Preparation of Polyethylene glycol Protected Nanoparticles with Variable Ligand Density

Marian E. Gindy, Shengxiang Ji, Thomas R. Hoye, Athanassios Z. Panagiotopoulos, and Robert K. Prud'homme

Supporting Information

I. Titration of BSA free thiol content

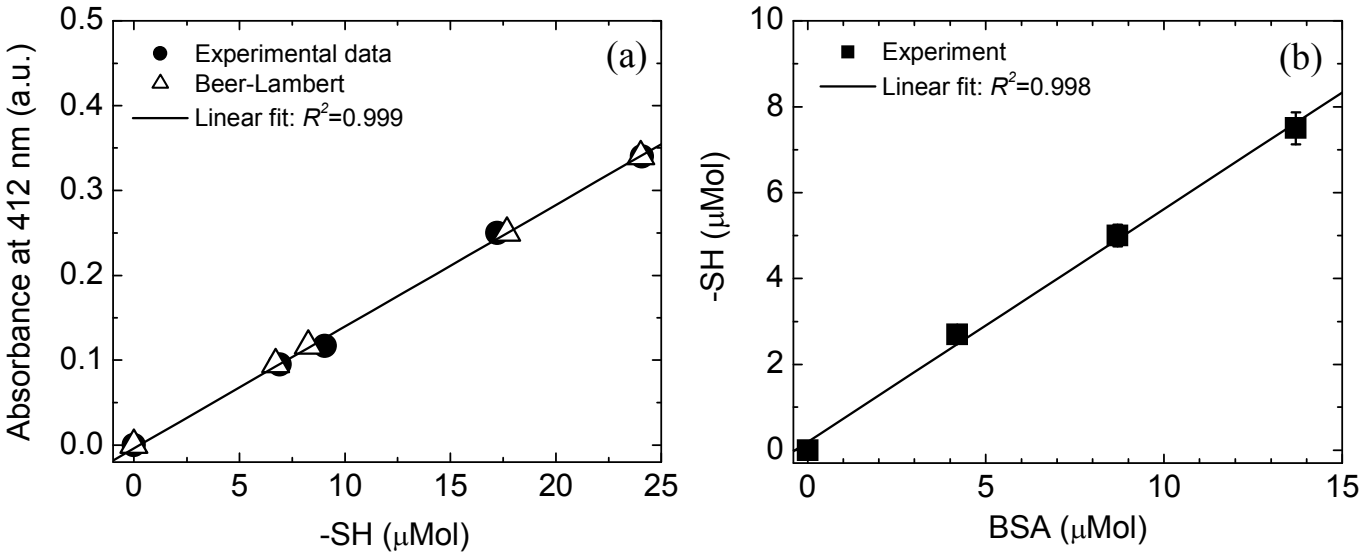

Figure SI-1. (a) Standard curve of -SH groups based on calibrated L-cysteine solutions (•) and fit of absorbance values to Beer-Lambert equation (Δ) using an extinction coefficient of 13,600 $M^{-1}cm^{-1}$. The solid line was fit by linear regression. (b) Titration of calibrated BSA solutions with Ellman's reagent and experimentally measured -SH concentration (■). Solid line was fit by linear regression. Slope of line represents the molar ratio of SH to BSA, estimated at 0.55 ± 0.02.

II. Calculation of maleimidePEG-BSA homogeneous reaction time

To estimate the time for maleimide-thiol reaction in solution (step iii of heterogeneous coupling reaction), the homogeneous reaction of maleimide-terminated PEG (*maleimide*PEG; 5 KDa; Nektar Therapeutics, Huntsville, AL) with BSA was independently studied. A summary of the results is shown in Figure SI-2.

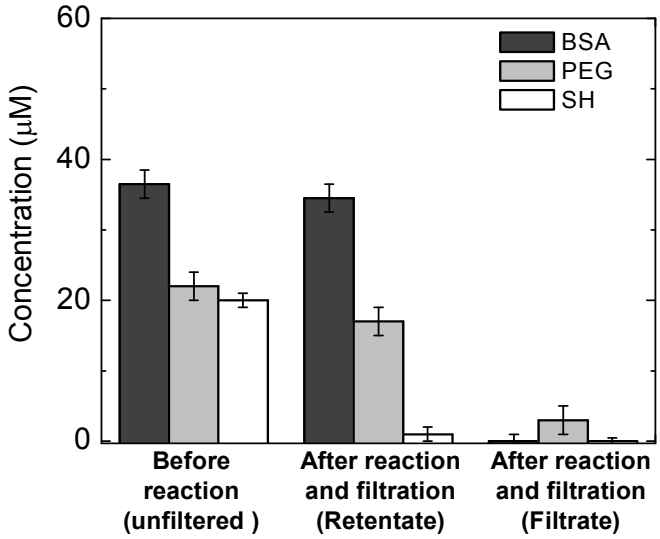

Figure SI-2. Homogenous reaction of *maleimide*PEG with BSA. The concentrations of BSA, free thiol (SH) and PEG in solution before reaction, in the retentate following reaction and purification, and in the filtrate were determined via BCA, Ellman's reagent and Beauleax assays, respectively. A reaction conversion of approximately 87 ± 6 % is estimated.

As seen from Figure SI-2, the molar concentration of free thiol (SH) (white column) and *maleimide*PEG (light gray column) are essentially equivalent in the solution prior to reaction (t=0 h). The concentration of SH in solution was via the concentration of BSA (dark gray column) as measured by BCA assay and by titration with Ellman's reagent. Assuming *maleimide*PEG has a functionality of nearly 100% (as per manufacturer specification), the ratio of maleimide:SH in the initial reaction solution corresponds to approximately 1:1 mole:mole%. Following a reaction time of 1 hour, a sample of the solution was filtered via 10KDa MWCO membrane allowing for the retention of BSA and BSA-PEG conjugate on the membrane surface (retentate) and passage of unreacted PEG in solution (filtrate). The retentate was re-suspended in 0.15M NaCl and analyzed for BSA, free thiol, and PEG concentrations. The reaction conversion was calculated based on the measured concentration of unreacted PEG in the filtrate and was estimated at ~87 ± 6%, in line with conversions of comparable *maleimide*PEG reactions as reported in literature.[3] Analysis of the retentate (second set of columns) indicates no free thiol present in the BSA and further confirms the mass balance of PEG and BSA in all samples analyzed. Thus, the homogenous reaction of maleimide-tethered PEG chains with BSA occurs on the order of minutes.

Methods

Reaction of maleimide-terminated PEG with BSA Reaction of BSA with maleimide-terminated PEG (*maleimide*PEG, 5KDa, Nektar Therapeutics, Huntsville, AL) was carried out as follows. *maleimide*PEG (10 mg, 1.7 μmol) and BSA (0.2 g, 3.1 μmol) were added to a glass vial equipped with septum and nitrogen purge. 50 mL of degassed 0.15M NaCl solutuion (pH 6.2-6.5, 2mM EDTA) was added via syringe. The solution was reacted at room temperature under mild agitation using a rotating mixer set at low speed. After 1 h, the solution (3 mL) was filtered via a 10KDa MWCO OMEGA nanoseparation centrifuge filter membrane (Pall Corporation, East Hills, NY) using a bench-top micro-centrifuge (Eppendorf 5415C, Hamburg Germany) rotating at 8,800 x g for 30 minutes. The retentate (material on membrane surface) was re-suspended in 0.15M NaCl solution (3 mL). The concentrations of BSA, free thiol and PEG in the solution before reaction, in the retentate, and in the filtrate were determined.

DTNB (Ellman's reagent) assay Quantification of BSA free thiol content was determined spectrophotometrically via the thiol-disulfide interchange reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) as follows. A solution of the protein (5-30 μM) was incubated at room temperature with an excess of DTNB dissolved in freshly prepared and degassed 0.1M sodium phosphate (pH 7.8) for a period of approximately 15 min. The reaction of DTNB was monitored spectrophotometrically at 415 nm with an Evolution 300 spectrometer (Thermo Electron Inc., Madison, Wisconsin) using a 0.1cm path length Quartz cell. All samples were run in duplicate and appropriate controls were recorded in all cases. Thiol content was calculated using an extinction coefficient of 13,600 $M^{-1}cm^{-1}$ for 5-thio-2-nitrobenzoate and additionally through comparison to a standard curve based on measurements of calibrate L-cysteine solutions.

Baleux assay The concentration of PEG was quantified according to an assay described by Baleux, where hydrogen bonding interactions of PEG with iodine result in the formation of a colored helix-complex. A standard curve was generated by incubation of PEG solutions (1 mL) with 25 μL of freshly prepared iodine-potassium iodide solution (0.04M I_2, 0.12M KI). The concentration of PEG in solution ranged from 0.1-2 mg/mL. After five minutes, the optical density of the solution at 500 nm was measured at ambient temperature using an Evolution 300 spectrometer (Thermo Electron Inc., Madison, Wisconsin). Appropriate controls were recorded in all cases.