Electronic Supplementary Material (ESI) for Biomaterials Science.
This journal is © The Royal Society of Chemistry 2014

SUPPLEMENTARY INFORMATION

1. Materials and Methods

1.1. *Synthesis, functionalisation and fluorescent labelling of SiHA*

Synthesis and functionalisation processes were carried out as described in [14]. Briefly, SiHA was synthesised via a wet chemical precipitation method using calcium nitrate, diammonium hydrogen phosphate and silicon tetraacetate at pH 10. Salt concentrations were set to maintain the Ca/(Si+P) ratio to approximately 1.67.

Fluorescent tagging was a two stage process first involving the modification of the SiHA surface with the (3-mercaptopropyl)trimethoxysilane (MPTS) via covalent bonding, which in turn presents a thiol functional group from the particle surface. The second process involves the attachment of the commercially available thiol reactive probe fluorescein-5-maleimide as shown in figure S1 below.

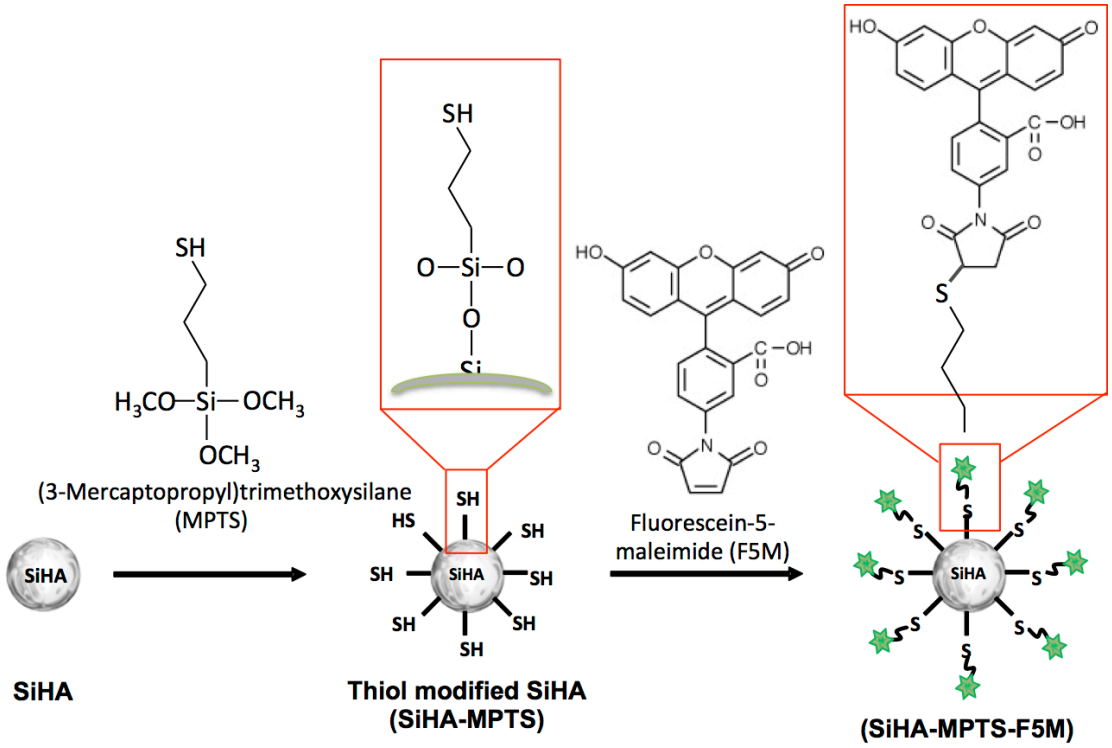

Figure S1: Schematic diagram of the thiol functionalisation process of SiHA using MTPS and the subsequent labelling with fluorescein-5-maleimide (F5M). Reproduced from [14].

Thiol functionalisation: 230mg of HA/SiHA in double distilled water was centrifuged at 4000rpm for 10mins and the supernatant removed. The particles were then resuspended in 16.4mL of absolute ethanol filtered with a 0.22µm pore size syringe filter before adding 100µL of MPTS. The samples were mixed using a ThermoMixer (ThermoMixer Comfort, Eppendorf AG., Hamburg, Germany) at 37°C for 3 hours at 1000rpm and then washed five times with double distilled water by centrifuging at 4000rpm for 10 minutes.

Fluorescent tagging: A stock solution of 5.2mg Fluorescein-5-Maleimide (F-150, Life Technologies Ltd., Paisley, UK) in 1mL $MgCl_2$ and $CaCl_2$-free PBS (Sigma-Aldrich Ltd., Dorset, UK.). 1mL of MPTS functionalised SiHA/HA (SIHA/HA-MPTS) (containing approximately 6mg of solid material) was centrifuged at 4000rpm for 10 min (ThermoScientific Inc., Waltham, MA, USA). The supernatant was removed and the pellet resuspended in 500µL of $MgCl_2$ and $CaCl_2$-free PBS before adding 470µL of the fluorescein-5-maleimide stock solution and mixed on a ThermoMixer for 2 hours at 37°C. Finally, the SiHA-MPTS-F5M particles were washed twice with absolute ethanol and five times with double distilled water (both filtered beforehand using a 0.22µm pore size filter).

1.2. *PAGE*

Polyacrylamide gel electrophoresis was performed using the OmniPAGE-mini electrophoresis system (Cleaver Scientific Ltd., Rugby, UK) Gel thickness was 1 mm and gels were cast in two layers: a stacking layer applied to concentrate the sample on a band and a running layer to resolve any possible multiple bands. The resolving layer was prepared by mixing 10mL of a 2x stock buffer solution (0.75 M Tris/HCl pH 8.3) with 10 mL of 30% Acrylamide/Bis solution 37.5:1 (Bio Rad, 161-0158) and polymerized by the addition of 200 mL of ammonium persulphate solution (A-3679, Sigma-Aldrich Ltd., Dorset, UK.) 80mg/mL in DI water and 10 mL of TEMED (T-9281, Sigma-Aldrich Ltd., Dorset, UK). The casting system was filled reserving a distance of approximately 1 cm from the bottom of the comb for the stacking layer. Once the resolving layer was set (approximately one hour), the stacking layer was prepared by mixing 1.5 mL of a 10x stock buffer solution (1.25 M Tris/HCl pH 6.8) with 10.5 mL of deionized water and 3 mL of acrylamide solution. 150 mL of the ammonium persulphate solution and 7.5 mL of TEMED were added for polymerization which lasted a minimum period of four hours. The electrode working buffer was prepared with deionized water from a 10x stock consisting of Glycine (G9888, Sigma-Aldrich Ltd., Dorset, UK) 150 g/L (or 2 M) and Tris 30 g/l (or 0.25 M). The gel tank was filled accordingly with manufacturer specifications. Samples consisting of SiHA-F5M (particles exposed to dye without thiol surface modification, SiHA-MPTS-F5M (thiol modified particles with dye attached) and F5M control (dye alone) were prepared 1:1 (v/v) with a glycerol/working electrode buffer (1:10 v/v) with 5µL of the final sample mix added to separate lanes cast within the gel. PAGE was run at a constant voltage of 120 V for 45 minutes. Images of the gels were acquired with a PhotoDoc-it™ Imaging System using a Visi-Blue™ transilluminator (Ultra-

violet Products Ltd., Cambridge, UK) with an excitation wavelength of 460/470 nm in the low setting and through a SYBR gold filter.

1.3. *Live-cell confocal fluorescence imaging*

1.3.1. Preparation of MC3T3 cells for live cell imaging

MC3T3 (passage 10) cells were seeded at a density of $3x10^4$ cells per quadrant in a 4-segmented live cell imaging dish (code: 627870, Greiner-Bio One Ltd., Gloucester, UK) and incubated overnight at 37°C and 5% in the supplemented culture media. Fluorescently tagged SiHA in supplemented media was prepared to concentration of 300µg/mL, mixed using a vortex mixer and sonicated for 5 minutes before being kept in a water bath at 37°C until required. After 24 hours, the media was removed and replaced with 1mL of supplemented media containing tagged SiHA at the desired final concentration while gently agitating the dish to ensure even distribution of the particles across the surface. The media was removed 24 hours after exposure to the particles, the cells in each quadrant washed three times with 1mL PBS and replaced with 1mL per quadrant of cell imaging media (A14291DJ, Life Technologies Ltd., Paisley, UK).

Lysosome labelling of the cells was carried out during the last 30 minutes of the 24 hour exposure to the labelled particles. A 1mM stock of LysoTracker Red® (L-7528, Life Technologies Ltd., Paisley, UK) was diluted in supplemented culture media to a working concentration of 75nM. 500µL of the working concentration dye solution was added to each quadrant of the imaging dish containing cells and incubated for 30 minutes at 37°C and 5% The cells were then washed prior to imaging as described above.

1.3.2. Confocal Microscopy

The confocal microscope system consisted of a Zeiss Axio Observer.Z1 inverted microscope attached to a Zeiss LSM 710 ConfoCor 3 confocal unit containing a 34-channel spectral detector with a Zeiss EC Plan-Neofluar x63 (NA = 1.40) oil objective lens. Two separate detection channels were created to detect the fluorescence from the Fluorescein-5-maleimide and LysoTracker Red® and employed the 488nm and 563nm laser lines respectively. These laser lines were found to create negligible cross-excitation of the fluorophores. Images for each detection channel were acquired sequentially i.e switching between 488nm and 563nm laser lines and corresponding wavelength detection windows after frame-by-frame.

Images were acquired at 1024x1024 pixels 12-bit depth with a 3µs pixel dwell time and 0.1% laser power to minimise photo-induced damage to the cells. Detector gains were altered within the Zeiss ZEN software and care was taken to avoid saturation of the

image in regions of interest such as the cells under investigation.

1.4. *Computational image analysis*

1.4.1. *Identification and size estimation of internalised structures*

Identification of structures within the cell was performed using an algorithm as summarized in the following workflow (and schematically shown in Figure S2):

1. The user selects an unlimited series of points around the perimeter of the cell(s) on an interactive bright field image, which leads to the creation of a polygon approximating the perimeter of the cell(s).

2. The thresholded fluorescence image corresponding to the brightfield image selected in (1) was selected. This image was in binary format such that all pixels with a value of one represented objects of interest to the user and pixel values of zero were classed as background and ignored. Objects in the image were identified by the program by grouping all pixels connected in at least one of eight possible geometries in two dimensions (x,y) - four geometries representing the four unique arrangements that two pixels can be in contact along their edges and a further four geometries representing contact of the corner of one pixel to the corner of another pixel. This approach is commonly termed as the '8-connected neighbourhood'. The program numbered each detected object and recorded the co-ordinates of the pixels the forming those objects.

3. Objects located outside of the cell(s) perimeter (defined in step 1 by the polygon) were removed by employing a logical AND operation between the polygon image and the image containing all detected objects. The resulting binary image contained the objects deemed to be located fully and partially within the cell perimeter.

4. Objects located partially within the cell(s) were removed by another logical AND operation with the inverse of the polygon image (which therefore highlights all points outside of the cell). The pixels remaining in the image were matched to the database of detected objects and removed.

5. The area of each object (in pixels squared) was calculated by summing the number of pixels assigned to each numbered object and then fitting an ellipse to the shape in order to determine major and minor axis lengths. In addition, the diameter of a circle with equivalent area to the object was computed. The estimated lengths were converted from pixel units to microns by extracting the pixel-micron scale factor from the original .lsm microscope data file.

6. A histogram of equivalent diameters of internalised objects was computed by binning the calculated diameters in 0.1µm wide bins with the total number of bins determined automatically by the program by calculating the upper range limit of sizes.

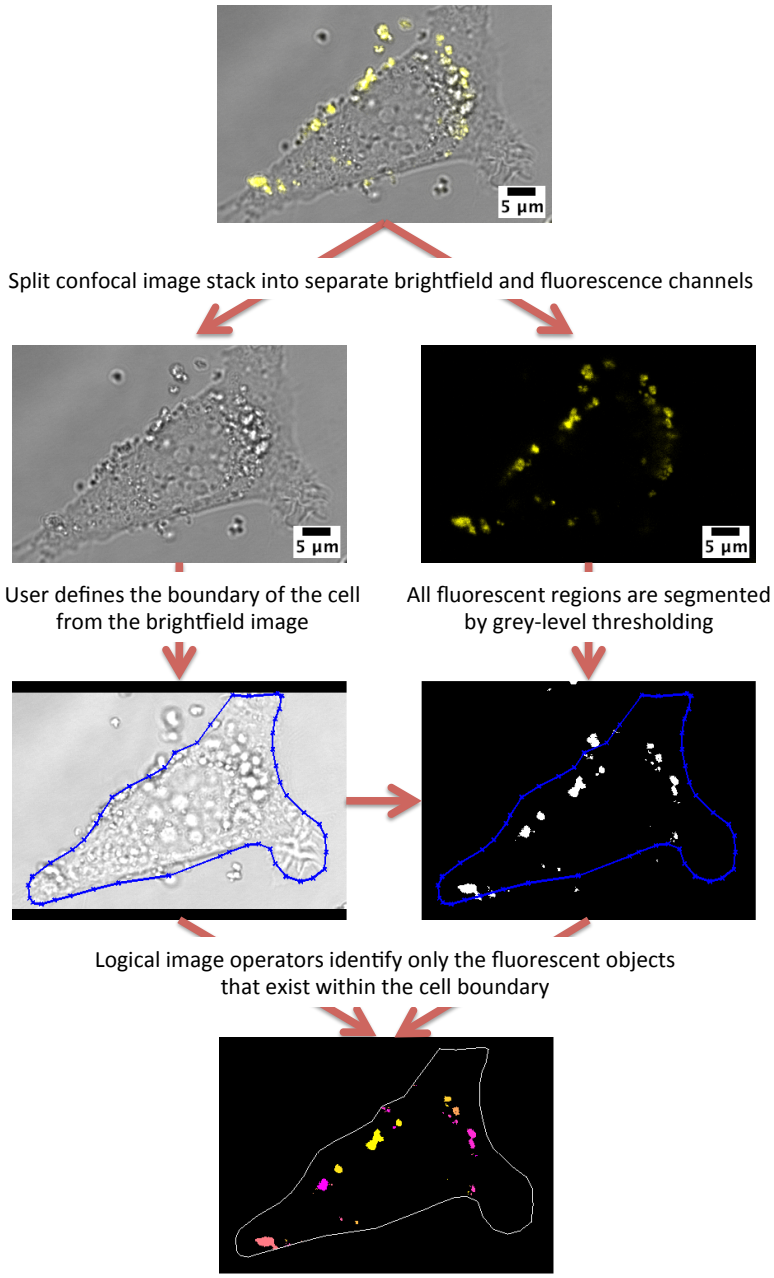

Figure S2: Workflow diagram of the image processing steps 1-4 showing an image of the result after each step with sub captions where appropriate. Once program identified each of the coloured objects in the last image, their size and area (in pixel units) were estimated using shape fitting and converted to µm

using the calibration factor in the original Zeiss data file.

1.4.2. *Estimation of internalised structure volume*

The volume of each slice through an image object was calculated by multiplying the area obtained using the algorithm outlined in section 1.3.1 by the slice thickness (which can be estimated from the user-defined confocal pinhole diameter recorded in the original Zeiss data file).

The total estimated volume of each object was then computed by multiplying the volume of each slice through the object by the inter-slice spacing set during the image acquisition process as shown in figure S3. This method of mass estimation was based on the following assumptions: i) that all detected objects in every two-dimensional image slice extended perpendicularly to the image plane by the same length equal to the inter-slice spacing and, ii) that all objects were solid and consisted only of the CaP material, which was homogeneously distributed throughout the volume of the objects. The perhaps more realistic situation of the internalised material being loosely compacted into discrete masses with air/fluid acting as filler rather than solid masses was considered, however these 'filler' regions could not be resolved at high magnification. This suggested that filler regions existed, but the average volume they occupied was insignificant in comparison to the overall size of the internalised object and therefore the total mass could be roughly considered to be evenly distributed.

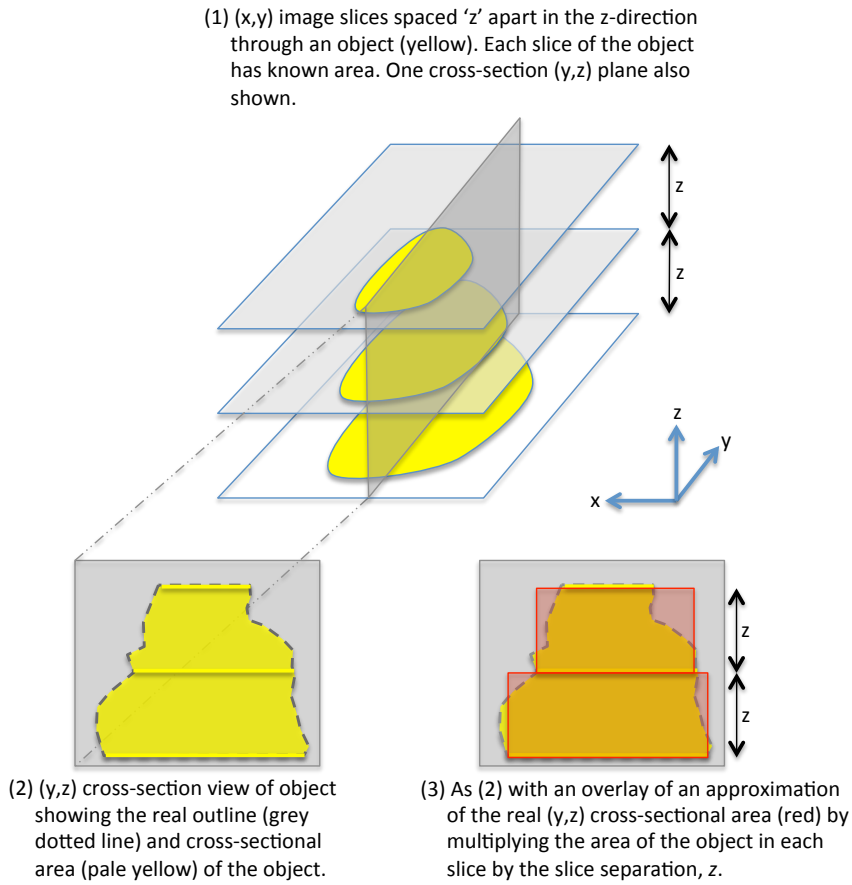

Figure S3: Schematic diagram illustrating how the volume of an object in a confocal microscopy image z-stack can be estimated. For clarity, this illustrated example uses a 2D cross-section (y,z) through the object. The total volume of the object can be reconstructed by multiplying the volume of each slice by the known slice spacing, *z*.

2. Supplementary Results

2.1. 1D PAGE analysis of free dye component of dye labeled thiol functionalised and non-functionalised SiHA

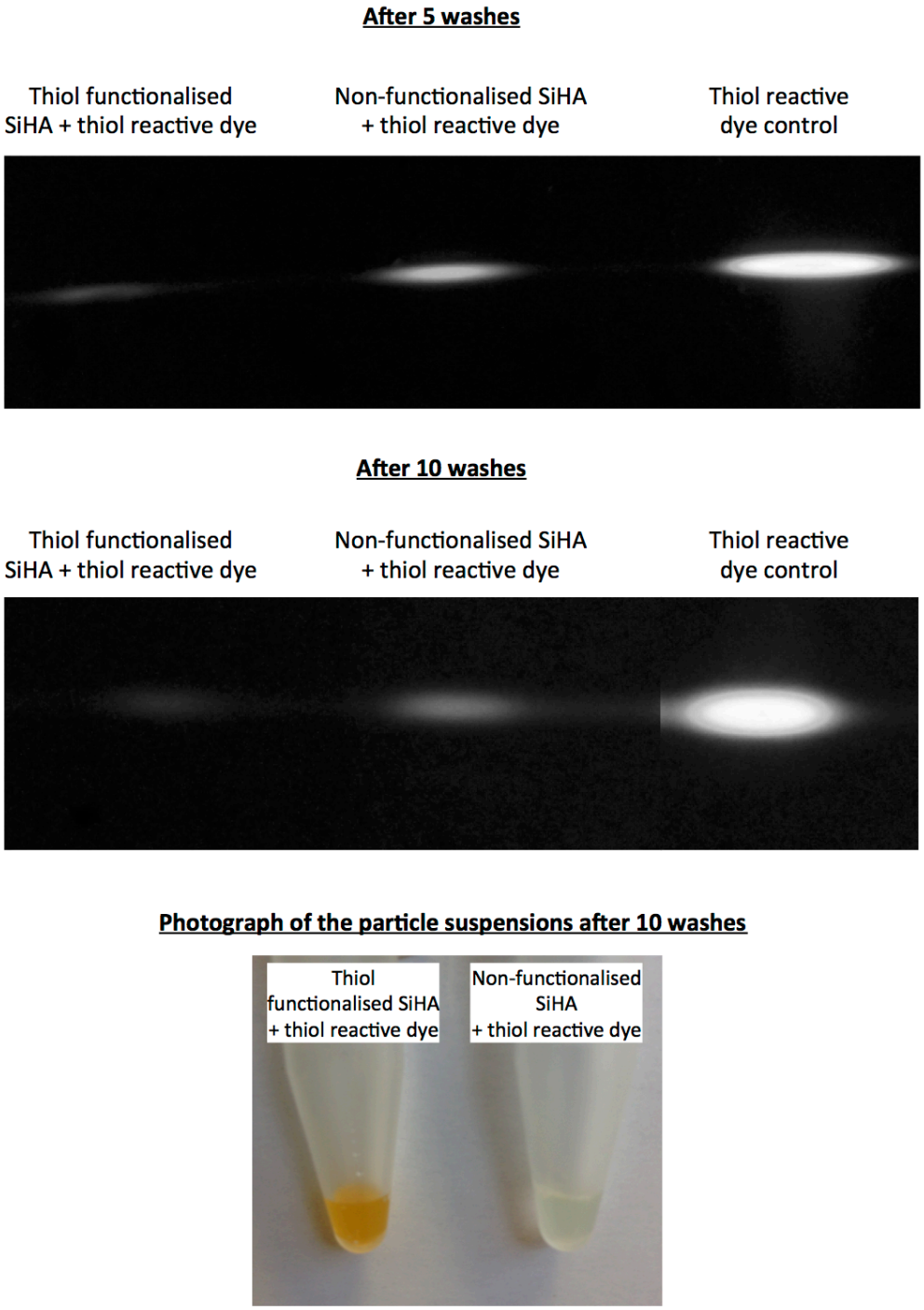

Figure S4: PAGE analysis of unbound dye during after the washing stage of the formulation process. Unbound dye from Thiol-functionalised/non-functionalised samples all subjected to the dye attachment

process would run in the gel and form a band inline with the free dye control, while dye attached to the thiol groups of the particle surface was expected to remain immobilized in the sample wells of the gel. After 5 washes (top row of images), a band was clearly identified for the non-functionalised sample, which indicated that presence of free dye in the sample suspension. A smaller band of much weaker intensity was detected in the thiol functionalised sample, which qualitatively suggests significantly less free dye was present in the sample compared to the non-functionalised particles. Unbound dye was still detected in the non-functionalised sample after 10 washes (second row of images), while a barely visible streak was observed in the thiol functionalised sample, indicating no further loss of dye from this sample. A photograph of the thiol functionalised and non-functionalised dye labeled samples after washing 10 times is shown at the bottom of the figure. The thiol functionalised sample clearly displays a yellow coloured appearance due to the presence of the dye, which is was not observed in the non-functionalised sample. The latter case, the dye has been removed from the particle surface leading to the detection of the intense bands in PAGE images. We can therefore conclude that the vast majority of the dye molecules are indeed bound to the thiol functionalised particles with negligible free dye in the final product.

3. Demonstration of SiHA-Lysosome colocalisation

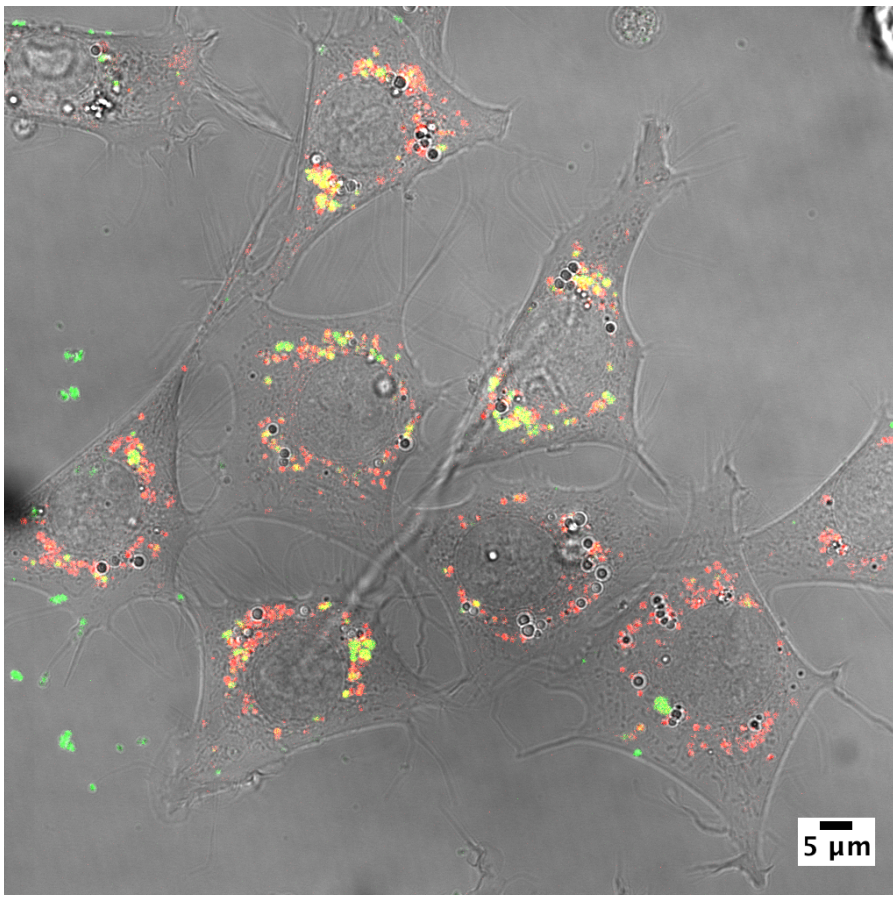

Figure S5: Combined bright field and confocal fluorescence image of a population of MC3T3 cells 24 hours after exposure to SiHA-MPTS-F5M particles (green) with the lysosomes labelled with LysoTracker Red® (red). Regions of colocalisation of the particles (or aggregates of) with the lysosomes is shown in yellow. The image further demonstrated that the labelling mechanism remains intact after 24 hours within the cellular environment after endocytosis and, up to the time point of this image, remained active within the acidic environment of the lysosome.

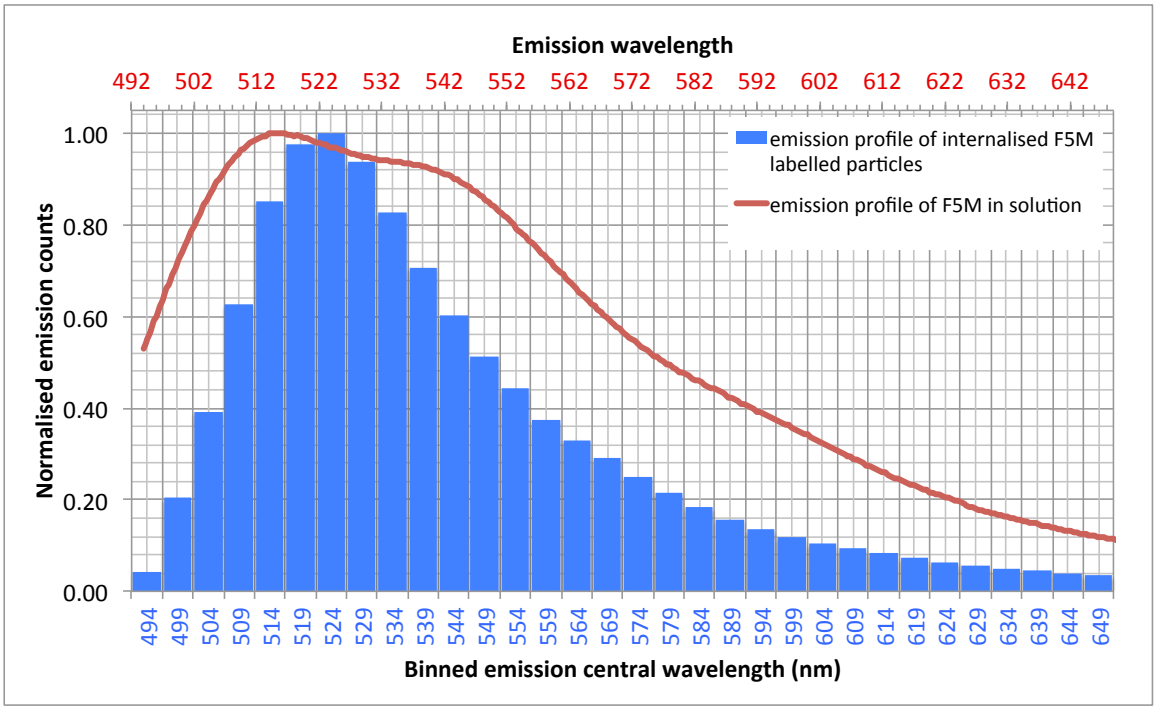

Figure S6: Fluorescence histogram of the fluorescence from the labeled particles during live cell imaging compared to the fluorescence profile of free fluorescein in solution as measured by UV-vis spectrophotometry. Intensity of both samples was normalised to their respective maximum intensity values. Both profiles appeared to have the same shape over the entire wavelength range and the emission maxima differed by only around 10nm, which may be due to differences in the pH of the environment in both samples.