Alkyne-tagged PLGA allows direct visualisation of nanoparticles *in vitro* and *ex vivo* by stimulated Raman scattering microscopy

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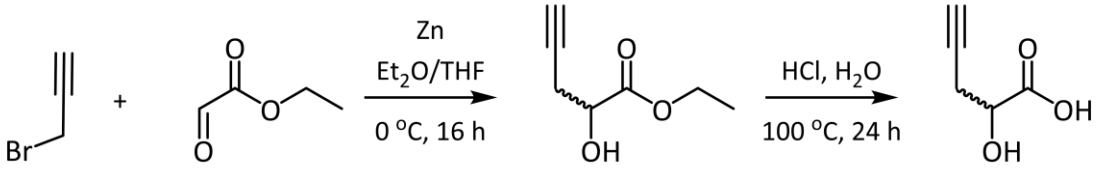
Supporting information

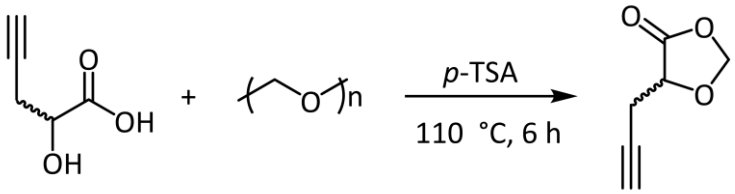
Materials and instruments: All chemicals were purchased from commercial vendors and used as received unless otherwise stated. Toluene, ether and THF were dried using an Innovative Technologies purification system consisting of aluminium and copper catalyst. All air-sensitive manipulations were performed in an MBraun LABmaster sp glovebox equipped with a -35°C freezer, [O_2] and [H_2O] were <0.1ppm according to built-in analysers or on a dual manifold Schlenk line using standard Schlenk techniques. ^{1}H and ^{13}C NMR spectra were obtained on Brucker instruments at the stated frequency using TMS as a reference and residual solvent as an internal standard. Gel permeation chromatography was carried out in THF at a flow rate of 1 mL min^{-1} at 35°C on a Malvern Instruments Viscotek 270 GPC Max triple detection system with 2×mixed bed styrene/DVB columns (300 × 7.5 mm). Data was processed using OmniSEC 5.0.

Synthesis of propargyl DOX: To obtain the propargyl α-hydroxy acid, propargyl bromide was reacted with ethyl glyoxalate in the presence of Zn in a Barbier reaction (Scheme S.1). This synthesis has been previously reported.[1,2] The ethyl ester was then hydrolysed to the carboxylic acid by acid hydrolysis. In all instances spectroscopic data matched that previously published.



Scheme S.1: Propargyl bromide was reacted with ethyl glyoxalate in the presence of catalytic Zn in ether/THF. The ethyl ester was then hydrolysed to the acid in HCl/H_2O.

The propargyl α-hydroxy acid (1 eq), paraformaldehyde (1.6 eq) and *p*-toluene sulfonic acid (0.1 eq) were dissolved in benzene and refluxed in a Dean-Stark apparatus to remove water, in a modification of our previously reported procedure,[3] to give the desired product in 66% yield (Scheme S.2). The ^{1}H NMR spectrum of the propagyl-DOX is shown in Figure S.1.



Scheme S.2: Propargyl α-hydroxy acid, paraformaldehyde and *p*-toluene sulfonic acid were refluxed in benzene for 6 hours removing water continually with a Dean-Stark apparatus (66 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.65 (1H, s, CH_AH$_B$), 5.49 (1H, s, CH$_A$$H_B$), 4.38 (1H, m, C*H*), 2.85-2.68 (2H, m, CH_2-C≡C), 2.09 (1H, t, *J* = 2.7 Hz, C≡C-*H*).

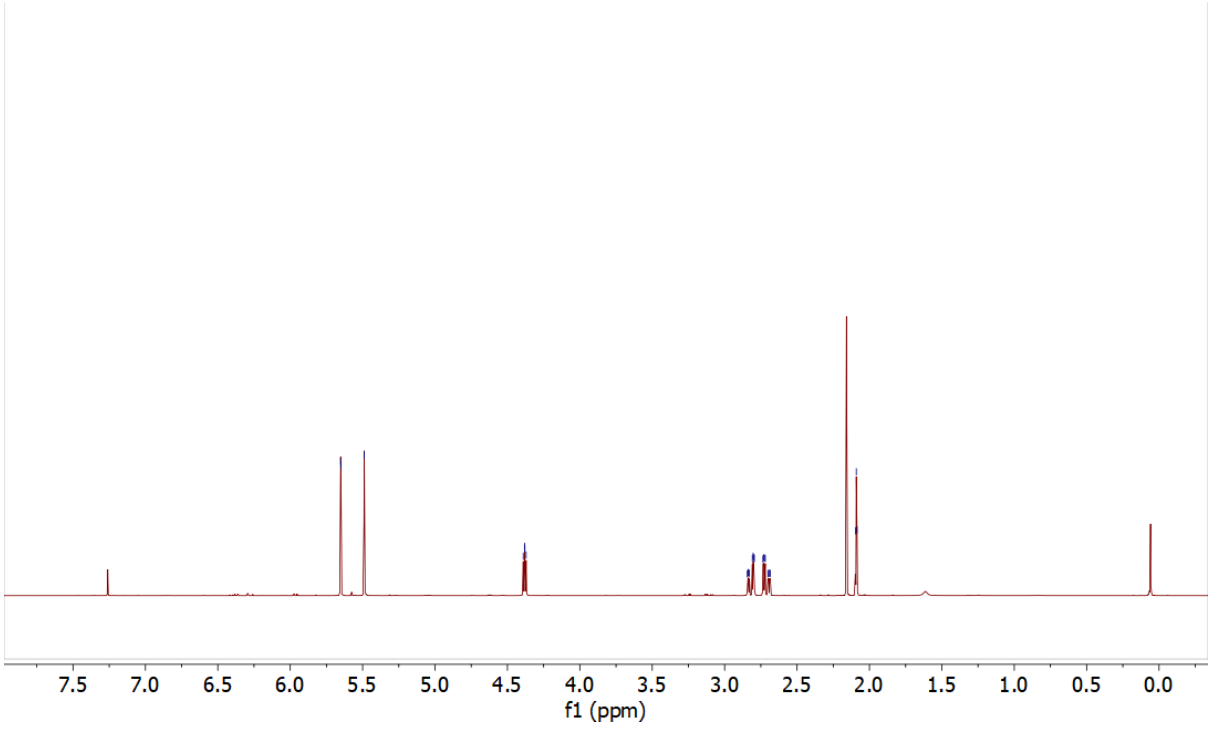


Figure S1. ^{1}H NMR of propargyl-DOX

Synthesis of PLGA-alkyne: Propargyl-DOX was reacted with pre-formed PLGA in a transesterification reaction. The ^{1}H NMR of PLGA-alkyne is shown in Figure S.2. To confirm that incorporation of the propargyl units into the PLGA had been successful, DOSY NMR was used (Figure S.2). The single cross peak in the DOSY NMR shows that the propargyl-DOX has been incorporated into the existing PLGA chains.

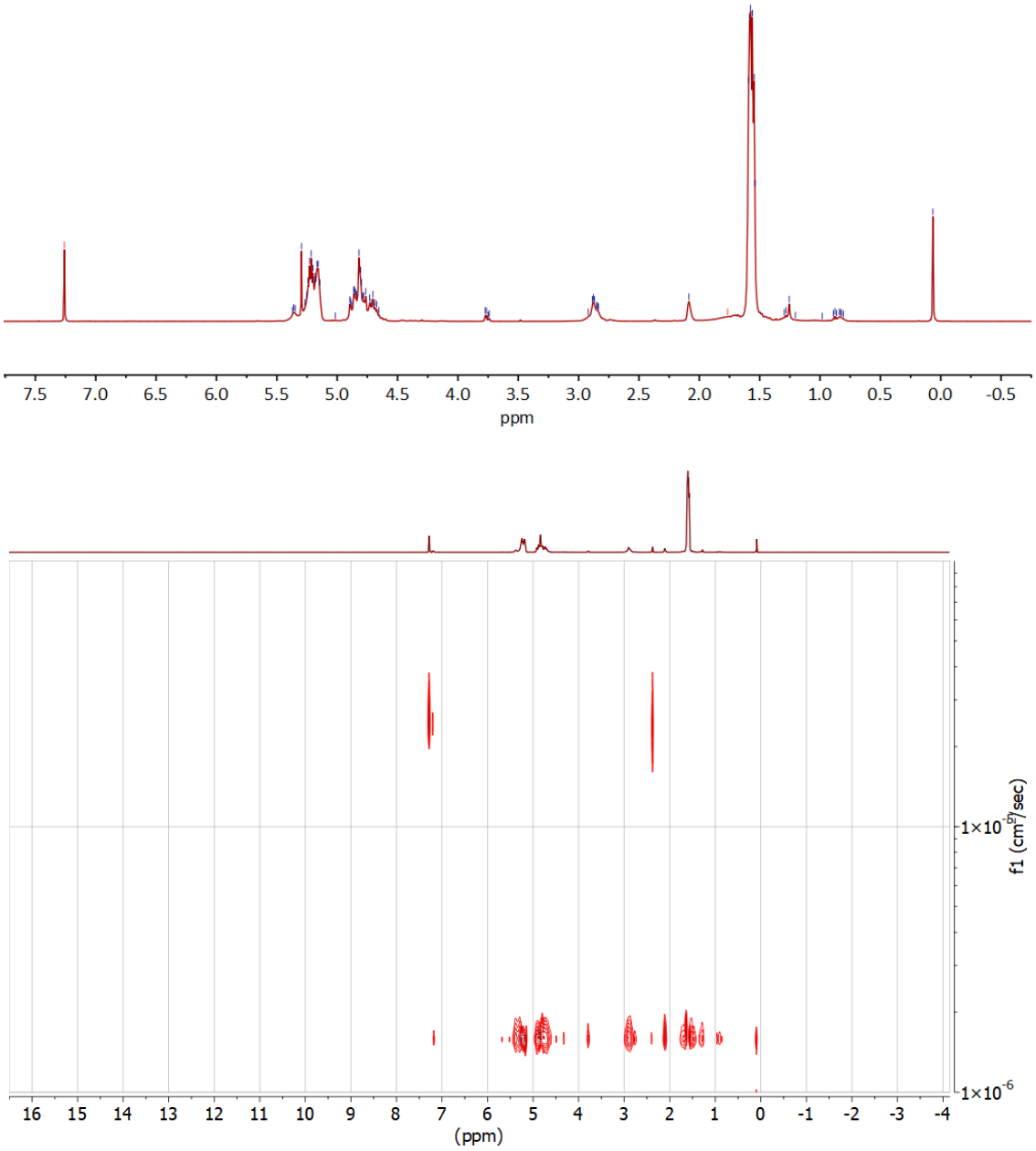


Figure S2. DOSY NMR analysis of PLGA-alkyne gives a single cross peak showing that the alkyne units were successfully incorporated into the polymer.

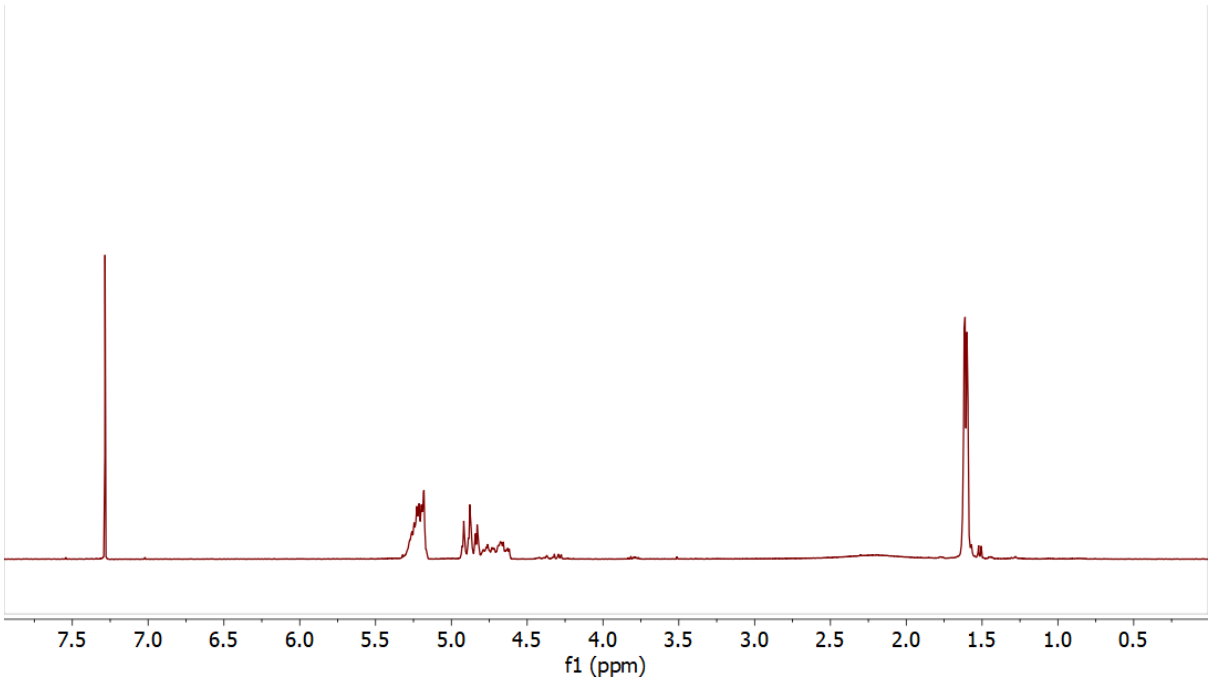


Figure S3. ^{1}H NMR spectrum of PLGA-D.

Aqueous Stability of the NPs: PLGA-D and PLGA-alkyne NPs were synthesized using the emulsification-evaporation method and after filtration through a 0.45 µm filter, their particle size was measured by DLS in water at 25°C. The NPs were then left at room temperature and at 2, 6 and 10 days after synthesis, the particle size was measured by DLS to quantify the particle aggregation over time. This showed that the PLGA-D NPs aggregated, but that the PLGA-alkyne NPs were stable over this timescale.

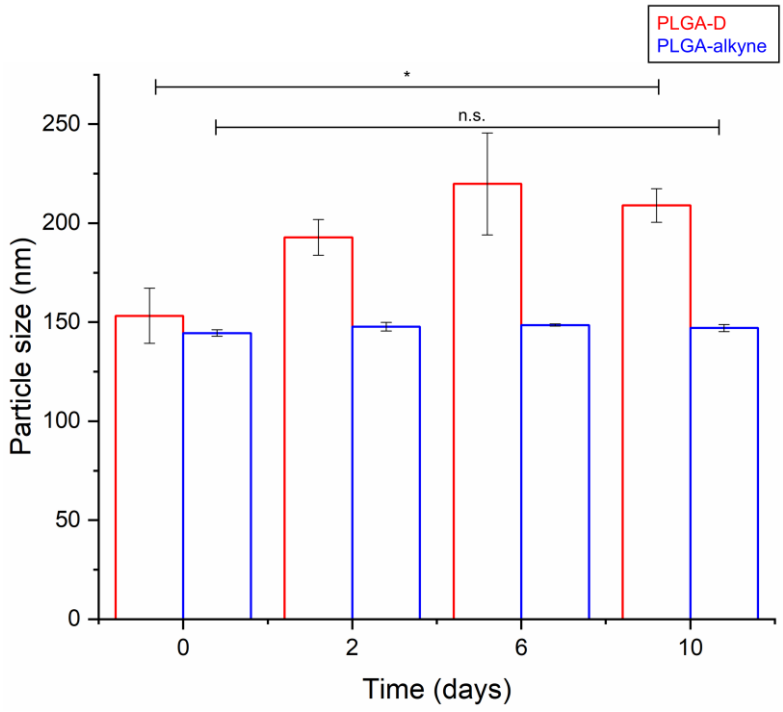


Figure S4: The aqueous stability of PLGA-D and PLGA-alkyne NPs was quantified over time by measuring their particle size in triplicate with DLS. Error bars show standard deviation, *: $p < 0.05$, n.s.: $p > 0.05$, one-way ANOVA.

Cell viability assay: Microglia were plated at 5×10^4 cells per well in a 96 well plate and left overnight to adhere. PLGA, PLGA-D and PLGA-alkyne NPs were then added at 1, 2 and 4×10^9 particles mL^{-1} and incubated for 24 hours. Each condition was carried out in triplicate. Viability was then assessed using the CellTiter-Glo ® assay. 100 µL of luciferase was added to each well, the plate was shaken for 2 minutes before a 10 minute incubation at room temperature. The amount of luminescence was then assessed with a plate reader (Promega), with higher luminescence associated with more ATP and therefore more living cells. Three biological replicates of the assay were carried out. Figure S4 shows that all of the NPs were non-toxic at all concentrations tested.

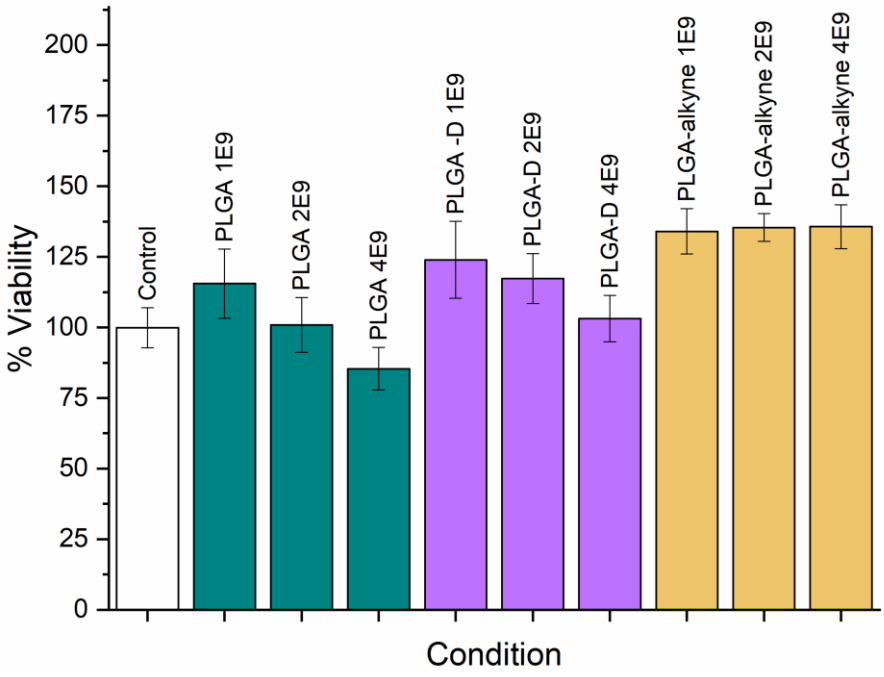


Figure S5. Cell viability assay of microglia incubated with PLGA, PLGA-D and PLGA-alkyne at 1, 2 and 4×10^9 particles mL^{-1}. Each condition was assessed in triplicate and three biological replicates of the assay were carried out, error bars show standard error of the mean.

Z stack analysis of alkyne NPs inside microglia: Z-stack images were aquired from the SRS microscope to assess the 3D location of the alkyne NPs within microglia.

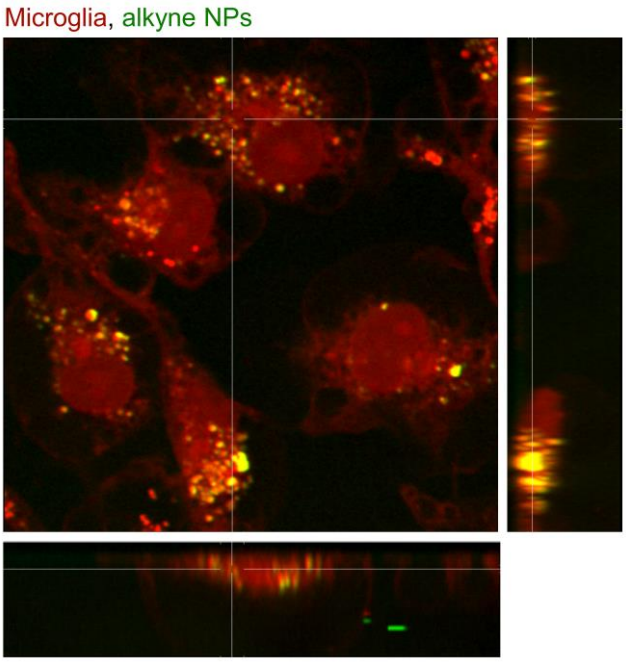


Figure S6. Orthogonal views of microglia (red) and alkyne NPs (green) shows internalization of the NPs inside the cell. Z stacks were acquired at the protein (2939 cm^{-1}) and alkyne stretches (2128 cm^{-1}) and then visualized using Imaris image analysis software.

Time dependent analysis: Microglia were cultured with PLGA-alkyne NPs at 2×10^9 particles mL^{-1} and at 0, 12 and 24 hours after NP addition, the cells were washed, fixed and imaged with SRS. Six fields of view were imaged for each time point over two biological replicates. The alkyne intensity for each cell in these fields of view was quantified using Image J. There were differences in the intensity of the alkyne between the two biological replicates, which could have been due to differences in the batches of primary cells, or day-to-day changes in the microscope. As such, the alkyne intensities for each biological replicate were normalised between zero and one; these values were then pooled for each time point and are plotted in Figure S7B. This shows a significant increase in alkyne intensity over time.

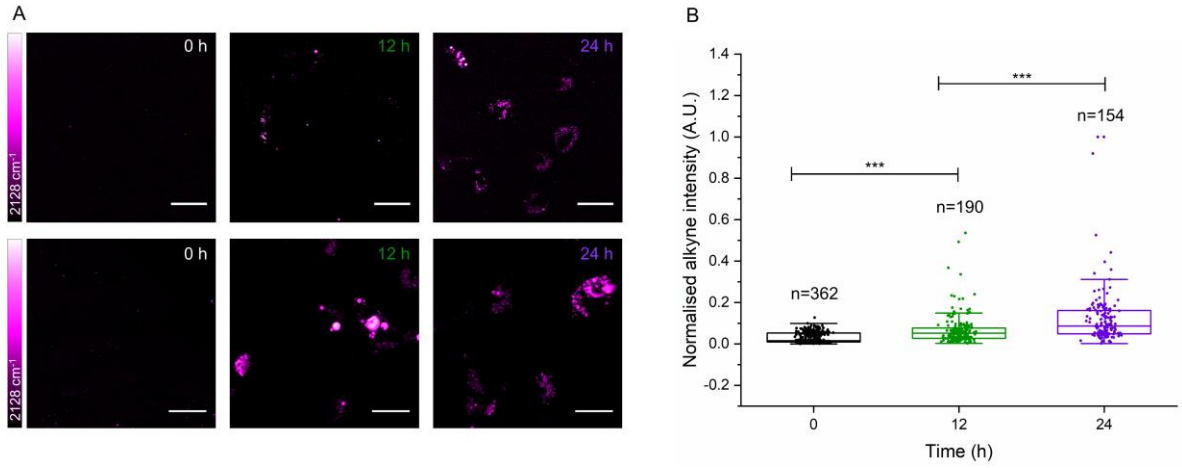


Figure S7: Time dependent analysis of PLGA-alkyne NPs in microglia. A) Representative images of microglia at 0, 12 and 24 hours from two biological replicates. Scale bars = 20 µm. B) Box plot showing the mean alkyne intensity per cell over time. Each dot represents one cell and n gives the total number of cells. ***: $p < 0.001$, Mann-Whitney U test.

Encapsulation experiment: PLGA-alkyne NPs were loaded with rhodamine by adding 2 mg of rhodamine 6G to the DCM phase in the standard emulsification-evaporation method. The NPs were then added to microglia at 2×10^9 particles mL^{-1} for 24 hours, before washing and fixing the cells. A multi-modal imaging approach was then used to visualize the NPs by SRS and two-photon fluorescence. The alkyne signal of the NPs and the fluorescent signal of the rhodamine were found to co-localize well in microglia. This shows the potential to use these Raman active NPs for encapsulation and delivery of a payload.

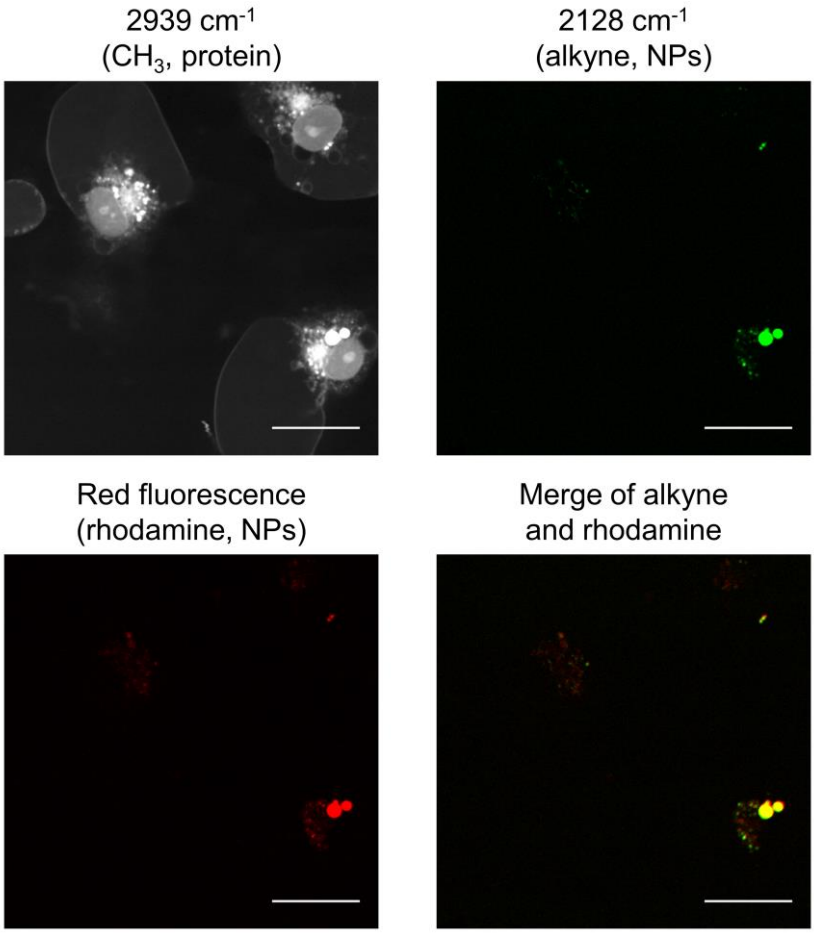


Figure S8: Encapsulation of rhodamine inside PLGA-alkyne NPs. A multi-modal imaging approach was used to visualize the microglia (protein, grey) and NPs (alkyne, green) with SRS and rhodamine (red) with two-photon fluorescence. The merged image of the alkyne and rhodamine signals (yellow) shows that they co-localize in the microglia. Scale bars = 20 µm.

References

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