Supplementary Information

Selective Targeting of *Mycobacterium smegmatis* with Trehalose-Functionalized Nanoparticles

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Materials and Methods

1,2-Hexadecanediol (85%), tetraethyl orthosilicate (TEOS), oleylamine (70%), dibenzyl ether, octadecylamine (90%), 1-octadecene (90%), ethanol (200-proof), ammonium hydroxide (25%), D-(+)-maltoheptaose (99%), D-(+)-trehalose dihydrate (99%), β-cyclodextrin (≥ 97%), D-(+)-glucose, 3-(trihydroxysilyl)propyl methylmethylphosphonate monosodium salt (50%, phosphonate-silane), tetramethylammonium hydroxide pentahydrate (97%, TMAH), potassium hexacyanoferrate(II) trihydrate (K₄[Fe(CN)₆]·3H₂O) (99.5%), hydrochloric acid (37%), formaldehyde solution (36.5%) were purchased from Sigma-Aldrich. Water used was from a Milli-Q water ultrapure water purification system. (3-Aminopropyl)trimethoxysilane (APTMS) was acquired from TCI America (Portland, OR). Iron (III) acetylacetonate was purchased from Acros Organics (Fair Lawn, NJ). PFPA-phosphate¹ and PFPA-silane² were synthesized following previously-developed procedures.

Middlebrook 7H9 broth with OADC enrichment was prepared from Middlebrook 7H9 (4.7 g, BD bioscience), glycerol (2.0 mL, Acros Organics), Tween® 80 (2.5 mL, 20%) and Milli-Q water (900 mL), and was sterilized by autoclaving (Tuttnauer EZ 10, Hauppauge, NY) before use. OADC enrichment (100 mL, BD Bioscience) was added to sterilized Middlebrook 7H9 broth when the temperature of the sterilized Middlebrook 7H9 broth reached at 45 °C. The phosphate buffered saline (PBS) (pH 7.4) was prepared by dissolving a PBS tablet (Sigma-Aldrich) in Milli-Q water (200 mL). Paraformaldehyde (5%) (Sigma-Aldrich) was prepared by dissolving (0.5 g) of paraformaldehyde in HEPES buffer (100 mM) (Sigma-Aldrich). Glutaraldehyde (8%),
OsO₄ (4%), Embed 812, Araldite 502, DDSA, DMP-30, 200-mesh Cu grids were purchased from Electron Microscopy Sciences (Hatfield, PA). M. smegmatis strain mc² 155, F-12K with L-glutamine, fetal bovine serum (FBS), A549 lung carcinoma cell line, DPBS 1X buffer were purchased from ATCC™ (Manassas, VA). Cell counting kit-8 was purchased from Dojindo Molecular Technologies, Inc (Rockville, MD). AlamarBlue® bacterial viability assay kit was purchased from Life Technologies (Grand Island, NY). BD BioCoat™ glass cover slips were purchased from BD Biosciences (San Jose, CA).

Dynamic light scattering (DLS) measurements were taken using Horiba SZ-100 particle size analyzer (Horiba Scientific Ltd., Kyoto, Japan). Transmission electron microscopy (TEM) images were obtained on a Phillips EM-400 TEM microscope operating at an accelerating voltage of 100 kV. Laser scanning confocal microscopy (LSCM) images were obtained on an Olympus LSCM FV300 microscope. Thin section samples were prepared using a Leica EM UC6 cryo-ultramicrotome. Scanning electron microscopy (SEM) images were obtained on a JEOL JSM 7401F microscope.

**Synthesis of Fe₃O₄ magnetic nanoparticles (MNP)s and phosphonate-silane coated MNPs (MNP)s.** The synthesis follows the previously reported procedure.³ Iron (III) acetylacetonate (0.706 g, 2.0 mmol), 1,2-hexadecanediol (2.584 g, 10.0 mmol), oleic acid (2.239 mL, 6.0 mmol) and oleylamine (2.820 mL, 6.0 mmol) in dibenzyl ether (20 mL) were stirred under a blanket of nitrogen. The mixture was then heated to 200 °C for 2 h followed by heating at 300 °C for 1 h. After cooling down to room temperature, ethanol (40 mL, 200-proof) was added and the mixture was centrifuged at 7000 rpm for 10 min. The black precipitate was re-dispersed in hexanes (30 mL) containing oleic acid (0.05 mL) and oleylamine (0.05 mL), and the mixture was centrifuged at 6000 rpm for 10 min.
The precipitate was discarded, and the supernatant was collected and ethanol was added. After centrifugation, the precipitate was re-dispersed in hexanes.

To introduce silane coating on MNPs, MNPs (30 mg) were dispersed in toluene (15 mL), and a solution of phosphonate-silane in methanol (3 mL, 0.01 M) and a solution of TMAH in methanol (3 mL, 0.01 M) were added, and the mixture was stirred at 80 °C for 3 h (Scheme S1). After cooling down to room temperature, the mixture was centrifuged at 12,000 rpm for 30 min and the solid precipitate was re-dispersed in ethanol. This step was repeated for 3 times, and finally the nanoparticles were dispersed in ethanol (15 mL).

**Synthesis of PFPA-functionalized MNPs.** A solution of MNPs (15 mL, 2 mg/mL) in methanol was added to a solution of PFPA-phosphate in CHCl$_3$ (3 mL, 12 mg/mL), and the mixture was stirred at room temperature overnight followed by centrifugation at 12,000 rpm for 30 min. The supernatant was discarded, and the pellet was consecutively re-dispersed once in hexanes, twice in methanol and once in ethanol. After the final centrifugation, the supernatant was discarded and the resulting PFPA-functionalized MNPs were re-dispersed in acetone/water (10 mL/20 mL). Concentration of the PFPA-MNPs was determined by measuring the dry weight of PFPA-MNPs in a known volume.

**Synthesis of PFPA-functionalized silica NPs (SNPs).** SNPs were synthesized following a slightly modified Stöber protocol. A solution of anhydrous ethanol (34 mL), ammonium hydroxide (2.1 mL, 6.25%) and TEOS (2.1 mL) was stirred at room temperature for 2 days to obtain SNPs. To synthesize PFPA-functionalized SNPs, a solution of PFPA-silane in toluene (8 mL, 12.6 mM) was directly added to the SNPs solution and the mixture was stirred overnight followed by refluxing at 78 °C for 1 h. The mixture was centrifuged at 12,000 rpm for 20 min and the precipitate was re-
dispersed and washed in ethanol for 3 times followed by in acetone for 3 times. Finally, the purified PFPA-SNPs were dispersed in acetone.

**Synthesis of PFPA-functionalized dye-doped silica nanoparticles (FSNPs).** FSNPs were synthesized following a previously developed procedures. Fluorescein isothiocynate (FITC) (1.5mL, 12 mM in ethanol) was stirred overnight with (3-aminopropyl)trimethoxysilane (APTMS) (20 μL) in anhydrous ethanol (15 mL) at 42 °C to obtain the FITC-APTMS precursor. Precursor solution (5.0 mL) was then added to anhydrous ethanol (34 mL) containing ammonium hydroxide (2.0 mL, 6.25%) and TEOS (2.0 mL). The mixture was stirred for 2 days to obtain FSNPs. To synthesize PFPA-functionalized FSNP, a solution of PFPA-silane in toluene (8 mL, 12.6 mM) was added to FSNPs solution and the mixture was stirred overnight followed by refluxing at 78 °C for 1 h. The mixture was centrifuged at 12,000 rpm for 20 min, and the precipitate was re-dispersed and washed in ethanol for 3 times followed by in acetone for 3 times. Finally, the purified PFPA-FSNPs were dispersed in acetone.

**Conjugation of carbohydrates on SNPs, FSNPs and MNPs.** To a glass bottle containing an acetone solution of PFPA-SNPs or PFPA-FSNPs (1 mL, 4 mg/mL) or PFPA-MNPs (1 mL, 1 mg/mL), an aqueous solution of trehalose (Tre), glucose (Glc), maltoheptaose (G7) or β-cyclodextrin (CD) (200 μL, 10.0 mg/mL) was added. A 280 nm long-pass optical filter (Schott Glass Technologies Inc., Duryea, PA) was placed on top of the glass bottle, and the mixture was irradiated with a 450 W medium-pressure mercury vapor lamp (Ace Glass Inc., Vineland, NJ) for 30 min while stirring (3.2 mW/cm² measured by a 254 nm sensor). The resulting carbohydrate-conjugated
nanoparticles were purified by centrifugation and re-dispersion in autoclaved Milli-Q water for 6 times followed by dialysis overnight.

**Determination of carbohydrate density via thermal gravimetric analysis (TGA).** All carbohydrate conjugated-nanoparticles and PFPA-functionalized nanoparticles were first heated to 100 °C and held at 100 °C for 10 min to remove volatiles, and then cooled to 30 °C using ultra-high purity argon gas (99.99 %). TGA experiments were conducted by heating at a rate of 10 °C /min to 800 °C for SNPs and FSNPs, and to 1000 °C for MNPs.

**Colony counting to determine the CFU/mL for *M. smegmatis***. Bacteria were grown in OADC-enriched Middlebrook 7H9 broth at 37 °C until an OD$_{650}$ of 0.3 was attained. Then a 10 μL aliquot of this bacterial suspension was taken and serially diluted 10$^5$ folds in OADC-enriched Middlebrook 7H9 broth. From the diluted solution, 20 μL was spread on OADC-enriched Middlebrook 7H10 agar plates. Colonies were counted after 36 h of incubation at 37 °C, and the colonies were counted. After multiplying the number of colonies by dilution factor it reported as log CFU/mL.

**Treatment of carbohydrate-conjugated nanoparticles with *M. smegmatis***. *M. smegmatis* strain mc$^{2}$ 155 was inoculated overnight in OADC-enriched Middlebrook 7H9 broth at 37 °C while shaking at 180 rpm. They were re-inoculated in fresh OADC-enriched Middlebrook 7H9 medium and were grown until an OD$_{650}$ of 0.3 was attained (~ 1 x10$^8$ bacteria cells/mL). The bacteria cells (30 mL, ~ 3 x 10$^9$ bacteria cells) were then harvested, centrifuged at 5,000 rpm, and re-dispersed in pH 7.4 PBS buffer (5 mL). The carbohydrate-conjugated nanoparticles (200 μL, ~1 mg/mL) were added to an aliquot of *M. smegmatis* (500 μL, ~ 3 x 10$^8$ bacteria cells), and the mixture was incubated at 37 °C for 6 h while shaking at 180 rpm. The mixture was then centrifuged at 1,500 rpm for
10 minutes, and the supernatant containing nanoparticles was discarded. The pellet was then re-dispersed in autoclaved PBS. This centrifugation redispersion step was repeated for 3 times.

**TEM measurements.** Samples for the TEM were prepared by dropping the suspension of nanoparticles (few drops) or nanoparticle-treated bacteria cells (few drops in PBS buffer) onto a Cu grid (200-mesh), and vacuum drying for few hours. The particle size was estimated by averaging the diameters of over 100 nanoparticles.

The thin section samples were prepared as follows. First, nanoparticle-treated bacteria cells suspension or untreated bacteria cells (1 mL) was centrifuged at 2000 rpm for 5 min and supernatant was slowly removed. A solution of glutaraldehyde in PBS (1 mL, 2.5%) was added to the pellet in an eppendorfTM tube. This was stored at 4 °C overnight. Then glutaraldehyde was removed and the pellet was washed with PBS for 3 times by incubating the pellet in PBS (~1 mL) at 4 °C for 10 min. Upon washing with PBS the pellet was then incubated in a solution of OsO₄ in PBS (1 mL, 2%) at 4 °C for 30 min. The excess OsO₄ was removed by washing the pellet in PBS (1 mL) at 4 °C for 3 times at 10 min each. Pellet was dehydrated by incubating it with 25%, 30%, 50% and 75% ethanol, respectively, followed by 95% ethanol (twice) and 100% ethanol (3 times) for 10 min each. Finally, the pellet was incubated in 1 mL of propylene oxide (twice) for 10 min each. Then portion of the pellet was transferred to a BEEM capsule.

The resin medium that was used to embed the pellet was prepared by mixing Embed-812 (5.0 mL), Araldite 502 (heated to 60 °C, 3.0 mL), DDSA (heated to 60 °C, 11.0 mL) and DMP-30 (1 mL), and the resulting resin mixture was kept in a vacuum oven to remove all the air bubbles. The pellet was then infiltrated sequentially in
propylene oxide/resin (3:1, 2:1, 1:1, 1:2 v/v) at room temperature for 15 min each. After a final infiltration in the resin, the sample was allowed to cure overnight at 60 °C in a vacuum oven. Finally the BEEM capsule was removed and the resin-embedded pellet was cut with an ultramicrotome (Leica EM UC6, Buffalo Grove, IL) into ~100 nm thick slices which were then placed on Cu grids (200-mesh) for TEM imaging.

**Treatment of carbohydrate-conjugated nanoparticles with A549 cells.** A suspension of A549 lung carcinoma cells was seeded in a T75 flask in the presence of F12K media supplemented with 10% FBS and 1% penicillin-streptomycin. This was incubated at 37 °C, 5% CO₂ until the monolayer of A549 cells reach 75% confluency. The media was removed and cells were washed with PBS buffer. Then this T75 flask was trypsinized, and cells were harvested by centrifugation at 1150 rpm for 7 min. The supernatant was removed, and fresh F12K media supplemented with 10% FBS and 1% penicillin-streptomycin (3 mL) was added. To determine the cell viability, cells were stained with trypan blue and counted using a countess® automated cell counter.

From the above cell suspension, 125,000 cells/well were seeded onto a 6-well plate, and was incubated at 37 °C, 5% CO₂ for 12 h. After the cells were attached to the bottom of the well plate, the media were removed and the cells were repeatedly washed with PBS buffer. Finally, F12K medium (800 μL) and carbohydrate-conjugated nanoparticles (Glc-NPs, Tre-NPs) (200 μL, 1 mg/mL) were added to each well, and the cells were incubated at 37 °C for 2 h.

**Prussian blue staining.** Nanoparticle-treated A549 cells were washed with PBS buffer for three times, and an aqueous formaldehyde solution (0.5 mL, 10%) was added. After incubation for 30 min to fixate the cells to the wells, the wells were washed with PBS
buffer for three times. Prussian blue staining was performed by adding to each well an aqueous solution containing $K_4[Fe(CN)_6] \cdot 3H_2O$ (0.5 mL, 4%) and HCl (0.5 mL, 2%), and incubating the samples at 37 °C for 3 h. The excess reagent was removed and wells were washed with PBS buffer for three times. The Prussian blue stained samples were observed by an inverted light microscope.

**SYTO® 61 staining of *M. smegmatis***. *M. smegmatis* (mc² 155) was grown until $OD_{650}$ of 0.3 ($1 \times 10^8$ bacteria cells/mL) was reached. The bacteria cells (10 mL, ~ $1 \times 10^9$ bacteria cells) were then harvested, centrifuged at 5,000 rpm, and re-dispersed in pH 7.4 PBS buffer (1.0 mL). Then SYTO® 61 (5 μL) was added and incubated at 37 °C for 1 h. To remove the excess dye, SYTO® 61 stained bacteria was centrifuged at 5000 rpm for 10 min and the supernatant was discarded. The pellet was redispersed in fresh OADC-enriched middlebrook 7H9 media (10 mL).

**Treatment of A549 cells with *M. smegmatis* followed by incubating with carbohydrate-conjugated SNPs or FSNPs.** A suspension of A549 cell (10⁶ cells/mL) was prepared in the antibiotic-free F12K medium supplement containing 10% FBS. From this, 125,000 cells/well were seeded into a 6-well plate containing a BD BioCoat™ cover slip and was incubated at 37 °C for 12 h. After the cells were attached to the cover slip, the media were removed and the cells were repeatedly washed with PBS buffer. SYTO® 61 stained *M. smegmatis* (1 mL) was diluted by 100 times with antibiotic-free F12K containing 1% FBS. From this bacterial suspension, 1 mL (~ $10^6$ CFU/mL) was added to each well. Number of bacteria per cell of mycobacteria to A549 cells was kept at 10. The cultures were then incubated at 37 °C for 2 h, after which, the media were removed and the cells were repeatedly washed with PBS buffer. Then F12K medium (800 μL) and
carbohydrate-conjugated nanoparticles (Tre-FSNP, CD-FSNP, Tre-SNP and CD-SNP) (200 μL, ~1 mg/mL) were added to each well, and were incubated at 37 °C for 6 h. Then cells were repeatedly washed with PBS buffer. Finally cells were fixed in paraformaldehyde (5%)

**Treatment of carbohydrate-conjugated FSNPs with rat macrophages (RAW 264.7).** A suspension of rat macrophage (RAW 264.7) were seeded into a T75 flask in the presence of DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin. This was incubated at 37 °C, 5% CO₂ until the monolayer of RAW 264.7 cells reached 75% confluency. Then media was removed and cells were washed in PBS buffer. T75 flask was trypsinized and cells were harvested by centrifugation at 1150 rpm for 7 min. Supernatant was removed and fresh DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin (3 mL) was added. To determine the cell viability, cells were stained by trypan blue and counted by using countess® automated cell counter. From this, 125,000 cells/well were seeded into the 6-well plate containing a BD BioCoat™ cover slip, and was incubated at 37 °C for 12 h. After the cells were attached to the cover slip, the media were removed and the cells were repeatedly washed with PBS buffer. Finally, DMEM medium (800 μL) and carbohydrate-conjugated nanoparticles (Tre-FSNP, Glc-FSNP) (200 μL, ~1 mg/mL) were added to each well, and the cells were incubated at 37 °C for 3 h.

**SYTO® 61 staining of RAW 264.7 rat macrophages.** Nanoparticle-treated RAW 264.7 cells were washed with PBS buffer for three times. Then cells were fixed in paraformaldehyde solution in 5% HEPES buffer at 37 °C for 30 min. After incubation for 30 min to fixate the cells on the cover slip, the cover slips were washed with 5% HEPES
buffer for three times. Then 1 μM solution of SYTO® 61 dye in 5% HEPES buffer was added and cells were stained at 37 °C for 30 min. Then cells were washed in 5% HEPES buffer for three times. Finally cells were observed under Olympus LSCM FV300 with the excitation of 633 nm for visualization of red color SYTO® 61 and 488 nm for visualization of FSNPs.

**Cell cytotoxicity assay (A549 cells) - WST-8 assay**

A549 lung carcinoma cell suspensions (10⁶ cells/mL) were prepared in F12K media supplement with 10% FBS. From this, 36,000 cells/well were seeded into the 96-well plate and was incubated at 37 °C for 12 h. After the cells were attached to the bottom of the well plate, the media was removed and the cells were repeatedly washed with PBS buffer. F12K media (80 μL) and glyconanoparticles Tre-SNPs, Glc-SNPs, CD-SNPs, G7-SNPs, Tre-FSNPs or CD-FSNPs (20 μL, 1 mg/mL) were added to each well and the cells were incubated at 37 °C for 6 h. Then WST assay kit (10 μL) was added to each well, and plate was incubated at 37 °C for 1 h. Finally, absorbance was measured at 450 nm using Biotek® Epoch microplate reader.

**Cell cytotoxicity assay (M. smegmatis) - alamarBlue® assay**

*M. smegmatis* (mc² 155) was grown until OD₆₅₀ of 0.3 (1 x 10⁸ CFU/mL) was reached. The bacteria cells (30 mL, ~3 x 10⁹ bacteria cells) were then harvested, centrifuged at 5,000 rpm, and re-dispersed in pH 7.4 PBS buffer (5.0 mL). The carbohydrate-conjugated nanoparticles (20 μL, ~1 mg/mL) were added to an aliquot of *M. smegmatis* (50 μL, ~4.5 x 10⁸ bacteria cells) in a 96 well plate, and the mixture was incubated at 37 °C for 6 h while shaking at 180 rpm. Then alamarBlue® (7 μL) were added and plate was incubated
at 37 °C for 4 h. Finally, florescence emission intensity was measured at 585 nm (excitation 570 nm) using TECAN Infinite® 200 PRO microplate reader.

**Scheme S1.** (a) Synthesis of MNPs. (b) Synthesis of PFPA-functionalized MNPs (c) Synthesis of PFPA-functionalized SNPs and FSNPs (d) Conjugation of carbohydrates to MNPs, SNPs and FSNPs.
Figure S1. (a) TEM image of silanized MNPs, (b) DLS graph of silanized MNPs.

Figure S2. (a) TEM image of SNPs, (b) DLS graph of SNPs.

Figure S3. (a) TEM image of FSNPs, (b) DLS graph of FSNPs.
Figure S4. TEM image of (a) PFPA-MNPs (b) PFPA-SNPs.

Figure S5. FTIR spectra of (a) PFPA-SNPs, (b) Tre-SNPs, (c) Glc-SNPs, (d) G7-SNPs, (e) CD-SNPs.
Figure S6. TEM images of (a) Tre-SNPs, (b) Glc-SNPs, (c) G7-SNPs, (d) CD-SNPs, (e) Glc-MNPs, (f) G7-MNPs, (g) CD-MNPs, (h) Tre-MNPs.
Figure S7. TGA curves of (a) SNPs (b) FSNPs (c, d) MNPs.
**Table S1.** Surface coverage of carbohydrates on nanoparticles.

<table>
<thead>
<tr>
<th>carbohydrate</th>
<th>surface coverage density (µg/nm²)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SNPs</td>
</tr>
<tr>
<td>Tre</td>
<td>11.5×10⁻¹⁶</td>
</tr>
<tr>
<td>CD</td>
<td>16.0×10⁻¹⁶</td>
</tr>
<tr>
<td>Glc</td>
<td>7.25×10⁻¹⁶</td>
</tr>
<tr>
<td>G7</td>
<td>7.96×10⁻¹⁶</td>
</tr>
</tbody>
</table>

Carbohydrate density was calculated according to the following equation

\[ \frac{W_x}{3W_y^{\rho r}} \]

where \(W_x\) is the percent (%) weight difference between carbohydrate-NP and PFPA-NP at the cutoff temperature, \(W_y\) is the percent (%) weight at the cutoff temperature, \(\rho\) is the density of the nanoparticles and \(r\) is the DLS radius of the nanoparticles. The cutoff temperature for SNPs, FSNPs and MNPs was 800 °C, 800 °C and 1000 °C, respectively. The density of SiO₂ or Fe₃O₄ was used for the density of SNPs or MNPs.

**Figure S8.** TEM images of thin section samples of *M. smegmatis* (mc²155) after incubating 6 h with Tre-MNPs.
**Figure S9.** Optical images of Prussian blue-stained samples of A549 cells treated with (a) Tre-MNPs and (b) Glc-MNPs.

**Figure S10.** (a) Viability of *M. smegmatis* (*mc²155*) after treating with carbohydrate-conjugated silica nanoparticles measured by alamarBlue® assay, (b) Viability of A549 cells after treating with carbohydrate-conjugated SNP or FSNP measured by WST-8 cytotoxicity assay. Each result is an average of 3 repetitions.
Figure S11. SEM images of (a) *M. smegmatis*-treated A549 cells, (b) *M. smegmatis*-treated A549 cells after incubating with Tre-SNPs, (c) *M. smegmatis*-treated A549 cells after incubating with CD-SNPs.

Figure S12. LSCM images of A549 cells treated with SYTO® 61 stained *M. smegmatis* and incubated for 6 h with CD-FSNPs. (a) At 633 nm excitation showing SYTO® 61 stained *M. smegmatis* (b) At 488 nm excitation (c) Transmission image (d) Merged image (c) of transmission image and confocal images of a and b.

Figure S13. Transmission image of paraformaldehyde fixed A549 cells.
REFERENCES