SUPPORTING INFORMATION

Bionanoconjugation via Click Chemistry: The Creation of Functional Hybrids of Lipases and Gold Nanoparticles

Jennifer L. Brennan, Nikos Hatzakis, T. Robert Tshikhudo, Nijole Dirvianskyte, Valdemaras Razumas, Shamkant Patkar, Jesper Vind, Allan Svendsen, Roeland J. M. Nolte, Alan E. Rowan* and Mathias Brust.*

Table of Contents

1. General Experimental Procedures.

Tetrahydrofuran (THF) was distilled under nitrogen from sodium/benzophenone. All other chemicals were used as received and deionised water was used throughout. Ligand **1**, N-(2-{2-[2-(2-{2-[2-(2-{2-[2-(2-{2-[2-(2-Azido-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethyl)-2-[aminoethyldisulfanyl-ethylcarbamoyl)-methoxy]-acetamide, was purchased from Encapson BV and used as received.

Citrate-stabilized gold nanoparticles of 14 nm diameter were synthesised using the classical Turkevich/Frens procedure (*1,2*). Briefly, an aqueous solution of sodium citrate (25 ml, 39 mM) was added to a boiling aqueous solution of $HAuCl_4$ (250 ml, 1 mM), and the reaction mixture was heated

under reflux for 30 min. It was allowed to cool to room temperature, stirred overnight, and filtered before use through a 0.45 μm syringe filter (Millipore).

The citrate-stabilized gold nanoparticles were functionalized with **1** by mixing of a molar excess of **1** (dissolved in water) with the gold nanoparticle suspension following a previous method (*3*). Briefly, 500 μl of an aqueous solution (17 mg, 20 μmoles) of **1** was mixed with 10 μL of 0.1 M dithiothreitol, and was left to stand at room temperature for 25 min. This mixture was then added to filtered citrate-stabilized gold nanoparticles (20 ml, 2.8 nM) under stirring. After mixing for 18 hours at room temperature, the particles were purified by repeated centrifugation (3 times at ~ 15,000g) and re-dispersion in water. As a final purification step, the particles were passed through a 5 ml Zeba Desalt spin column (Pierce). A molar extinction coefficient of 4.2×10^8 M^{-1} cm^{-1} (at 522 nm) based on gold nanoparticles of 14±1 nm diameter was used to calculate a final concentration of 1.4 Nm (*4*,*5*). The nanoparticle diameter was determined by transmission electron microscopy (TEM) using a JEOL 2000 EX transmission electron microscope operating at 200 kV. Specimens for TEM were prepared by the slow evaporation of one drop of an aqueous solution of the nanoparticles onto a carbon-coated copper mesh grid. TEM results are depicted in Figure S1. Elemental analysis was carried out by ICP-AES (gold and sulphur) and combustion analysis (carbon, hydrogen and nitrogen). For a typical preparation of AzNP a composition of gold = 95.8%, sulphur = 0.48%, carbon = 2.90%, hydrogen = 0.42% and nitrogen = 0.33 % was found. This elemental analysis indicates that approximately 60% of ligands are the long chain azide-containing portion of **1**.

Lipase from *Thermomyces lanuginosus*, engineered to contain one solvent-accessible lysine NH_2 group (Lys 46), was prepared by Novozymes A/S using in-house protein engineering and expression techniques (M.W. 30,000 g mol^{-1}). It was labelled with an acetylene group using 4-pentynoic acid, and EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) as a coupling reagent. (*6*). The reaction was carried out in 20 mM phosphate buffer, pH 7.0 (PB). 600 μL of lipase solution (0.0225 mM in water), 6.7 μL of 4-pentynoic acid solution (100 mM in 50% THF/PB), 13 μL of EDC solution (50 mM in PB), 150 μL THF and 130 μL of PB were mixed in a 10 ml plastic culture tube, yielding a final reaction volume of 1500 μL (10% v/v total THF content). The reaction mixture was shaken overnight at room temperature and the THF was allowed to evaporate off in a chemical fume-hood. The remaining mixture in 100% PB was dialyzed three times against 500 ml PB for a total of 36 hrs using a molecular weight cut-of of 12 kDa. Lipase concentration was determined using UV-vis spectroscopy (molar extinction coefficient 3.7×10^4 M^{-1} cm^{-1} at 280 nm).

To verify the successful acetylene labelling of the lipase, a click chemistry reaction was carried out with an azide modified coumarin dye (7-Acetoxy-4-(azidomethyl)coumarin, synthesis described in Section 2). To 100 μL of an acetylene-lipase solution (0.0084mM in PB) was added 1.1 μL of 7-Acetoxy-4-(azidomethyl)coumarin (75 mM in THF), 10 μL of catalyst (10 mM $CuSO_4 \cdot 5H_2O$, 50mM ascorbic acid in PB), 50 μL of THF and 89 μL of buffer, to a final volume of 250 μL (20 % v/v total THF content). The reaction mixture was shaken overnight at room temperature and dialyzed as before. The product was analyzed by fast protein liquid chromatography (FPLC) using a Pharmacia *SMART* high performance liquid chromatograph, equipped with a Superose 12 PC 3.2/30 column (optimal separation 1 – 300 kDa) (Pharmacia) with PB as eluent and UV detection (λ = 280 nm and λ = 350 nm). FPLC traces (Figure S2) of lipase and coumarin-dye-functionalized-lipase at λ = 280 nm and λ = 350 nm were recorded. Unmodified lipase showed no coumarin absorption at λ = 350 nm, while for the lipase click coupled with the coumarin dye, the opposite was true. The FPLC trace is shown in Figure S2. This demonstrates the successful click coupling labeling of the lipase with coumarin and hence, the presence of an accessible acetylene group in the lipase which may be utilized for other click reactions.

Azide-functionalized gold nanoparticles were labelled with the acetylene-lipase using click chemistry. To 192 μl of a solution of azide-nanoparticles (AzNP, 13.0 nM in H_2O) was added 36 μl of acetylene-lipase (69 μM in PB) and 2.5 μl of catalyst (10 mM $CuSO_4 \cdot 5H_2O$, 50mM ascorbic acid in H_2O), to a final volume of 1 ml in H_2O. This reaction mixture was stirred at room temperature for 3 days, after which the nanoparticles were purified from copper catalyst and excess lipase by centrifugation into a pellet (30 min at ~ 10,000g), removal of supernatant and re-dispersion of the pellet in PB. This procedure was repeated eight times. The supernatants were retained and tested for the presence of lipase using a lipase activity assay (described below). Agarose gel electrophoresis of the nanoparticles was carried out using a Biorad Sub-Cell GT agarose gel electrophoresis system. Nanoparticles (in 10% glycerol) were loaded onto a 1.5% agarose gel, which was run for 60 min at 100 V in tris-borate-EDTA (TBE) buffer.

Lipase activity was determined using a fluorescence-based activity assay adapted from that reported by Guilbault and Hieserman (*7*). A new non-fluorescent substrate, 5-O-palmitoylindole (synthetic details in Section 2) was employed. In the presence of lipase, the palmitoyl chain of the substrate is cleaved at the ester bond, yielding a fluorescent 5-OH-indole product. Briefly, 30 μL of 5-O-palmitoylindole in

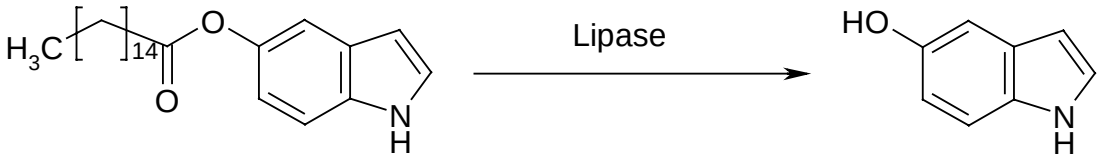
2-methoxyethanol was mixed with 70 μL of PB, and 20 μL of lipase (or nanoparticle) sample was added, while a stop clock was started simultaneously. The emission intensity of the assay mixture at the start and end of 5 minutes incubation was monitored using a Perkin Elmer LS50B luminescence spectrometer (λ_{ex} = 300 nm, λ_{em} = 333 nm). The rate of change of emission intensity w.r.t. time (min^{-1}) is converted to enzyme activity units (U ml^{-1}) using the molar fluorescence of 5-OH-indole (1.15×10^9 ml mol^{-1} in this assay). 1 activity unit (U) is equivalent to 1 micromole of 5-OH-indole product liberated per minute. The blank rate of the assay (auto-hydrolysis of the substrate) was determined by substituting 20 μL of PB for the 20 μL of lipase sample in the assay mixture. This control experiment demonstrates that auto-hydrolysis of the substrate was negligible under these assay conditions. As nanoparticles absorb strongly at both the excitation and emission wavelength of 5-OH-indole, it is likely that the fluorescence quantum yield of the 5-OH-indole fluorophore will be lower when AzNPs are present in the lipase assay solution (due to the nanoparticles absorbing both the excitation and emission energy). To quantify this "quenching" effect, a Stern-Volmer-type quenching constant for the AzNPs and 5-OH-indole was determined. K_{SV} for this system is 5.57×10^7 M^{-1}. All quoted nanoparticle-lipase activity rates have been corrected using this K_{SV}. The equivalent molar concentration of lipase in the lipase-labelled AzNP sample was determined using a calibration curve of activity versus solution free acetylene-labelled lipase concentration (Figure S4). The number of enzymes per nanoparticle was estimated by dividing this value by the molar concentration of nanoparticles in the same sample, assuming that lipase activity is neither increased nor decreased upon binding to the nanoparticle. Experiments to independently determine the exact concentration of lipase bound per nanoparticle (following nanoparticle decomposition using 1M KI solution) were carried out using a fluorescent protein-specific dye (NanoOrange Protein Quantitation Kit, Invitrogen), but were not successful. The limit of detection of the assay when carried out using acetylene-labelled *T. lanuginosus* as the protein standard was 500 nM, and the assay is therefore not sensitive enough to detect the quantities of lipase bound to nanoparticle (low nanomolar range).

2. Synthesis of 7-acetoxy-4-(azidomethyl)coumarin

7-acetoxy-4-(bromomethyl)coumarin (0.51 g, 1.72 mmol) was suspended in ethanol (9 ml) and a solution of sodium azide (0.37 g, 5.74 mmol) in water (1 ml) was added. The mixture was heated at reflux for 11 hrs. The volume of the solution was reduced *in vacuo* and the precipitate thus formed was collected by filtration and air dried to yield 7-hydroxy-4-(azidomethyl)coumarin (0.25 g, 56 %) as a pale orange solid.

- **UV-Vis:** λ_{max} (MeOH) 326 (log ε 4.17) nm.
- **^{1}H NMR** (MeOD), δ (ppm): 4.68 (s, 2H, CH_2), 6.29 (s, 1H, vinylic), 6.73 (s 1H, Ar), 6.82 (d, *J* = 8.0 Hz, 1H, Ar), 7.55 (d, *J* = 8.0 Hz, 1H, Ar).

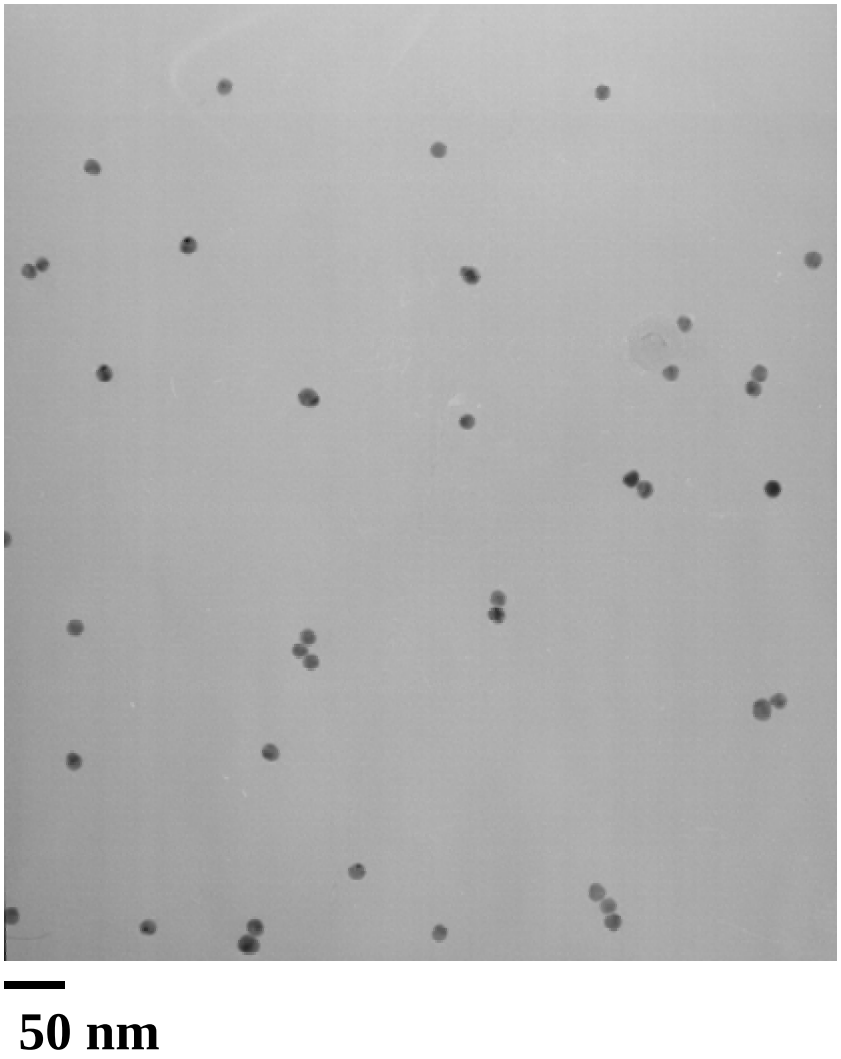
3. Synthesis of 5-O-palmitoylindole



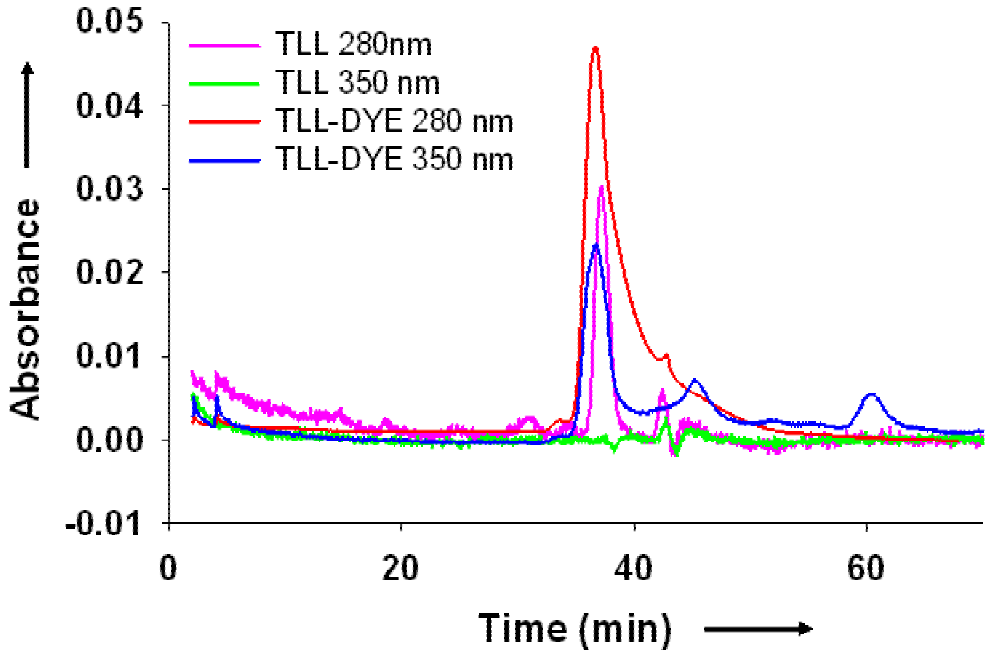
To a vigorously stirred solution of 0.13 g (1 mmol) 5-hydroxyindole in 4 ml $CHCl_3$, 0.36 g (1.3 mmol) of palmitoyl chloride, and 0.17 ml (1.3 mmol) of triethylamine were added at 0 ^oC. The mixture was stirred at room temperature for 2 h, and washed three times with ice-water. The separated organic layer was dried over anhydrous $MgSO_4$, evaporated *in vacuo*, and the solid residue was purified over silica gel column (eluent hexane-CH_2Cl_2) and finally re-crystallized from hexane (0.2 g, 53%).

- **m.p**. 88-91 ^oC.
- **IR** (KBr), ν (cm^{-1}): 1581 - 1624 (C=C), 1735(COO), 2848 - 2917 $(CH_2)_n$, 3389 (NH).
- **^{1}H-NMR** ($CDCl_3$), δ (ppm): 0.88 (3H, t, *J* = 6.9 Hz, CH_3), 1.23-1.26 (24 H, m, $(CH_2)_{12}$), 1.78 (2H, q, *J* = 7.4 Hz, $C\underline{H}_2CH_2CO$), 2.57 (2H, t, *J* = 7.5 Hz, CH_2CO), 6.50 (1H, m, CH), 6.87 (1H, dd, *J* = 2.3 Hz, Ar), 7.17 (1H, t, *J* = 2.8 Hz, Ar), 7.27 - 7.30 (2H, m, CH, Ar); 8.22 (1H, bs, NH).

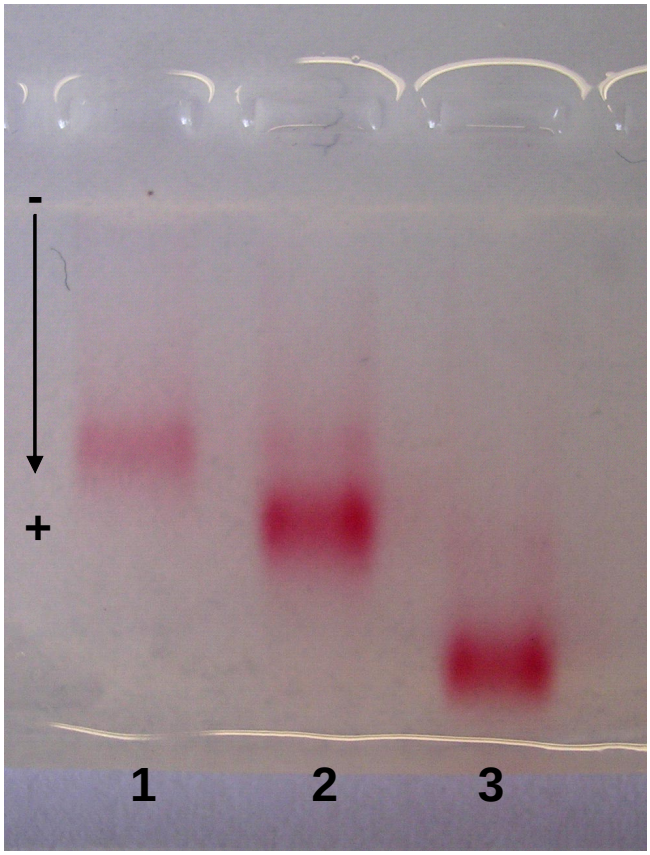
4. **Figure S1** Transmission electron micrograph of gold nanoparticles stabilized with thioalkylated azide PEG ligand **1**.



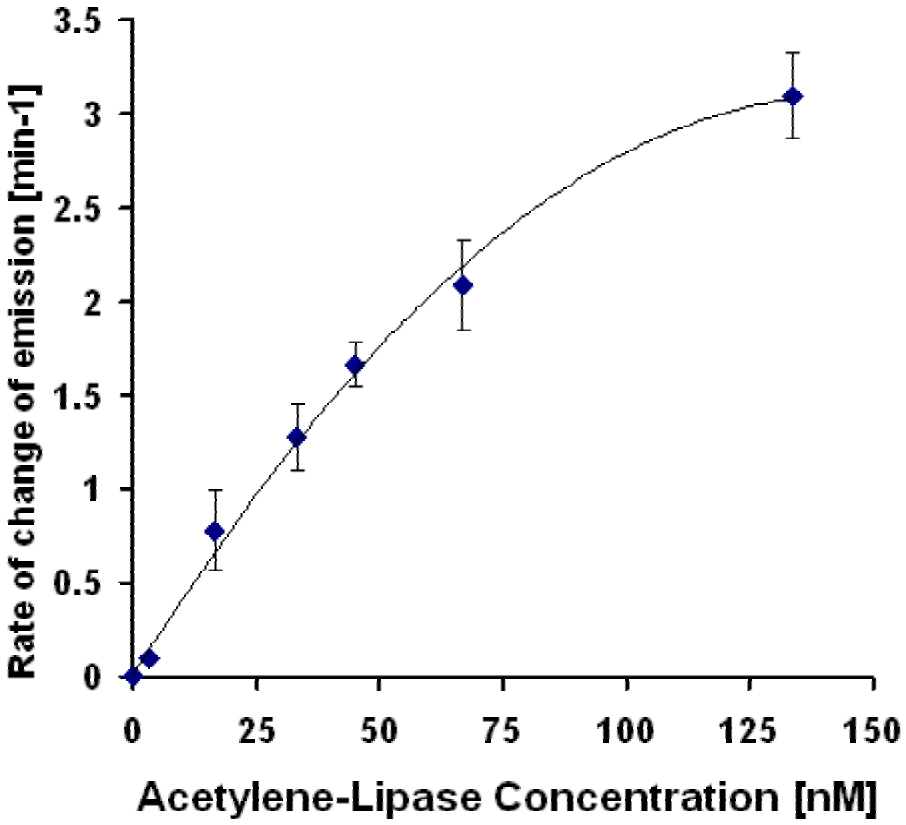
5. Figure S2: Fast protein liquid chromatography (FPLC) of *Thermomyces lanuginosus* lipase (TLL) - coumarin dye conjugate. FPLC traces of TLL and coumarin-dye-functionalized-TLL at λ = 280 nm and λ = 350 nm are depicted. Unmodified TLL showed no dye absorption at λ = 350 nm (green line), while for the TLL coupled with the coumarin dye (blue line), the opposite was true.



6. **Figure S3:** 1.5% agarose gel electrophoresis of 14 nm gold nanoparticles stabilized with **1** (AzNPs), immediately after click reaction with acetylene-lipase, prior to washing to remove excess lipase. Lane 1: AzNPs. Lane 2: AzNPs reacted with acetylene-lipase without copper catalyst (blank). Lane 3: AuNPs reacted with acetylene-lipase with copper catalyst (sample).



7. **Figure S4:** Calibration curve of lipase activity vs. lipase concentration for solutions of acetylene-labeled *Thermomyces lanuginosus* lipase.



LITERATURE CITED

(1) Turkevich, J., Stevenson, P. C., and Hillier, J. (1951) A study of the nucleation and growth processes in the synthesis of colloidal gold. *Diss. Faraday Soc. 11*, 55-57.

(2) Frens, G. (1973) Controlled nucleation for regulation of particle-size in monodisperse gold suspensions. *Nature Phys. Sci. 241*, 20-22.

(3) Tshikhudo, T. R., Demuru, D., Wang, Z., Brust, M., Secchi, A., Arduini, A., and Pochini, A. (2005) Molecular recognition by calix[4]arene-modified gold nanoparticles in aqueous solution. *Angew. Chem. Int. Ed. 44*, 2913-2916.

(4) Link, S., Wang, Z. L., and El-Sayed, M. A. (1999) Alloy formation of gold-silver nanoparticles and the dependence of the plasmon absorption on their composition. *J. Phys. Chem. B 103*, 3529-3533.

(5) Mulvaney, P., Giersig, M., and Henglein, A. (1992) Surface chemistry of colloidal gold: deposition of lead and accompanying optical effects. *J. Phys. Chem. 96*, 10419-10424.

(6) Hermanson, G. T. Bioconjugate Techniques, Academic Press, New York, 1996, p.170.

(7) Guilbault, G. G., and Hieserman, J. (1969) Fluorometric substrate for sulfatase and lipase. *Anal. Chem 41*, 2006-2009.