Engineering of lipid-coated PLGA nanoparticles with a tunable payload of diagnostically active nanocrystals for medical imaging

Aneta J. Mieszawska,[a] Anita Gianella,[a] David P. Cormode,[a] Yiming Zhao,[b] Andries Meijerink,[b] Robert Langer,[c] Omid C. Farokhzad,[d] Zahi A. Fayad,[a] and Willem J. M. Mulder,[a,b]*

* Corresponding author. Willem Mulder, e-mail: willem.mulder@mssm.edu; tel: +1 212 241 7717; fax: +1 212 534 2683.
[a]Translational and Molecular Imaging Institute and Imaging Science Laboratories, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029
[b]Condensed Matter and Interfaces, Utrecht University, The Netherlands
[c]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA
[d]Laboratory of Nanomedicine and Biomaterials, Department of Anesthesiology, Brigham & Women's Hospital, Boston, MA, USA

Materials.

1-dodecanethiol, 11-mercaptoundecanoic acid, 11-mercapto-1-undecanol, 4-dimethylaminopyridine, N,N'-dicyclohexylurea, Poly(D,L-lactide-co-glycolide) lactide:glycolide (50:50), mol wt 30,000-60,000, chloroauric acid, sodium borohydride, methyl trioctylammonium chloride, and cis-9-octadecene-1-thiol were purchased from Sigma-Aldrich (St. Louis, MO). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (PEG2000-DSPE) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Soybean lecithin was purchased from MP Biomedicals (Solon, OH).

The synthesis of nanocrystals.

1-dodecanethiol coated 1-3 nm Au nanocrystals (AuNCs), were synthesized according to Brust et al.[1] Oleic acid coated quantum dots (QDs) were synthesized according to Xie et al.[2] These methods produce AuNCs and QDs capped with hydrophobic ligands that are soluble in organic solvents such as toluene and chloroform.

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AuNCs and QDs ligand exchange.

The CH_3 terminated dodecanethiol ligands of AuNCs (108 ligands per one AuNC) and oleic acid ligands of QDs were exchanged to COOH terminated mercaptoundecanoic acid (MUA) or OH terminated 11-mercapto-1-undecanol (MUD) terminated ligands by the Murray method.[3] Shortly, 200 mg of dodecanethiol capped AuNCs were dissolved in 50 ml of toluene. 100 mg (25 ml) were mixed with 140 mg MUA and 100 mg (25 ml) with 130 mg of MUD (exchange ligands were added at 5 x molar excess with respect to dodecanethiol ligand). The exchange reactions were allowed to proceed for 96 hrs with gentle stirring. After 96 hrs toluene was evaporated using round bottom flask and rotary evaporator with 70°C water bath. The AuNCs were resuspended in 100 ml of acetonitrile (ACN) via brief bath sonication and collected on a frit (Opti Chem) under bench top vacuum. The AuNCs were washed with 100 ml of ACN, dried, and collected. AuNCs-MUA and AuNCs-MUD are soluble in polar solvents i.e. ethanol, methanol or dimethylformamide (DMF).

QDs (1 nmol) in 1 ml of chloroform were mixed with 3 mg of MUD and stirred for 24 hours at room temperature. The QDs solution was centrifuged for 60 min at 14.5K rpm to pellet the QDs. The supernatant was discarded and QDs were washed with 1 ml of chloroform and centrifuged again. The washing was repeated three times. The final QDs pellet was resolubilized in 200 µl DMF.

PLGA modification with nanocrystals (Steglich esterification)[4] and formation of lipid-PLGA nanoparticles.

Coupling of AuNCs to PLGA and formation of lipid-PLGA-AuNCs nanoparticles.
2 ml of a 5 mM PLGA solution (average MW of 60,000 g/mol) in DMF was mixed with 2.5 mg of Au-OH nanocrystals (filtered through 0.2 µm syringe filter) in 500 µl DMF (1:2 molar ratio of PLGA to OH ligands). The catalysts: N,N'-dicyclohexylurea (DCC), and 4-dimethylaminopyridine (DMAP) were added at 20 mM concentration (both)

and the reaction was allowed to proceed for 3 hrs with gentle stirring. After 3 hrs the solvent (DMF) was evaporated and PLGA was dissolved in ACN at concentration of 20 mg/ml and stored overnight in -20°C freezer to precipitate unreacted AuNCs. After 24 hrs the solvent was removed from 80 mg of PLGA-AuOH (4 ml of the supernatant) and redissolved in 1 ml DMF, mixed with 6.5 mg of Au-COOH nanocrystals in 500 µl DMF (syringe filtered), and 20 mM DCC and DMAP. The reaction proceeded for 3 hrs with gentle stirring. After 3 hrs the solvent was evaporated, PLGA dissolved in ACN at a concentration of 20 mg/ml of PLGA, stored in -20 °C freezer overnight to precipitate unreacted AuNCs. Steps 4 through 6 were repeated using Au-OH for the 3rd round of esterification reaction (PLGA-AuNCs x3). The ACN PLGA solution should be brownish/reddish and clear after the Au coupling reactions. 3 mg of PLGA-AuNCs in 1.5 ml of ACN was dripped to stirred and heated at 75°C solution of 0.6 mg PEG:Soybean lecithin (3:7 eq mol) in 3 ml of 4% ethanol. The PLGA-AuNCs-PEG-Soybean lecithin solution was sonicated for 3 min with a tip sonicator and stirred overnight under the fume hood. After overnight stirring the lipid-PLGA-AuNCs nanoparticle solution was filtered through 0.22 um syringe filter to remove any residual DCC precipitate and washed 3 x with water using 10 kDa MWCO Vivaspin 6 filter to remove residual DMAP.

Coupling of QDs to PLGA and formation of lipid-PLGA-QDs nanoparticles.

20 mg of PLGA was dissolved in 1 ml of DMF and mixed with DCC and DMAP (20 mM both) and 200 µl of QDs solution. The esterification reaction was allowed to proceed for 3 hrs with gentle stirring. Next, the DMF was evaporated under a rotary evaporator and PLGA-QDs was solubilized in 10 ml of ACN (2 mg/ml of PLGA) and left at -20°C overnight to precipitate unreacted QDs. The lipid-PLGA-QDs nanoparticles were prepared through nanoprecipitation method described above.

Determination of nanoparticle diameters by DLS.

After the synthesis of lipid-PLGA nanoparticles, 100 μl of each sample was suspended in 900 μl of Millipore water and the hydrodynamic diameter was measured by dynamic light scattering (DLS) device (Malvern Instruments, Worcestershire, United Kingdom). Each formulation was measured three times and the average diameter was calculated.

Measurements of absorption, excitation and emission spectra.

Absorption spectra were recorded on a Perkin-Elmer Lambda 950 UV/vis/NIR spectrometer. Emission and excitation spectra were recorded on an Edinburgh Instruments FL920 fluorescence spectrometer equipped with a 450 W Xe lamp and a double excitation monochromator with gratings blazed at 300 nm or 500 nm. Emission spectra were monitored with a Hamamatsu R928 PMT detector with a grating blazed at 500 nm, with sample excited at 400 nm. Excitation spectra were recorded by monitoring emission at 620nm.

Cell culture methods.

Murine macrophage J774A1 (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen) and grown in a 5% CO_2, water saturated atmosphere at 37 °C. For in vitro imaging experiments, 6×10^5 cells were seeded in 6 cm tissue culture dishes. After 48 hours the cells were incubated with lipid-PLGA-AuNCs, lipid-PLGA-QDs nanoparticles, and controls.

In vitro incubation experiments.

Macrophage cells J774A.1 were incubated with a lipid-PLGA-AuNCs nanoparticle solution at a concentration of 0.25 mg Au per dish (the volume of nanoparticle solution was adjusted to not exceed 10% of the media volume) for 4 hours. The

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concentration of lipid-PLGA-QDs nanoparticles for incubation with J774A.1 was matched to lipid-PLGA-AuNCs nanoparticles by adjusting the phosphate content. The cells were incubated with lipid-PLGA-QDs nanoparticles for 1 hour. The control incubations included Isovue (0.25 mg Iodine per dish) and media only. After removing the media, the cells were washed three times with PBS and then fixed with 2% glutaraldehyde and either collected as pellets or left in a dish for confocal imaging.

In vitro fluorescence imaging.

For confocal imaging nuclei were stained using DAPI mounting media (Vector Laboratories, Burlingame CA). Stack images were acquired in oil immersion method (63 X magnification) using a Leica SP5 DM scanning microscope (Bannockburn, IL). Fluorescence imaging of cell pellets was performed using the IVIS-200 imaging system (Calipex, Xenogen, Alameda, CA). The QD's detection was obtained using Ex filter = 465 nm, Em filter = 620 nm, 4 s exposure time and FOV = 6.5.

CT scanning.

The aqueous solutions of lipid-PLGA-AuNCs nanoparticles and Isovue as well as cell pellets were imaged on 256-slice Brilliance iCT scanner (Philips Medical Systems Nederland B.V., The Netherlands) at 140 keV and 250 mA with slice thickness of 0.67 mm at 0.3 mm increment and field of view of 200 mm. The matrix size was 768x768 and data was reconstructed using smooth reconstruction algorithm, pitch of 0.252 and 0.76 s rotation time. The CT images of all the samples were analyzed using the Osirix v.3.7.1 software. The attenuation of Au and Iodine expressed in Hounsfield (HU) units was derived from the images by drawing the regions of interest and recording the values (average of three for each sample) using the instrument's software. The concentration of Au was calculated from predetermined formula based on Au standards: [Au] (mg/mL)=$((HU_{SAMPLE}*1000/(HU_{H2O}-HU_{AIR}))-HU_{H2O})/29.7$.

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Transmission electron microscopy (TEM).

The TEM images were acquired using a Hitachi 7650 TEM operated at 80 kV and coupled to a Scientific Instruments and Applications (SIA) digital camera controlled by Maxim CCD software. The nanoparticle solutions were transferred to acetate buffer (0.125 M CH_3COONH_4 2.6 mM $(NH_4)_2CO_3$ 0.26 mM tetrasodium EDTA at pH 7.4) using Vivaspin columns (MWCO 10,000) and negatively stained with 2 % phosphotungstic acid. The 10 µl solution of nanoparticles was cast on a 100 mesh Formfar coated nickel grid (Electron Microscopy Sciences), dried in air and imaged.

To image cells by TEM after incubation with lipid-PLGA-AuNCs nanoparticles, the cells washed 3x with PBS and collected as pellets. The cells were fixed in 2% glutaraldehyde solution, stained with osmium tetraoxide and embedded in epoxy resin. The thin slices of 60 nm were post stained with 4% uranyl acetate and lead citrate, mounted on a grid and imaged by TEM.

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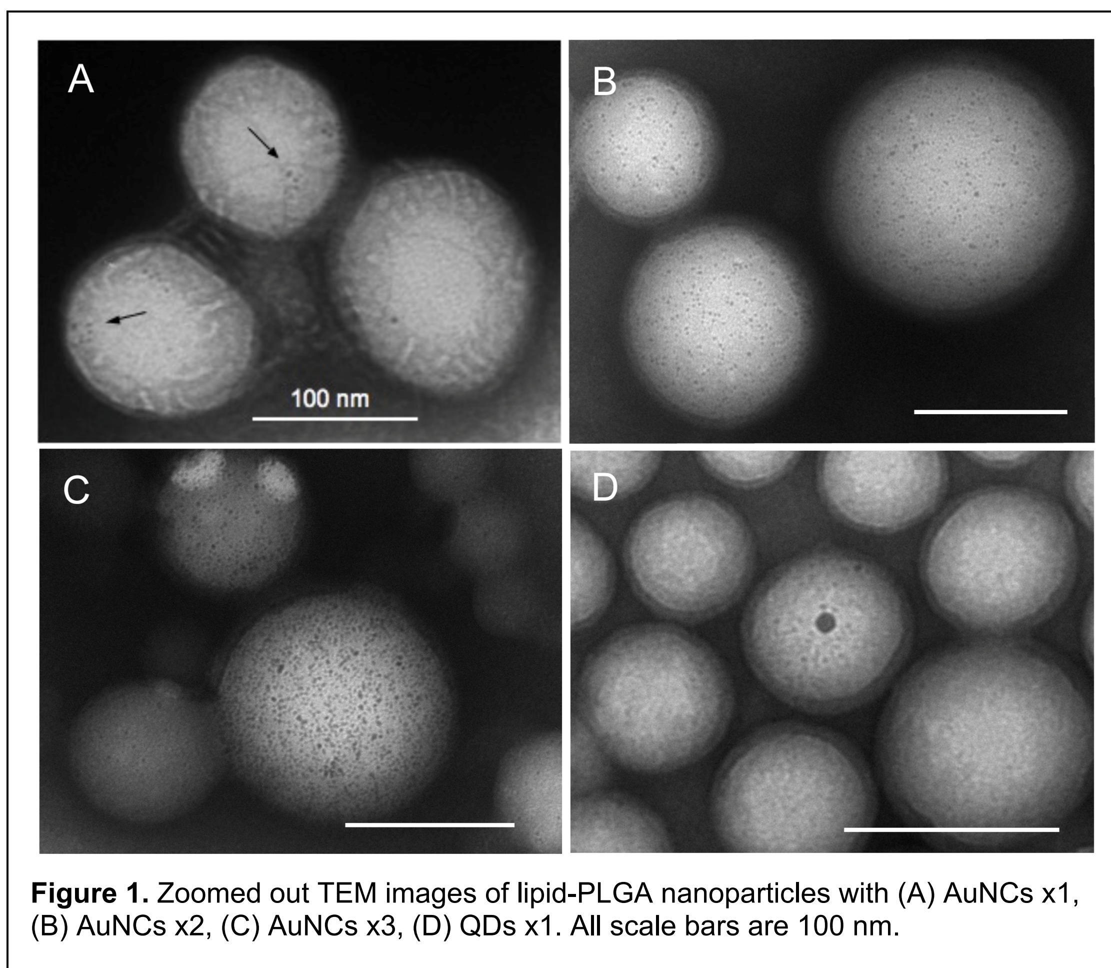

Figure 1. Zoomed out TEM images of lipid-PLGA nanoparticles with (A) AuNCs x1, (B) AuNCs x2, (C) AuNCs x3, (D) QDs x1. All scale bars are 100 nm.