Electronic Supplementary Material (ESI) for ChemComm.

Supplemental Information

Thiol-modified Gold Nanoparticles for the Inhibition of *Mycobacterium smegmatis*

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Materials & Methods

SYNTHESIS OF PMBA-AU NANOPARTICLES. pMBA-modified, 2 nm diameter gold nanoparticles were synthesized as described. Approximately 136 mg of $HAuCl_4 \cdot 3H_2O$ (Sigma Aldrich) was dissolved in 20 mL methanol. Simultaneously, approximately 210 mg para-mercaptobenzoic acid (pMBA) (TCI-America) was dissolved in 15.4 mL ultrapure H_2O and 0.64 mL 10 M NaOH by vigorous shaking. The pMBA solution was then added to the stirring gold solution. This polymer formation reaction is allowed to proceed overnight with constant stirring. The solution was then split into three flasks and diluted with 62 mL methanol and 178 mL ultrapure H_2O. A 7.5 mL 0.25 M solution of $NaBH_4$ (Sigma Aldrich) was freshly prepared, and 2.4 mL was added to each of the three reaction flasks. The solution was diluted further with 24 mL ultrapure H_2O then the nanoparticle formation reaction was allowed to proceed for another 24 hours with constant stirring. To harvest the nanoparticles, 2 mL 5 M NaCl and 150 mL methanol was added to each flask to cause precipitation. The precipitated nanoparticles are then pelleted in 50 mL conical tubes by centrifugation at 3200 g for 5 minutes. The pellets are left to air-dry, before resuspending in filter-sterilized ultrapure H_2O and washing six times on 10k M.W.C.O. filters (Millipore). The nanoparticle concentration was determined by UV-visible spectroscopy using the ε_{510} of 409,440 $M^{-1}cm^{-1}$. Nanoparticle size and distribution is observed by 15% tris-borate-EDTA polyacrylamide gel electrophoresis and transmission electron microscopy.

PLACE EXCHANGE REACTIONS. Four milliliter exchange reactions were started by first diluting 29.6 nmol of pMBA-Au nanoparticles in 4 mL of sterile 20 mM Na_2HPO_4 pH 9.5. Ligands 6 (cysteamine), 8 (3-mercapto-1-propane sulfonate) and 9 (2-diethylaminoethane thiol) were added in specific molar feed ratios (46x molar amount of gold for all three ligands on the most potent *M. smegmatis* nanoparticles) from 20 mM frozen stocks in water. Reactions are shaken at 19°C for 24 hours. The place exchange nanoparticle products are then harvested by the addition of 2 mL 5 M NaCl and 9 mL methanol. Precipitated nanoparticles are collected by centrifugation at 3200 g for 5 minutes. Pellets are resuspended in 4-6 drops filter-sterilized ultrapure H_2O then precipitated again with 0.5 mL 5 M NaCl and approximately 8 mL methanol. Centrifugation was repeated and pellets were dried overnight then resuspended and washed eight times with filter-sterilized ultrapure H_2O over 10k M.W.C.O. filters (Millipore). Purified nanoparticles are quantified the same as before, using UV-visible spectroscopy and a ε_{510} of 409,440 $M^{-1}cm^{-1}$.

BACTERIAL GROWTH INHIBITION ASSAYS. Overnight cultures of *M. smegmatis* (ATCC 700084) were started from 4 isolated colonies picked off of Middlebrook 7H10 Agar (Fisher) with a pipette tip and grown in 3 mL of Middlebrook 7H9 Broth (Fisher) at 37°C and 225 rpm. Cultures are then diluted to 2 x 10^6 CFU/mL in 7H9 and combined with equal amounts of nanoparticles (also diluted in 7H9 to the desired concentration). Samples were incubated for 72 hours at 37°C and 225 rpm. Percent inhibition values were determined by serially diluting cultures in 1x PBS and plating on 7H10 agar, grown for 3 days at 37°C and performing colony counts. For specificity tests, similar methods were followed for *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC BAA-2146), methicillin-resistant *S. aureus* (ATCC BAA-44), *M. abscessus* (ATCC 19977) and *M. avium* (MAC104WT).

TRANSMISSION ELECTRON MICROSCOPY – NANOPARTICLE DIAMETER MEASUREMENTS. Nanoparticle samples were dropped onto 300-mesh copper grids, and allowed to adhere for 10 – 20 seconds before wicking away the liquid. TEM imaging was conducted using a Philips CM100 microscope with an 80 kV acceleration voltage. Individual nanoparticles within each image were then measured in ImageJ to provide average diameters for different particle types.

MEMBRANE PERMEABILITY ASSAY. Protocol for the LIVE/DEAD BacLight Bacterial Viability kit (Invitrogen, L-7007) was followed exactly. In brief, two nucleic acid stains, green-fluorescent SYTO 9 and red fluorescent propidium iodide, are able to penetrate bacterial cell walls differentially and therefore indicate changes in membrane permeability under varying conditions.

MEMBRANE POTENTIAL ASSAY AND FLOW CYTOMETRY. Protocol for the BacLight Bacterial Membrane Potential kit (Invitrogen, B34950) was followed exactly. In brief, the carbocyanine dye $DiOC_2$ (3,3'-diethyloxacarbocyanine iodide) fluoresces green in all bacterial cells, but shifts towards red fluorescence in the presence of larger membrane potentials. The positive control CCCP (carbonyl cyanide 3-chlorophenylhydrazone) molecule provided with the kit removes all membrane potential by destroying the proton gradient. *M. smegmatis* was treated with pMBA-only, LAL-3346 nanoparticles and CCCP, as well as left untreated, then all cultures were stained with $DiOC_2$ and analyzed by a Benton Dickinson FACScan

Cytometer with FACScan software. Comparisons of green vs. red fluorescence for each resulted in an indication of the effect each particle type has on the membrane potential.

HEMOLYSIS ASSAY. Mechanically-defibrinated sheep's blood (Hemostat Labs) was incubated with our nanoparticle conjugates for up to 24 hours. An aliquot of blood cells was washed three times with 1x PBS and then used in a 1:10 dilution. Nanoparticles were added to blood cells in a range of concentrations to determine an IC_{50} value. After 24 hours, the remaining cells were washed with 1x PBS, lysed with 0.1% Triton and the release of heme was measured by UV-visible spectroscopy at 410 nm.

LUNG CELL TOXICITY ASSAY. The LAL-3346 conjugate was incubated with MRC-5 human lung cells (ATCC CCL-171) to investigate potential *in vivo* toxicity. Cells were grown in adherent Corning T75 flasks in EMEM until confluent. Media was aspirated, the adhered cells were washed twice with 8 mL 1x D-PBS and then 1 mL trypsin was added. After 15 minutes at 37°C, the trypsinized cells were resuspended in 9 mL EMEM and centrifuged at 1,000 rpm for 5 minutes. The cell pellet was resuspended in 1 mL RPMI and cell number was counted on a haemocytometer after a 1:10 dilution in media. Cells were seeded at 2.5 x 10^5 cells/well and allowed to attach for 30 minutes at 37°C. After this incubation, LAL-3346 nanoparticles added to the cells in a range of concentrations and allowed to incubate for 24 hours at 37°C. Cells were again washed, trypsinized and counted to determine percent surviving post incubation.

TRANSMISSION ELECTRON MICROSCOPY – BACTERIAL IMAGES. *M. smegmatis* was cultured in Middlebrook 7H9 media (Fisher) and adjusted to an optical density of 1 at 600 nm. pMBA-only or LAL-3346 nanoparticles were added to the mycobacterial solution at a concentration of 2 μM. After 24 hours, the mixture of mycobacteria and LAL-3346 was centrifuged at 12,000 rpm for 15 minutes. The cell pellets were resuspended in 1x PBS and centrifuged again. Washed cells were then fixed with 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C and post-fixed with 1% osmium tetraoxide in 0.1 M sodium cacodylate buffer at room temperature for two hours. After washing with cacodylate buffer, fixed pellets were dehydrated with increasing concentrations of ethanol as follows: 70%, 80%, 90%, 95% and 100% for 10 minutes each, then 100% acetone twice for 10 minutes each time. Dehydrated cell pellets were then infiltrated with a mixture of propylene oxide and Spurr's resin in 1:2, 1:1 and 2:1 volume ratios for 1 hour each, then 100% resin overnight. Finally, pellets were embedded in resin at 60°C for 24 hours to 48 hours. Sections, 60 nm to 70 nm thick, were cut using a Leica UC6 Ultramicrotome and collected onto 300-mesh copper grids. Grids were stained with 2% uranyl acetate and 1% lead citrate solutions. TEM imaging and analysis were conducted using a Philips CM100 microscope with an 80 kV acceleration voltage.

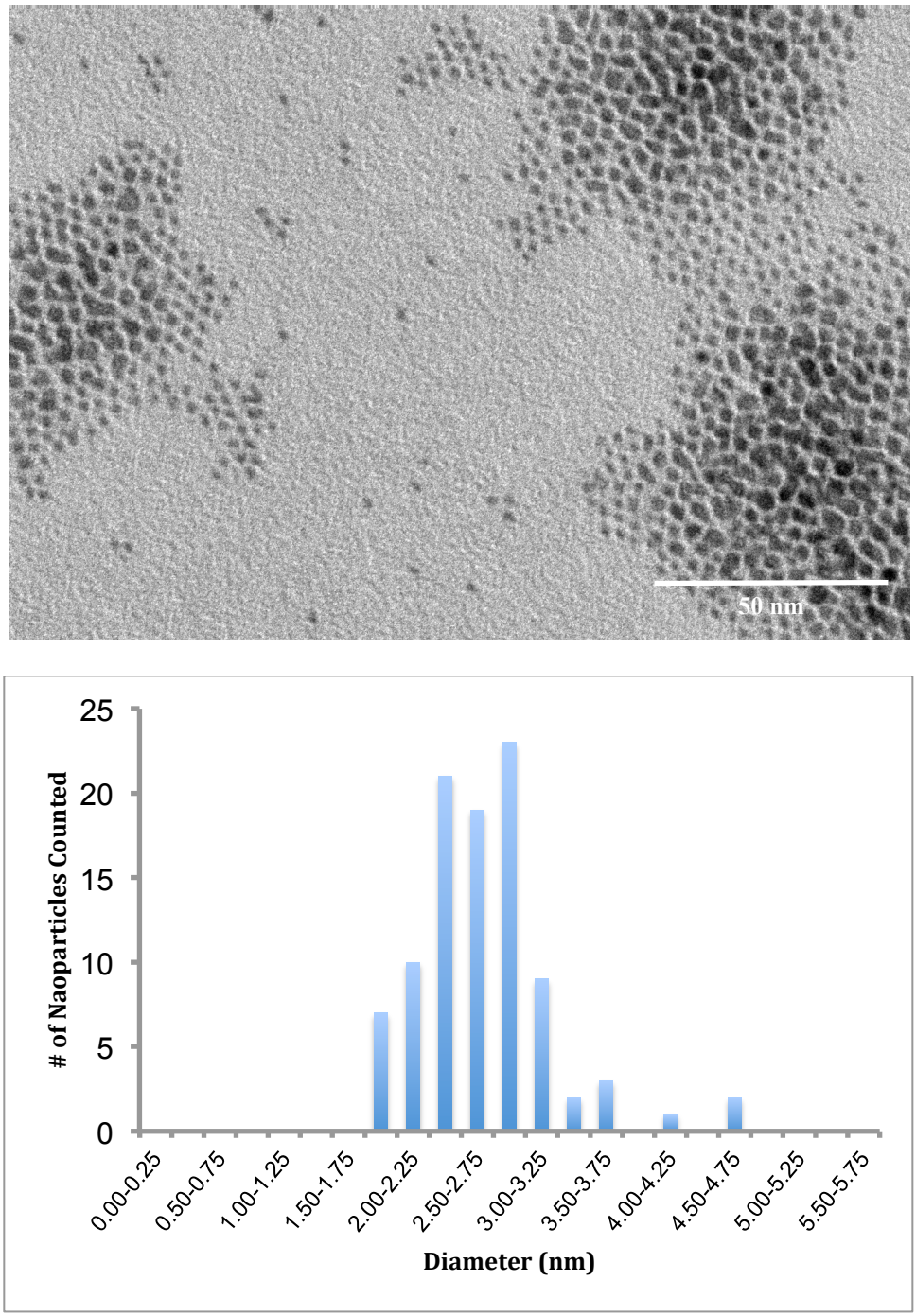


Figure S1A. TEM images of pMBA nanoparticles and accompanying size distribution measurements of those nanoparticles. Measurements were made in ImageJ software. The resulting average size is reported to be 2.7 nm ± 0.8 nm (sample size = 98).

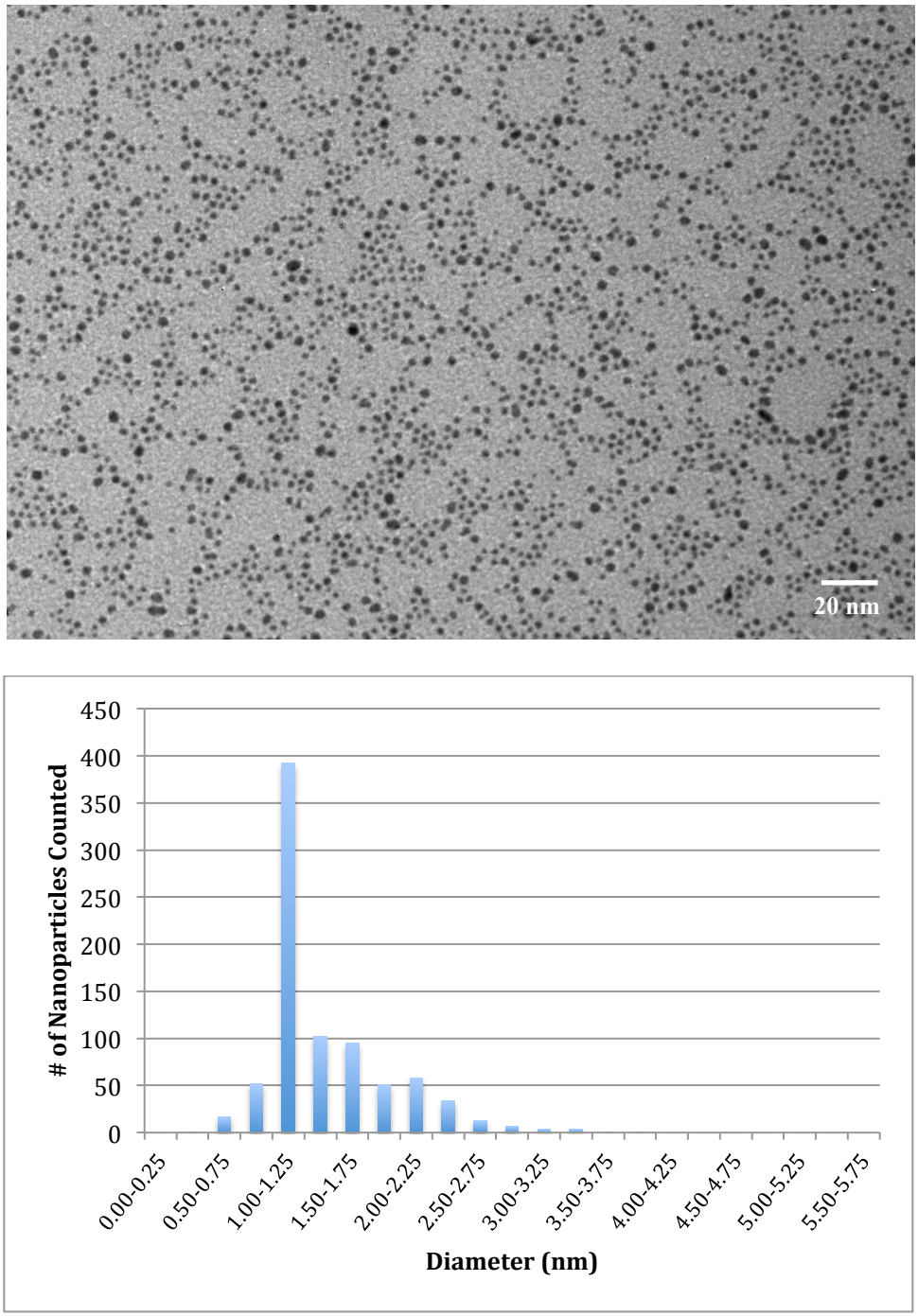
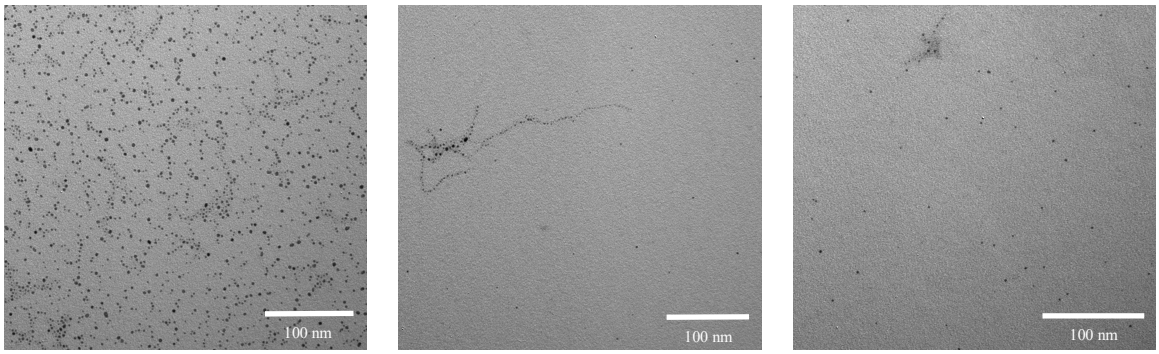


Figure S1B. TEM images of LAL-3346 nanoparticles and accompanying size distribution measurements of those nanoparticles. Measurements were made in ImageJ software. The resulting average size is reported to be 1.6 nm ± 0.6 nm (sample size = 832).



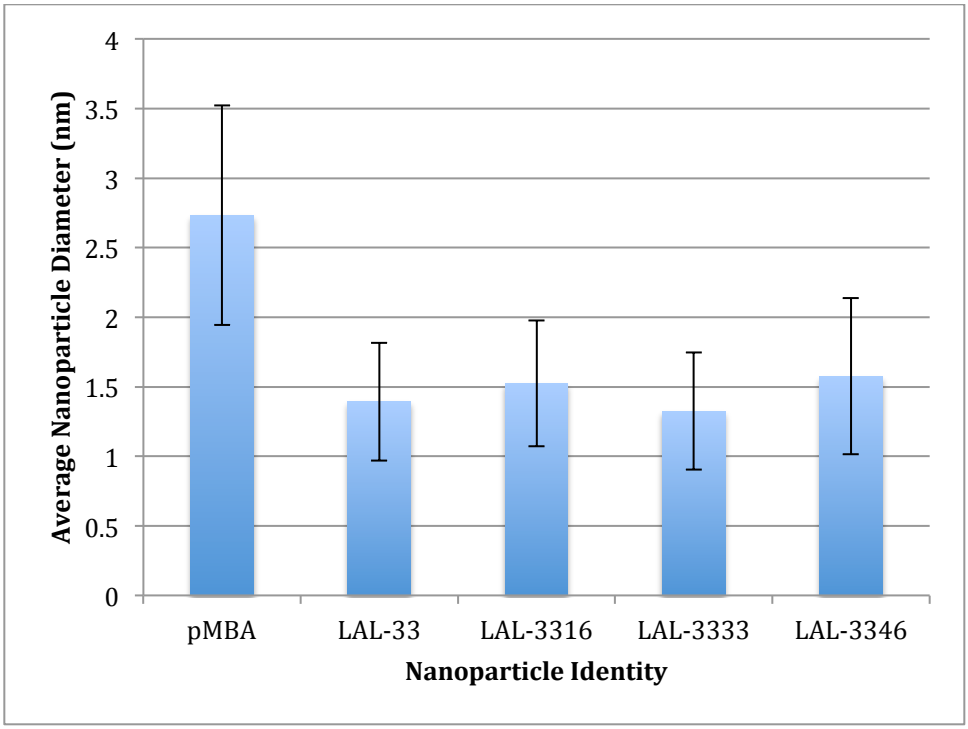


Figure S1C. Size distribution of pMBA, LAL-33, LAL-3316, LAL-3333 and LAL-3346 nanoparticle conjugates as determined by measurements on transmission electron microscope images in ImageJ software. The resulting average sizes with standard deviation were 2.7 nm ± 0.8 nm, 1.4 nm ± 0.4 nm, 1.5 nm ± 0.5 nm, 1.3 nm ± 0.4 nm and 1.6 nm ± 0.6 nm, respectively. All averages were based on measurements of at least 98 nanoparticles per sample. Example TEM images from which these measurements were made are shown for LAL-33, LAL-3316 and LAL-3333, in that order from left to right. For pMBA and LAL-3346 nanoparticles, see Figures S1A and S1B, respectively.

Table S1. *M. smegmatis* membrane permeability assay by LIVE/DEAD BacLight Bacterial Membrane kit from Invitrogen in the presence of 1 μM to 16 μM LAL-3346 nanoparticles.

Particle Concentration (μM)	Membrane Permeability, %
0.25	-5 ± 12
1	11 ± 18
4	15 ± 2
16	17 ± 16
1% Triton-X	58 ± 4

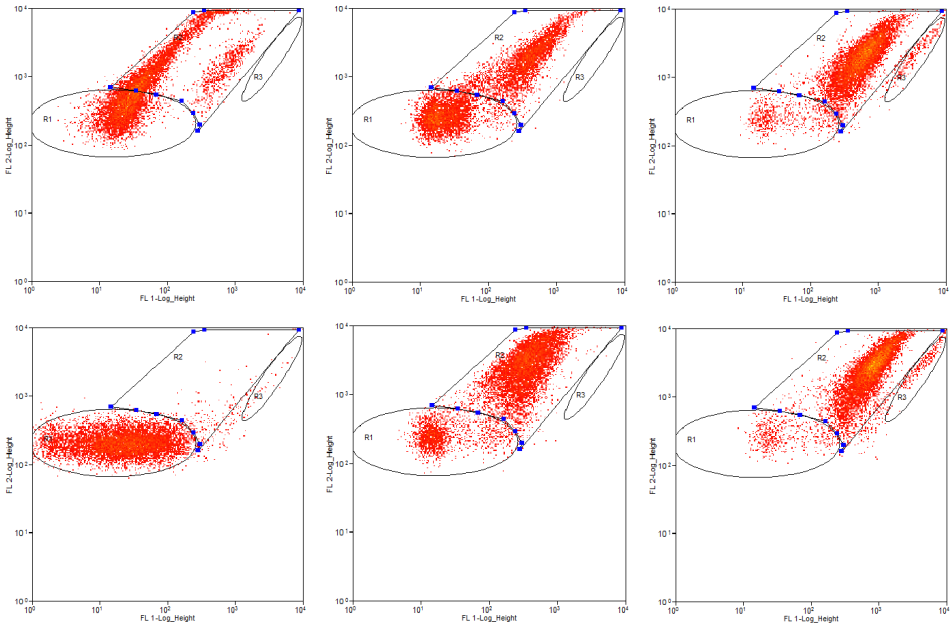


Figure S2. Changes in *M. smegmatis* membrane potential as measured by flow cytometry due to incubation with carbonyl cyanide 3-chlorophenylhydrazone (positive control, panel a), 8 μM pMBA base nanoparticles (b), and 6 μM and 8 μM LAL-3346 nanoparticles, respectively (c) and (f), after staining with carbocyanine dye $DiOC_2$. Negative controls with mycobacterium only (d) and no treatment but stained with $DiOC_2$ (e) are also shown for comparison. Regions 1, 2 & 3 for reference in Figure S3 are included in each panel.

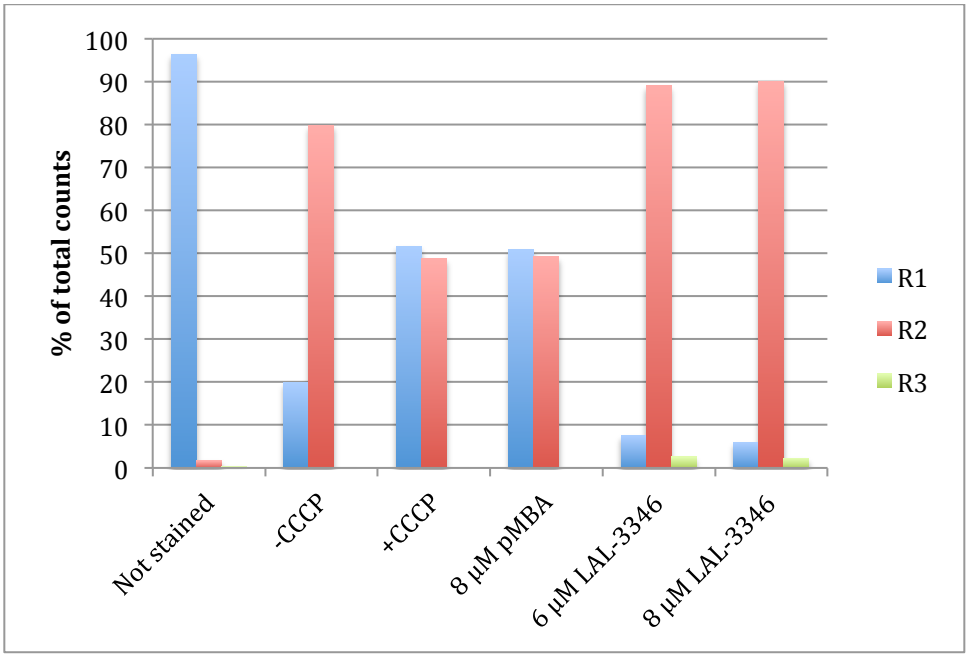


Figure S3. Quantitative comparison of the number of fluorescent counts in each of the three regions previously identified and outlined in the flow cytometry plots in Figure S2.

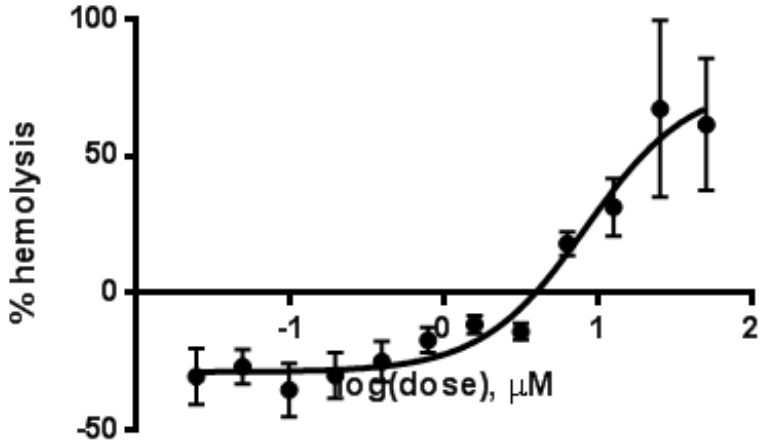


Figure S4. EC_{50} graph for hemolysis of sheep's blood cells after a 24-hour incubation with LAL-3346. This resulted in an EC_{50} of 8.3 μM with a 95% confidence interval between 4.0 μM and 17 μM, as determined by the "log(agonist) vs. response – variable slope (four parameters)" best fit function in the Graph Pad Prism 6 program. For this fit, the Hill coefficient was 1.287 and $R^2 = 0.9708$.

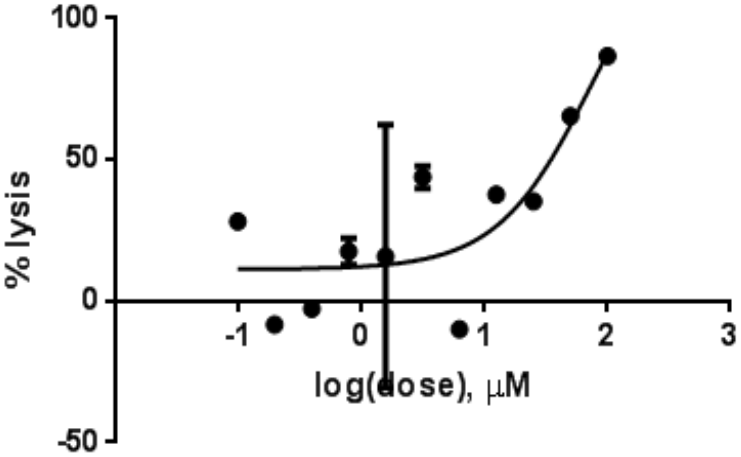


Figure S5. EC_{50} graph for lysis of human lung cells (MRC-5) after a 24-hour incubation with LAL-3346. An exact EC_{50} could not be determined from this data, but it is estimated to be greater than 50 μM, using the "log(agonist) vs. response – variable slope (four parameters)" best fit function in the Graph Pad Prism 6 program. For this fit, the Hill coefficient was 1.128 and $R^2 = 0.7015$.