Glucan Particles loaded with a NIRF agent for imaging monocytes/macrophages recruitment in a mouse model of rheumatoid arthritis

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SUPPORTING INFORMATION

Materials and Methods

Characterization of Glucan Particles

To better characterize Cy5-$(C16)_2$/Rho-Glucan Particles, size and morphology were investigated by means of AFM microscopy. Atomic Force Microscopy (AFM) measurements were performed in the intermittent-contact regime (tapping mode AFM, pyramidal cantilever with a tip radius < 6 nm) by using a modified Nanosurf Easyscan2 AFM instrument, equipped with a 70 μm and a high resolution 10 μm scan heads, placed on a high performance anti-vibration platform and shielded by an acoustically insulated enclosure. Before analysis, the sample was diluted in distilled water (1-10 mg/ml) and gently sonicated in a bath sonicator to disperse particles in the solution and to minimize the aggregation for 5 min. The solution was then dropped on freshly cleaved mica and dried for 30 min.
Cy5-(C16)2/Rho-GPs were then lyophilized to acquire the IR spectrum.

To check the entrapment of both Rho-DOPE and Cy5$(C16)_2$, excitation and emission spectra were acquired. To this purpose $2.7x10^8$ GPs were suspended in 3 mL of HEPES buffer. Excitation spectra were acquired fixing the emission at 590 nm (acquisition from 450 to 578 nm) or 675 nm (acquisition from 450 to 665 nm), respectively. Emission spectra were acquired exciting the sample at 560 nm (acquisition from 572 to 750 nm) or 643 nm (acquisition from 653 to 750 nm), respectively. Slit of entrance and exit were maintained at 5 nm.

DLE and DLC determination

To investigate the loading ability of GPs to dyes, the content of Rhoda-DOPE and Cy5(C16)$_2$ in each particle was estimated. To this end, dyes were extracted from particles adding methanol (220 µL to 20 µL of sample). Glucan shells were then discarded by means of centrifugation (10 min, 6000 rpm) and the colored supernatant was diluted 1:11 in methanol to be read at the spectrophotometer (Hitachi U-28000). A spectrum was acquired from 350 to 750 nm and the ratio between the absorbance peak at 642 nm and 561 nm was calculated (Figure 1). Then, a standard solution containing Rho-DOPE and Cy5(C16)$_2$ in methanol, at mutual concentrations resulting in a ratio between the absorbance peaks (643 nm/561 nm) comparable to that obtained with the GPs extract, was prepared. The standard solution was then diluted in methanol at different concentrations to prepare a calibration curve to precisely quantify the content in Rho-DOPE and Cy5(C16)$_2$.

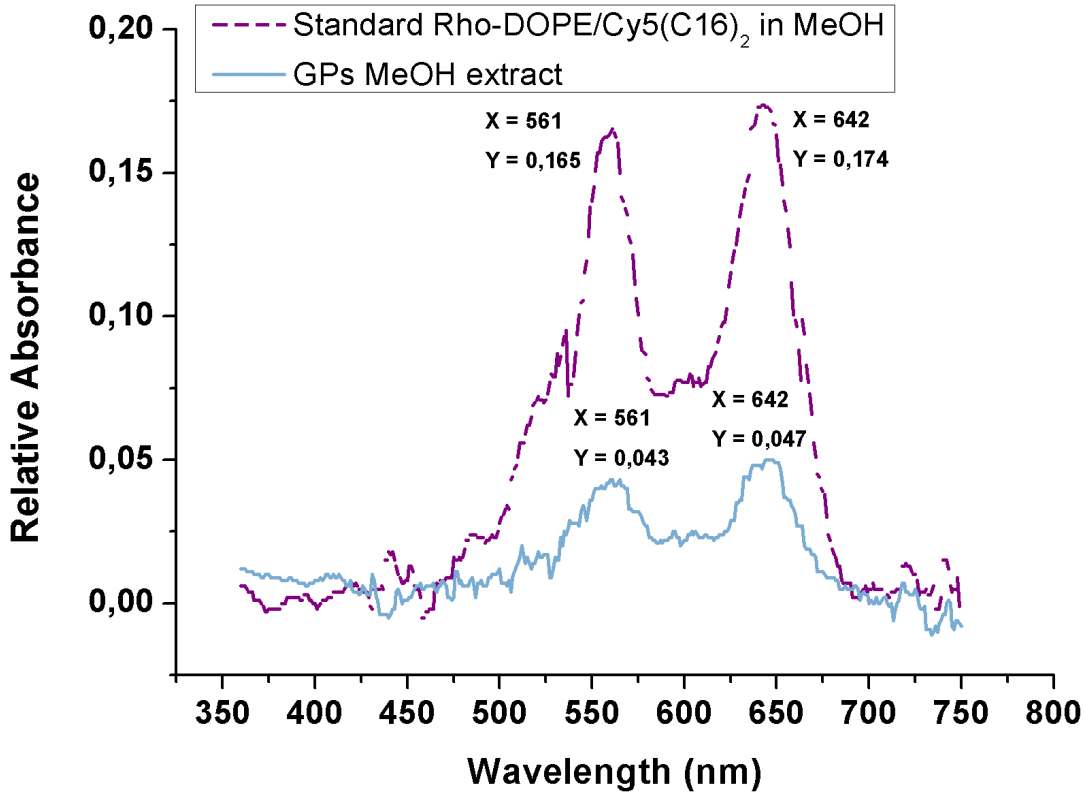

Figure 1. Absorbance spectrum of the extract of the inner content of GPs (straight line) and of a standard solution of Rho-DOPE and Cy5(C16)$_2$ in methanol (2 point segment line). The ratio between the absorbance values at 642 nm and 561 nm (reported in the graph) was calculated in order to estimate the ratio between Rho-DOPE and Cy5(C16)$_2$ in the microcarriers. Then a standard solution displaying the same ratio between the two absorbance peaks was prepared and used to draw calibration curves. In this way it was possible to overcome interactions between Rho-DOPE and Cy5(C16)$_2$, eventually resulting in over-/under-estimation of the GPs dye content.

Finally, following the quantification of Rho-DOPE and Cy5(C16)$_2$ performed by means of measurements at the spectrophotometer, and according to the calibration curves prepared from

standard solutions, Drug Loading Content (DLC) and Drug Loading Efficiency (DLE) for the two dyes were estimated as follows:

$$DLC(wt.\%) = \frac{mass\ of\ dye\ entrapped\ in\ GPs}{mass\ of\ GPs} x\ 100 \quad (1)$$

$$DLE = \frac{mol\ of\ dye\ entrapped\ in\ GPs}{mol\ of\ dye\ initially\ added} x\ 100 \quad (2)$$

Analysis of Cellular Uptake

Confocal microscopy

To demonstrate the uptake of Cy5-$(C16)_2$/Rho-GPs in J774A.1 cells, confocal laser scanning microscopy was performed. More in details J774A.1 cells (1×10^5) were seeded in sterile ibidi open µ-Slide (chambered coverslip) with 8 wells (dimensions of wells $w \times l \times h$ in mm, 9.4 × 10.7 × 6.8), tissue culture treated (ibidi GmbH, Planegg, Germany). After 24 h culture medium was discarded and cells were incubated for 90 min with $5.95x10^5$ GPs/µL DMEM. At the end of incubation, cells were washed five times with PBS and fixed with 4% formalin at room temperature for 15 min. Cells were profusely washed again; staining with 8×10^{-5} mg/mL Hoechst in PBS was performed for a few minutes, followed by 5 min washing in PBS at 4 °C. Finally cells were imaged through a Leica TCS SP5 confocal microscope (Leica Microsystems s.r.l.), magnification 40× ($laser_1$ wavelength = 405 nm, $laser_2$ wavelength = 561 nm, $laser_3$ wavelength = 633 nm to visualize Hoechst, Rho-DOPE and Cy5$(C16)_2$, respectively).

FACS analysis

In order to perform FACS (Fluorescence Activated Cell Sorting) analysis, adherent J774A.1 cells were seeded in 6 cm diameter culture dishes ($1.4x10^6$/dish). Cells were grown in DMEM until 80% confluence. The medium was then removed and cells were incubated for 90 min with: dish 1) $5.95x10^5$ Cy5-$(C16)_2$/Rho-GPs/µL DMEM; dish 2) $5.95x10^5$ empty GPs/µL DMEM; dish 3) DMEM only. At the end of the incubation cells were profusely washed with PBS, detached and counted using a cell sorting chamber (Burker-Turk chamber). Cells were then resuspended in PBS-0.1% BSA at a concentration of $1x10^3$ cells/µL, centrifuged and finally resuspended at a concentration of 300 cells/µL. Finally cells were analyzed for their PE/APC fluorescence on a flow cytometer (Becton Dickinson, FACS Calibur). Their PE/APC fluorescence was analyzed using the CELLQUEST PRO program.

Results

Characterization of Glucan Particles

Morphology and surface properties of modified Glucan Particles (Cy5-$(C16)_2$/Rho-GPs) have been investigated by AFM. By selecting the appropriate dilution (≈1-10 mg/ml), Glucan Particles may form regions ranging from isolated particles to near a monolayer coverage, being immobilized on the mica support (Figure 2, left panel). In this figure, particles with diameters in the 3-5.5 µm range (with a mean size of 4.4 ± 0.2 µm), are illustrated. Surface properties of an isolated Glucan Particle

have been obtained by high-resolution AFM imaging (Figure 2, right panel and insert therein). In this figure, a more irregular surface with the presence of subunits is emerging. A fine structure of these subunits, composed by fibril-like bundles (d≈30-50 nm) (inset of Figure 2, right panel), is plausibly associated with the underlying molecular structure of the β-glucan cell wall. The particle wall structure, as an interwoven network of β-glucan, well corroborates with the atomic structure imaged by Scanning Tunneling Microscopy (STM).[1] The FT-IR analysis (Figure 3) revealed an intense absorption band centered around 3300 cm^{-1}, which is a distinctive sign of axial stretching of hydroxyl groups (-OH). The existing signal in the range of 2900 cm^{-1} is related to the presence of -CH grouping in axial strain. The analysis also revealed absorptions around 1300–1800 cm^{-1}, characteristic of carbohydrates. Finally, a band between 1000 and 1100 cm^{-1} was detectable. This band is typical of the presence of β-glucans due to O-substituted glucose residues.

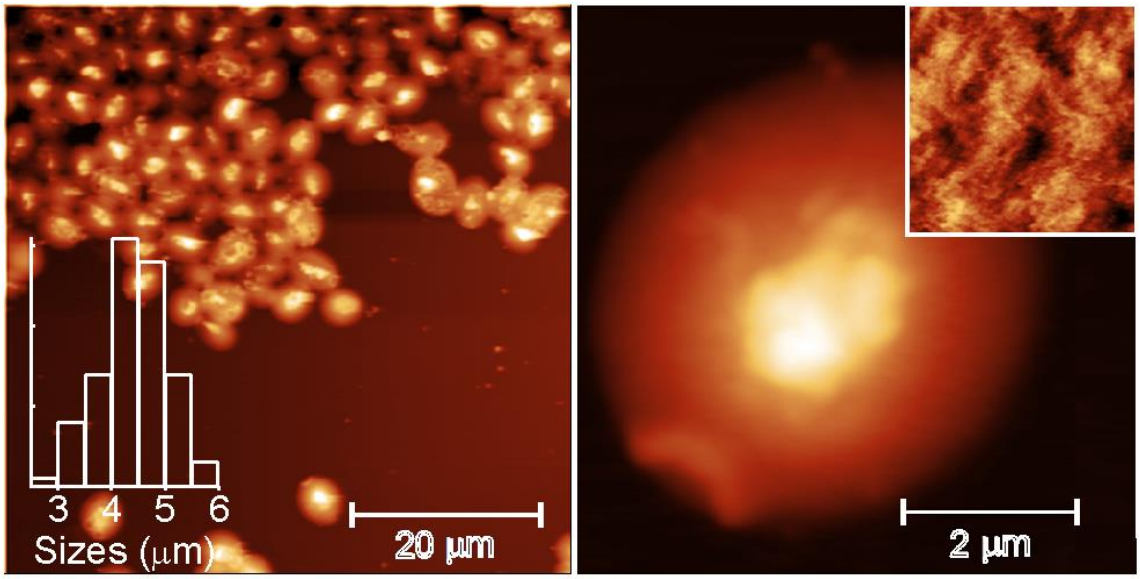

Figure 2. Left: 50x50μm AFM image (topography) of Cy5-$(C16)_2$/Rho-GPs on the mica support. Particle size distribution, as obtained by equivalent-spherical diameter model, is given in the same panel. Right: 6.5x6.5μm AFM image (topography) of a single Cy5-$(C16)_2$/Rho-GP, in the top-right inset a 400x400nm region is AFM imaged (phase signal).

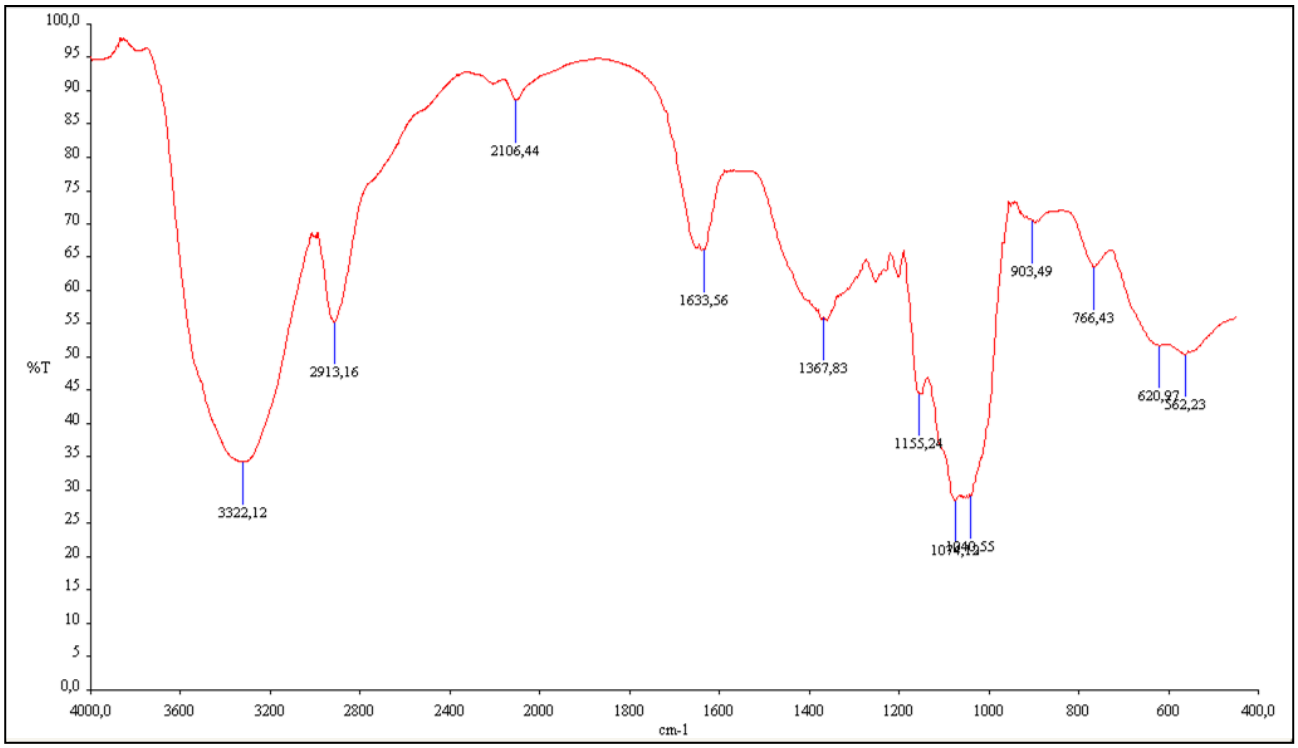

Figure 3. Infrared Spectrum of Cy5-$(C16)_2$/Rho-GPs, following particles lyophilization.

Excitation and emission spectra prove the internalization of both Rhodamine-DOPE and Cy5$(C16)_2$ in the microcarrier (Figure 4). Moreover, the profiles obtained showed the presence of interactions between the two molecules, probably due to Fluorescence Resonance Energy Transfer (FRET). The determination of the concentration of each compound thus requires a tricky procedure, in order to avoid over/under-estimation of the real content.

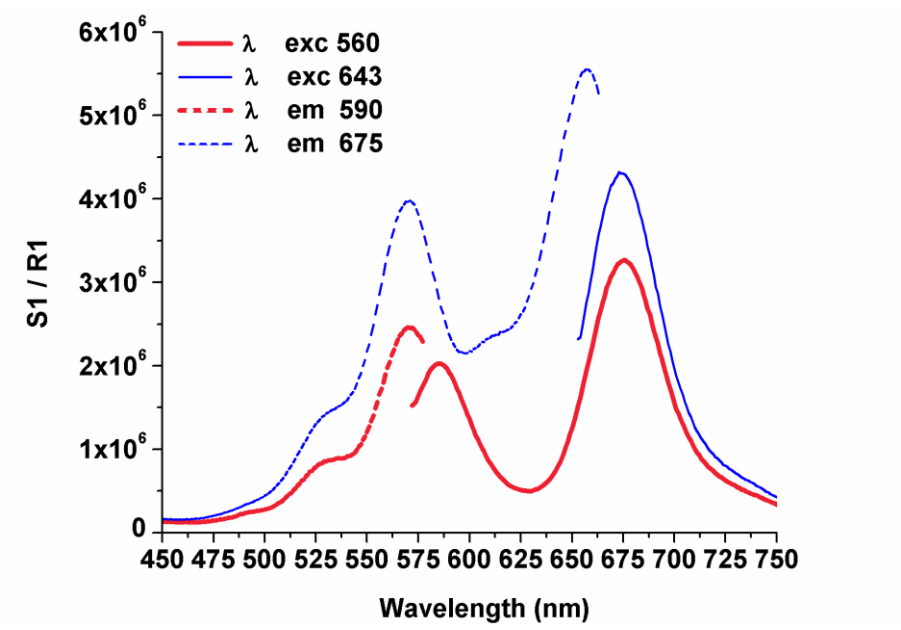

Figure 4. Excitation and Emission spectra of GPs in HEPES buffer, acquired with the spectrofluorometer.

DLE and DLC determination

To this purpose, the fluorescent content was extracted from the particles using methanol, and then diluted 1:11 in methanol to be measured. The absorbance spectrum was acquired from 360 to 750 nm and the ratio between the peak at 642 nm (corresponding to the absorbance of $Cy5(C16)_2$) and 561 nm (corresponding to the absorbance of Rho-DOPE) proved to be 1.09±0.05. Then absorbance spectra of different blends of Rho-DOPE and $Cy5(C16)_2$ standard solutions were acquired keeping fixed the concentration of rhodamine and varying the amount of cyanine, until a ratio between the peaks at 642 nm and 561 nm around 1.1 was obtained. The so-obtained standard solution was then diluted at different concentrations, maintaining constant the ratio between the two dyes, in order to prepare a calibration curve for the quantification of both Rho-DOPE and $Cy5(C16)_2$. The concentration of the two dyes in the extract was then extrapolated from the curves obtained. Rho-DOPE concentration attested at 55.1±1.4 μM, while $Cy5(C16)_2$ entrapped was around 46.1±1.7 μM. DLC, calculated according to the equation (1), was around 0.07% for Rho-DOPE and 0.06% for $Cy5(C16)_2$, while DLE attested at 72±2 % and 62±2 %, for the Rhodamine and Cyanine containing dye, respectively. Results obtained prove good yield of internalization.

Analysis of Cellular Uptake

Confocal microscopy

Results obtained by confocal microscopy demonstrated the fast and efficient uptake of fluorescent GPs by monocytes/macrophages (Fig.5). A strong signal was detectable when the sample was excited at 561 nm (Figure 5b, red), while as expected, signal coming from cyanine could be just slightly appreciated (Fig. 5c, green) due to its spectral properties.

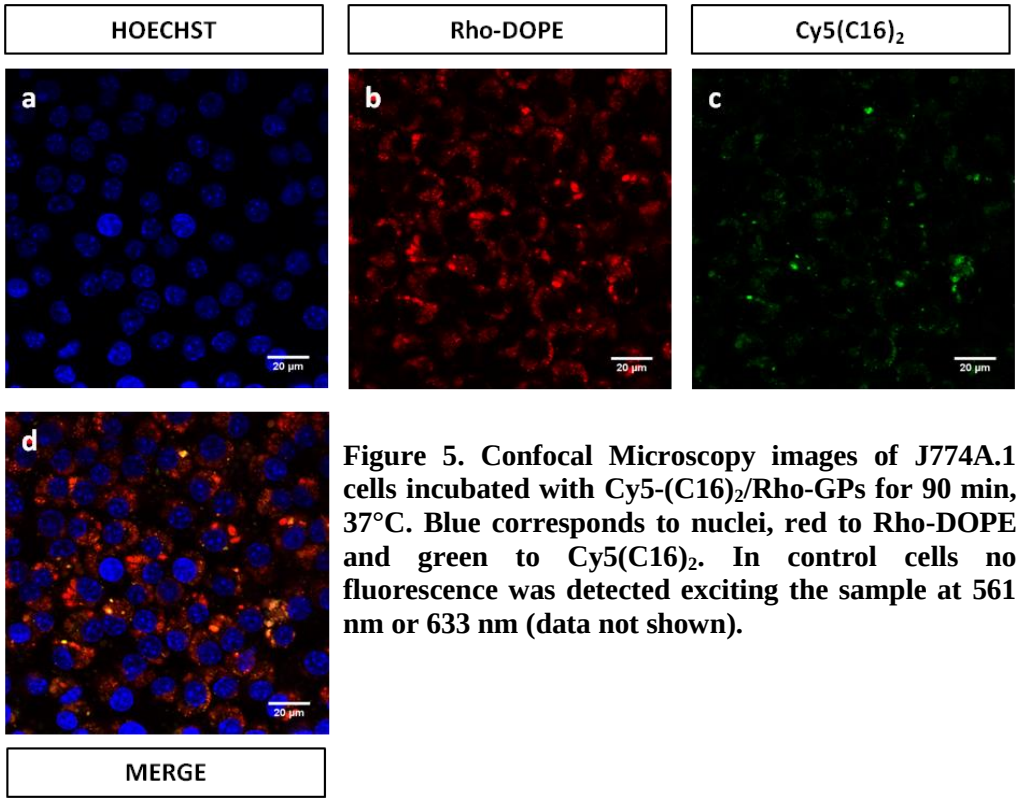

Figure 5. Confocal Microscopy images of J774A.1 cells incubated with Cy5-$(C16)_2$/Rho-GPs for 90 min, 37°C. Blue corresponds to nuclei, red to Rho-DOPE and green to $Cy5(C16)_2$. In control cells no fluorescence was detected exciting the sample at 561 nm or 633 nm (data not shown).

FACS analysis

Uptake of fluorescent GPs in monocytes/macrophages was also determined by flow cytometry, carried out by incubating cells (90 min) with Cy5-$(C16)_2$/Rho-GPs. Cells incubated with either DMEM only (Fig. 6a) or empty GPs (Fig. 6b) were used as control. From fluorescence-activated cell sorting (FACS) analysis (Figure 6) it was possible to evaluate that more than 95 % of J774A.1 cells were labeled with both Rho-DOPE and Cy5$(C16)_2$ (Fig 6c, 6d).

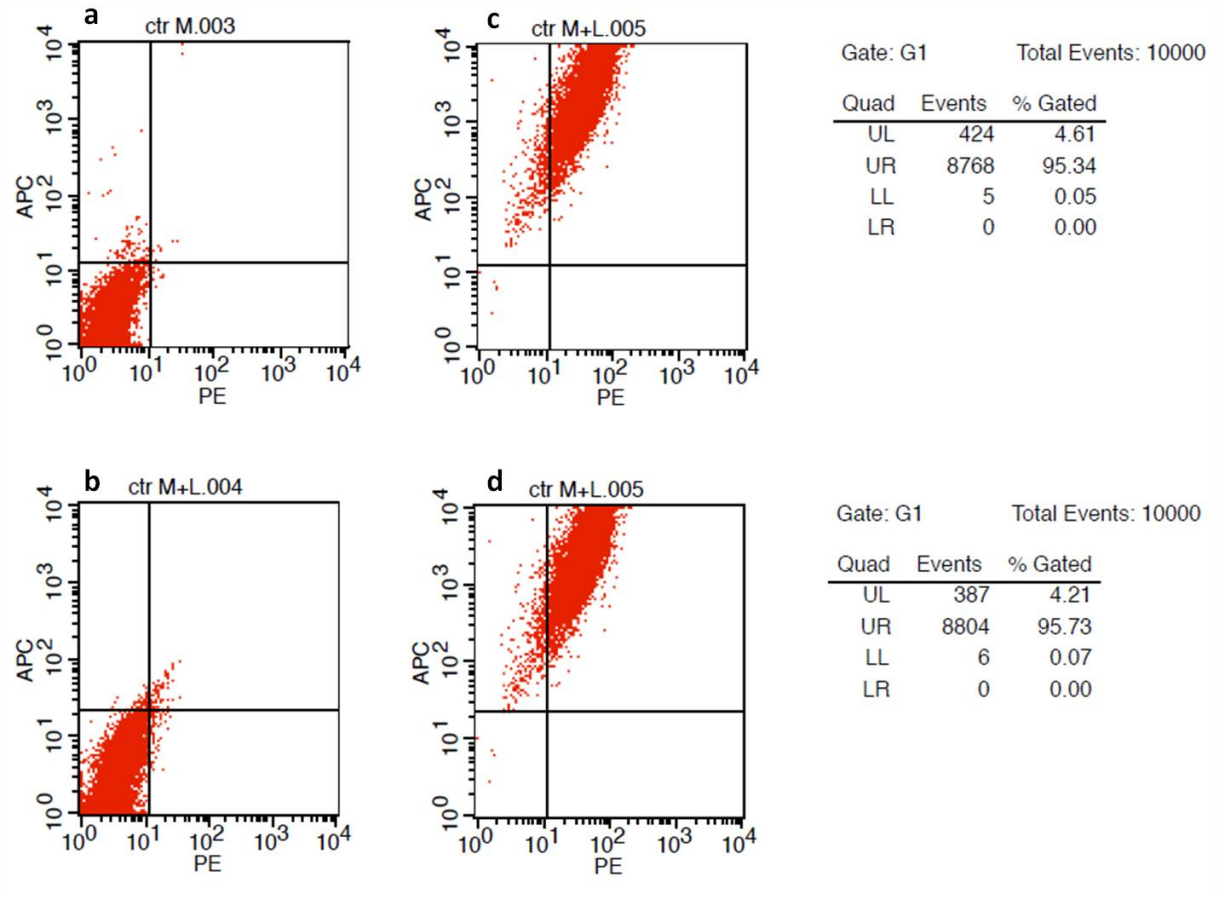

Figure 6. FACS analysis of J774A.1 cells incubated with fluorescent GPs. Data were analyzed using the CELLQUEST PRO program. In the panel (a) analysis of cells incubated with DMEM only is reported, in (b), analysis of cells incubated with empty GPs is represented. In panel (c) and (d) analysis of cells incubated with Cy5-$(C16)_2$/Rho-GPs normalized with respect to (a) cells incubated with DMEM only or (b) cells incubated with empty GPs, respectively, is reported.

Reference

1. A.A. Garcia, P Oden, U. Knipping, G. Ostroff, R. Druyor, Characterization of a β-Glucan Particle Using the Scanning Tunneling and Atomic Force Microscopes (pp. 131-144, Ch 4), In: Synthetic Microstructures in Biological Research, 1992, J.M. Schnur and M. Peckerar Eds., Springer, New York.