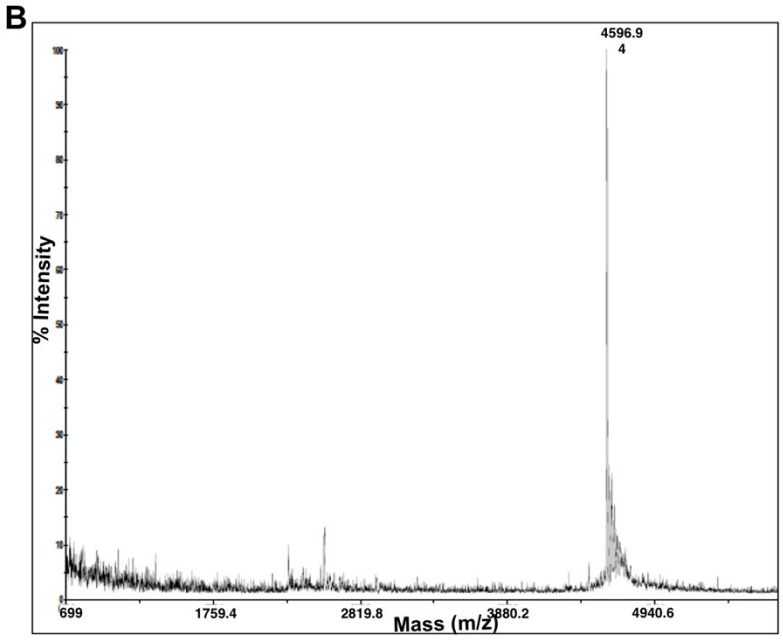


Figure S5- (A) HPLC and (B) mass spectroscopy data of soluble LL37 peptides. For HPLC measurement, buffer A (0.05% TFA + 2% CH3CN) and buffer B (0.05% TFA + 90% CH3CN) were used as eluent. Both data were provided by Caslo Laboratory Denmark, who has synthesized LL37 peptides.