Electronic Supplementary Information (ESI)

Real-time assessment of nanoparticle-mediated antigen delivery and cell response

Carlota A. Cunha-Matos†, Owain R. Millington‡, Alastair W. Wark§, Michele Zagnoni*†

†Department of Biomedical Engineering, University of Strathclyde, 106 Rottenrow, Glasgow, G4 0NW, UK.
‡Centre for Biophotonics, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow, G4 0RE, UK.
§Centre for Molecular Nanometrology, WestCHEM, Department of Pure and Applied Chemistry, Technology and Innovation Centre, University of Strathclyde, 99 George St, Glasgow, G1 1RD, UK.
ǁCentre for Microsystems and Photonics, Electronic and Electrical Engineering, University of Strathclyde, 204 George Street, Glasgow, G1 1XW, UK.

* Corresponding author: michele.zagnoni@strath.ac.uk

Contents:

Materials and Methods

Figures S1 to S7

Supplementary Videos:

- Video S1. Time-lapse video acquired at 12 frames/hour for 8 hours showing trapped single-cells within three regions exposed to different NR concentrations, as shown in Figure 3. Red represents NR fluorescence measured at 625 nm excitation and green represents DQOVA fluorescence measured at 488 nm excitation.
- Video S2. Time-lapse video acquired at 1 frame/min for 120 min, showing dendritic cell activity in static conditions within a well plate after a nanoparticle suspension was pipetted into the well.
Materials and Methods

**Microfluidic device design and prototyping.** Microfluidic devices were fabricated in polydimethylsiloxane (PDMS) using standard soft lithography procedures. In short, patterned silicon wafers were fabricated by photolithography using negative photoresist, SU-8 3035 [Chestech, UK] (25 µm thickness) and exposed to UV light through a chrome-on-glass mask [JD Photo-Tools, UK]. The wafers were silanised by vapour deposition of 1H,1H,2H,2H-perfluorooctyl-trichlorosilane [Sigma Aldrich, UK] for 1 hour. Devices were then produced by casting PDMS [Sylgard 184 Silicone Elastomer, Dow Corning, Michigan] in a ratio of 10:1 w/w elastomer base/curing agent onto the wafers to achieve 5 mm thickness. PDMS was then degassed and cured at 80°C for at least 2 hours. The mould was then removed from the silicon master and fluidic inlets and outlets punched manually using a flattened-tip needle. Fabrication quality assessment was achieved through PDMS microtrap visualisation using a Hitachi S-3000N Environmental Scanning Electron Microscope (SEM). PDMS devices and microscope coverslips [VWR, UK] were washed and dried prior to oxygen-plasma treatment and irreversibly bonded together. Two PDMS devices were typically bonded onto one coverslip so that two experiments could be carried out simultaneously. Metal connectors were made by cutting and filing G22 hypodermic needles [Fisher Scientific, UK] which were then inserted manually into the device inlets and outlets and connected to tubing.

**Gold nanorod preparation and characterization.** Synthesis of gold nanorods was performed using an adapted version of the seed-mediated growth method using cetyltriethylammonium bromide (CTAB). Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich [UK] and used as received. All solutions for the nanorod synthesis were prepared using ultrapure water. All glassware was previously cleaned with aqua regia for at least 2 hours and thoroughly rinsed with deionised water.

**Seed solution preparation:** Under vigorous stirring, a CTAB solution (5 ml, 0.2 M) was mixed with HAuCl₄ (5 ml, 0.5 mM), to which NaBH₄ (0.6 ml, 0.01 M) prepared immediately before was added. This produced a light brown solution which was kept at 25-30°C and used within 30 min. **Growth solution preparation** (targeting a longitudinal plasmon resonance of 780nm): HAuCl₄ (200 ml, 1.0 mM) was added to CTAB (200 ml, 0.2 M), as well as AgNO₃ (10 ml, 4 mM), and the solution gently mixed by inversion. Ascorbic acid (2.8 ml, 0.08 M) was then added and the solution mixed again, changing from orange to colourless. Freshly prepared seed solution (0.25 ml) was added to the growth solution, which was gently mixed again and kept in a water bath at 37°C for 48h. The nanorod solution was washed by centrifuging at 7500 rpm for 1 hour and resuspended in 1 mM CTAB three times. Nanorod longitudinal plasmon resonance could be adjusted by changing the amounts of AgNO₃ and seed added to the growth solution. **Dye incorporation and polyelectrolyte wrapping:** 3,3′-diethylthiadicarbocyanine iodide (DTDCI) incorporation into the CTAB layer of the gold nanorods was achieved by adding 10 ml of 100 µM DTDCI solution (diluted in deionised water from 10 mM stock in methanol) to 90 ml of rod stock solution, mixing gently and leaving overnight. Polyelectrolyte coating was based on established procedures. For the present study, poly-(sodium 4-styrenesulfonate) (PSS, MW=70,000) and polydiallyldimethylammonium chloride (PDDAC, MW≤ 100,000) were used. Aliquots of polyelectrolyte solutions (10 mg/ml in 5 mM NaCl, 0.25 ml per 1 ml of nanorod solution) were added dropwise to the nanorod solution under vigorous stirring and left stirring for a further 10 minutes. The coated nanorod sample was centrifuged at 7500 rpm for 1 hour (7000 and 6500 rpm for second and third layer coatings) and resuspended in deionised water. The final sample was centrifuged a further two times to wash out any remaining dye from the solution.

Particle characterisation was based on UV-NIR spectroscopy (400-1100 nm) using a Thermo Spectronic Unicam UV300 spectrophotometer with Vision 32 software. Stock nanorod concentrations were calculated
using an estimated extinction coefficient of $4.9 \times 10^9$ M$^{-1}$cm$^{-1}$ based on a previously reported study$^8$ and the optical density measured at the longitudinal $\lambda_{max}$. Bulk Raman spectra for the nanorod samples containing DTDCI were obtained at 633 nm using a Renishaw InVia Raman inverted microscope system with an incident laser power of $\sim 1$ mW focused through a transparent bottom micro-titre plate with a 300 µl aliquot in individual wells. A 10 s signal collection time for spectra acquisition was used. Full characterisation of the AuNR-dye complex has been previously published$^6$.

**Assessment of gold nanorod stability in culture medium.** Nanorods with encapsulated DTDCI and coated with a) PSS-PDDAC and b) PSS-PDDAC-PSS polyelectrolyte layers as above were tested for stability in complete RPMI. Samples were prepared at similar concentrations (approximate optical density of 1). These were then centrifuged at 7500 rpm for 30 minutes using an Eppendorf Minispin centrifuge, resuspended in complete RPMI, mixed and left at room temperature for one hour, after which a UV-Visible spectrum was acquired. At this point, solutions were transferred to new tubes (leaving any potential larger aggregates that had formed during centrifugation behind) and centrifuged again, resuspended in water and measured for UV-Visible absorption. Comparison of the extinction data in Figure S1e and Figure S1f demonstrates that for NR with a PSS-PDDAC coating (positively charged outer layer) partial aggregation of the colloid occurs when a concentrated aliquot of rods is added to the culture medium. This is indicated by the broadening and significant red-shift of the localised SPR peak. On the other hand, for a PSS-PDDAC-PSS coating (negatively charged outer layer), only a small shift was observed associated with the adsorption of serum proteins onto the outer PSS surface and the colloid also remained stable on further centrifugation and resuspension in water.

**Gold nanorod bioconjugation.** Conjugation of nanorods with encapsulated DTDCI and coated with PSS-PDDAC-PSS to DQ™ Ovalbumin (DQOVA) [Life Technologies, UK] or Ovalbumin (OVA) [Sigma-Aldrich, UK] was achieved by adding 10 µM of the protein to the gold nanorod solution (achieving a bulk nanorod optical density of $\sim 1$), mixing well by shaking and incubating at 4°C overnight. Samples were then washed by centrifuging at 6000 rpm for 30 minutes and resuspended in deionised water three times, before checking the UV-Vis spectrum and concentrating the samples accordingly for addition to the cells. In order to obtain an estimate of the concentration of ovalbumin molecules delivered by the nanorods, it was considered that the NR were two-dimensional rectangular particles and that OVA molecules covered all the sides of the particles. This led to an estimated 20 OVA molecules/NR, value that was used as order of magnitude for comparing results between NR and soluble OVA control experiments.

**Off-chip testing of NR conjugates.** Bone marrow-derived dendritic cells were generated from BALB/c or Ly5 mice bone marrow as previously described$^9$. Differentiated dendritic cells were harvested, counted using a haemocytometer and diluted to obtain a 5x10$^5$ cells/ml concentration in complete RPMI (RPMI 1640 with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine [all Life Technologies, UK]).

**NR biocompatibility assessment:** Cells were replated into 24-well Costar® plates [Corning, USA] (0.5 ml/well) and incubated with PSS-PDDAC-PSS-wrapped NR at different concentrations for 24 h, while a cell-only sample was used as negative control, camptothecin (CAM, apoptosis inducer) as a positive control for apoptosis and permeabilisation buffer (HBSS + 0.05% Tween-20) as positive control for necrosis. Samples were stained using Annexin-V FITC and Propidium Iodide [eBioscience, UK] according to manufacturer’s instructions and analysed using a BD FACSCanto™ with BD FACSDiva™ software.

**NR-DQOVA testing:** Dendritic cells were inoculated with 25 pM NR or 25 pM DQOVA-NR, with unpulsed cells used as a negative control and 5 µg/ml soluble DQOVA as a positive control. All samples were prepared in
triplicates for each time point, incubated at 37°C and 5% CO₂ and harvested at 30, 60, 120 and 360 minutes after addition of nanoparticles. Samples were analysed using a BD FACSCanto™ with BD FACSDiva™ software.

For confocal imaging, dendritic cells were replated at 5x10⁵ cells/mL into one 8-well glass slide [Lab-Tek II Chamber Slide, Nunc] and inoculated as above, in duplicates. Samples were fixed at 30 and 360 minutes with 4% paraformaldehyde [Sigma-Aldrich, UK], gently washed with HBSS [Life Technologies, UK] and a coverslip mounted onto the slide using VectaShield mounting medium with DAPI [Vector Laboratories, UK]. These slides were imaged with a Leica SP5 confocal microscope, using 488 nm for the DQOVA excitation and 633 nm for the NR (DTDCI) excitation.

**NR-OVA testing for T cell activation assessment:** Dendritic cells were inoculated with different concentrations of soluble OVA, NR or NR-OVA conjugates, in triplicates. Lymph nodes were recovered from donor OT-II mice (containing OVA-specific CD4+ T cells⁹) into a small amount of cRPMI. Cells were obtained by gently disrupting the tissue through a cell strainer [BD Biosciences, UK] using the plunger of a 1 ml syringe, after which they were counted using a haemocytometer and added to the dendritic cells at a ratio of 5:1. The cell mixture was incubated at 37°C and 5% CO₂. At 72h, 400 µl of medium from each well was removed into 1.5 ml centrifuge tubes [Star Labs, UK], centrifuged at 13000rpm for 5 minutes and the supernatant transferred into clean 1.5 ml centrifuge tubes for ELISA. Cells were then harvested from the wells and stained with FITC-conjugated Anti-CD4 and PE-conjugated Anti-CD69 [BD Biosciences, UK] according to manufacturer's instructions and analysed using a BD FACSCanto™ with BD FACSDiva™ software.

For ELISA of supernatants, flat-bottomed 96-well plates were coated with 50 µl of 2 µg/ml Anti-IFNγ (in pH 9 PBS) overnight at 4°C. Plates were washed three times in wash buffer (0.05% Tween 20 in pH 7.4 PBS) after each incubation step. Blocking was done for 1 h at 37°C using 10% FCS in pH 7.4 PBS. Samples were then added to appropriate wells (undiluted, 30 µl/well) as well as serial dilutions of recombinant IFNγ as standard and the plates incubated for 2 h at 37°C. Plates were washed again and incubated for 1 h at 37°C with 50 µl biotin-conjugated Anti-IFNγ at 0.5 µg/ml 10% FCS in PBS. Horseradish peroxidase (HRP)-conjugated streptavidin was then added at 1/2000 in FCS/PBS and incubated for 45 min at 37°C. Development was achieved using tetramethylbenzidine (TMB) substrate [KPL, US] and the catalysis of HRP was stopped when the colour change was sufficient for detection by adding 10% H₂SO₄. The absorbance was measured at 450 nm using a spectrophotometer.
Supplementary figures

Figure S1. Design and characterization of functionalized gold nanorods. a) Schematic representation showing sequestering of the reporter dye into the surrounding surfactant bilayer followed by polyelectrolyte wrapping and DQOVA conjugation. b) Representative SERRS spectrum (background corrected) of a colloidal solution of the polymer wrapped nanorod-DTDCI conjugate (633 nm excitation) with insert showing the dye chemical structure. c) Intrinsic fluorescence of DTDCI-containing nanorods following uptake by a dendritic cell (excitation at 633 nm and emission > 650 nm). Scale bar = 10 μm. d) Flow cytometry data following 24 hours exposure of dendritic cells to different nanorod concentrations showing good biocompatibility when compared to camptothecin (CAM – apoptosis inducer) and permeabilisation buffer (Perm) as positive controls. e) UV-Vis measurements obtained for gold nanorods with e1) PSS-PDDAC and e2) PSS-PDDAC-PSS polyelectrolyte coating when exposed to serum-containing medium for 1 hour compared with the same nanorod sample in water only. In (e2) additional spectra are shown following the centrifugation and water resuspension of the colloid after exposure to medium, while for (e1) this could not be acquired as the colloid was completely aggregated after centrifugation.

Figure S2. Analysis procedure used for flow cytometry data. a) Gating for cells according to forward and side scatter data, to exclude small non-cell like objects. b) Gating for specific surface marker CD11c positive cells (dendritic cells) according to conjugated PE fluorescence. c) Comparative analysis of activation marker CD40 fluorescence (APC conjugated) between unstimulated dendritic cells (DC), nanoparticle-pulsed dendritic cells (NP-DC) and lipopolyssacharide-stimulated dendritic cells (LPS-DC).
Figure S3. To estimate the flow rate needed to achieve a suitable nanorod concentration gradient, a 3D numerical model was used. First, to validate the model, the diffusion of fluorescein molecules against active transport within the microfluidic trap array chamber was simulated and compared to experimental results. COMSOL 3.5 was used to solve the Navier-Stokes equations to model pressure driven fluid transport together with Fick’s law to model diffusive transport. a) Experimental image showing signal from a fluorescein concentration gradient (increasing from left to right from 0 to 100 μM) generated using the two lateral inlets of the device (one injecting a fluorescein solution at 0.5 μl/min and the other injecting water at 0.5 μl/min). The graph shows the comparison between normalized fluorescent intensity profiles obtained from the finite element model solution (blue) and the experiment (red) in steady state condition across the full width of the array (as indicated by the red dashed line), confirming the accuracy of the numerical model. b) Experimental image showing fluorescence of a nanorod concentration gradient generated using the two lateral inlets of the device (one injecting a nanorod solution in culture medium at 0.5 μl/min and the other injecting culture medium at 0.5 μl/min). The graph shows the comparison between normalized fluorescent intensity profiles obtained from the equivalent finite element model solution (blue) and the experiment (red) in steady state condition across the full width of the array. From our simulations and as previously reported, the average flow velocity in regions outside the traps is an order of magnitude greater than that inside the traps (300-1100 μm/s outside versus 50-150 μm/s within traps, depending on the flow condition). Therefore, cells within the traps were exposed to a much smaller shear stress than those outside the traps. Variation of the NR concentration gradient profile along the flow direction in the trapping chamber was also assessed to evaluate possible changes of the profile due to lateral molecular diffusion. According to our simulation and to experimental results, the variation of the NR distribution (i.e., gradient profile) between the first and the last rows of traps is negligible, obtaining a variation of < 1% in the central region and ~3% at the side of the chamber.
Figure S4. Analysis of nanoparticle-induced toxicity within the microfluidic chamber. Dendritic cells were trapped and exposed to a concentration gradient of nanorods for 2 h, followed by perfusion of medium. 24 h after nanoparticle exposure, viability dyes were perfused for 15 minutes. The graphs show the mean fluorescence intensity (MFI) of (a) Annexin-V FITC (apoptosis marker) and (b) Sytox Blue (necrosis marker), as measured from fluorescence microscopy images of six contiguous fields of view across the width of the trapping array. Positive control samples (separate devices) were exposed to isopropanol on-chip to induce cell death and stained the same way. Each data point shows the mean ± S.E.M of between 56 and 99 cells per field of view, with the estimated NR concentration calculated from the average NR MFI in the same cells.
Figure S5. Multimodal imaging within the microfluidic device. (a) Composite bright-field image of the trap array showing trapped dendritic cells with overlay of representative normalized values of the average MFI of nanoparticles inside cells after nanorod delivery (red points). This was obtained by defining six array regions (along x-axis) across the width of the trap array and averaging the nanorod MFI from all the cells within each region. Cells were identified via DAPI staining of the nucleus. Scale bar = 100 µm. (b) Composite fluorescent image of the same region in the array chamber. Scale bar = 100 µm. (c1-3) Representative fluorescence images of single trapped cells (as highlighted with a red square in (b)) in regions of low, medium and high nanoparticle concentration areas spanning the width of the microfluidic array, and (d1-3) corresponding bright-field images and graphic maps of DTDCI-specific SERRS signal intensity (measured in the 1560-1620 cm⁻¹ region) of the same cells following fixation. Scale bar = 10 µm. (e1-3) SERRS spectra (background corrected) obtained as indicated by the red dot in the respective bright-field images, corresponding to the highest intensity signal from within each trapped cell. The arrows indicate the position of DTDCI-specific peaks, whose magnitude followed qualitatively the same trend observed with fluorescence microscopy.
Figure S6. Off-chip assessment of NR-mediated DQOVA delivery and processing. Dendritic cells were exposed to soluble DQ-OVA, unconjugated NR or NR-DQOVA conjugates at a range of concentrations. At various times, cells were harvested and analysed by flow cytometry to quantify (a1) NR fluorescence at 633 nm excitation and (a2) DQOVA fluorescence at 488 nm excitation. b) Confocal fluorescence imaging of fixed dendritic cells following a 6 hour incubation with 25 pM NR-DQOVA, where (b1) shows NR fluorescence, (b2) shows DQOVA fluorescence and (b3) shows the overlay of (b1) and (b2) with DAPI showing the cell nuclei. Scale bar = 20 µm.
Figure S7. On-chip assessment of soluble DQOVA delivery and processing. Dendritic cells were trapped following the same protocol used for NR-conjugate experiments, and exposed to a gradient of 0 to 222 nM soluble DQOVA.

(a) Composite image of the trapping array (Scale bar = 100 µm) showing DQOVA processing by dendritic cells. 

(b) Representative plots of averaged single-cell responses following soluble DQOVA uptake and processing. ROIs were drawn within individual cell-containing traps and data acquired every 5 minutes over 8 hours. For analysis purposes, three regions were defined according to different antigen concentrations: (b) null concentration (N=91), (c) intermediate concentration (N=123) and (d) high concentration (N=111) regions. Graphs show average DQOVA fluorescence intensity (background corrected) measured at 488 nm excitation ±S.E.M. for all the traps in each of these regions.

References