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Towards Bigger Nanogold: Preparation of Covalent 3nm Gold – Fab' Probes

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The use of gold cluster compounds such as the 1.4 nm Nanogold label [1], which are cross-linked selectively to specific chemical groups rather than adsorbed to their conjugate biomolecule, have extended gold labeling to molecules such as oligonucleotides, lipids, and peptides that cannot be labeled directly with conventional colloidal gold. Because additional stabilizing macromolecules are not required, conjugates are smaller than conventional colloidal gold probes, and penetration into samples and labeling of restricted antigens is significantly higher [2]. Larger labels with the same reactivity are highly desirable, because they would be more easily visualized in the electron microscope: they could be used to visualize labeling distributions in whole cells, or in the presence of other electron-dense materials, without autometallographic enhancement. A 3 nm gold – Fab' conjugate would be no larger than an IgG molecule, yet significantly more visible in the electron microscope than Nanogold. We are therefore developing methods for preparing covalent 3 nm gold conjugates using particles cross-linked by coordinated organic ligands.

Different chromatographic and centrifugation separation methods were investigated using 3nm gold conjugates of Cy5-labeled Fab' fragments. The absorption of the Cy5 label at 650 nm (where absorption from the gold particles is relatively low), and that of the gold particles at 420 nm, were used to indicate the relative amounts of Fab' in the conjugates. 3 nm gold particles, prepared by thiocyanate reduction [3], and functionalized with a 10 : 1 mixture of bearing hydroxyl- and t-Boc-protected amino- akanethiols, were deprotected (0.3 M HCl in isopropanol), activated with *sulfo*-succinimidyl-4-N-maleimido-cyclohexane-1-carboxylate (*sulfo*-SMCC), and reacted in a 1 : 1 ratio with Cy5-labeled goat anti-rabbit Fab' fragments prepared by Cy5-F(ab')$_2$ reduction.

Separation of conjugates was evaluated chromatographically (a) over hydroxyapatite type I, a combination gel filtration and hydrophobic interaction gel, eluted with a linear gradient of 0 to 100% buffer B (0.4 M sodium phosphate, pH 6.8, in 10% isopropanol/water), mixed with buffer A (5 mM sodium phosphate, pH 6.8, in 10% isopropanol/water), and (b) over Superose-6 gel filtration media eluted with 0.02 M sodium phosphate buffer with 0.15 M sodium chloride. Hydroxyapatite chromatography separated the reaction mixture into two minor species eluted before the introduction of buffer B; these were shown by UV/visible spectroscopy and scanning transmission electron microscopy (STEM) to contain mostly gold particles with little associated antibody. The two major peaks were eluted at close to 80 % and 95 % B: UV/visible spectroscopy and STEM indicated these to be unconjugated gold particles and partially labeled Fab' respectively (Figure **1**). Gel filtration over Superose-6 produced a single, broad peak; UV/visible spectra of individual fractions indicated that this contained multiple overlapping species. Contrary to expectations, a significantly higher Cy5, and hence Fab', content was found in fractions from its trailing side. STEM indicated a broader particle size distribution, and some aggregates, in fractions from the leading side of this peak.

Density gradient ultracentrifugation over a 10 to 30 % sucrose gradient gave three layers (Figure **2**); an upper band containing relatively little gold but significant amounts of antibody; a middle band containing both gold and antibody, consistent with a conjugate; and a lower band, containing little or no antibody. Hydroxyapapatite chromatography holds promise for conjugate separation from excess gold, and density gradient ultracentrifugation for separation from free Fab'.

References:

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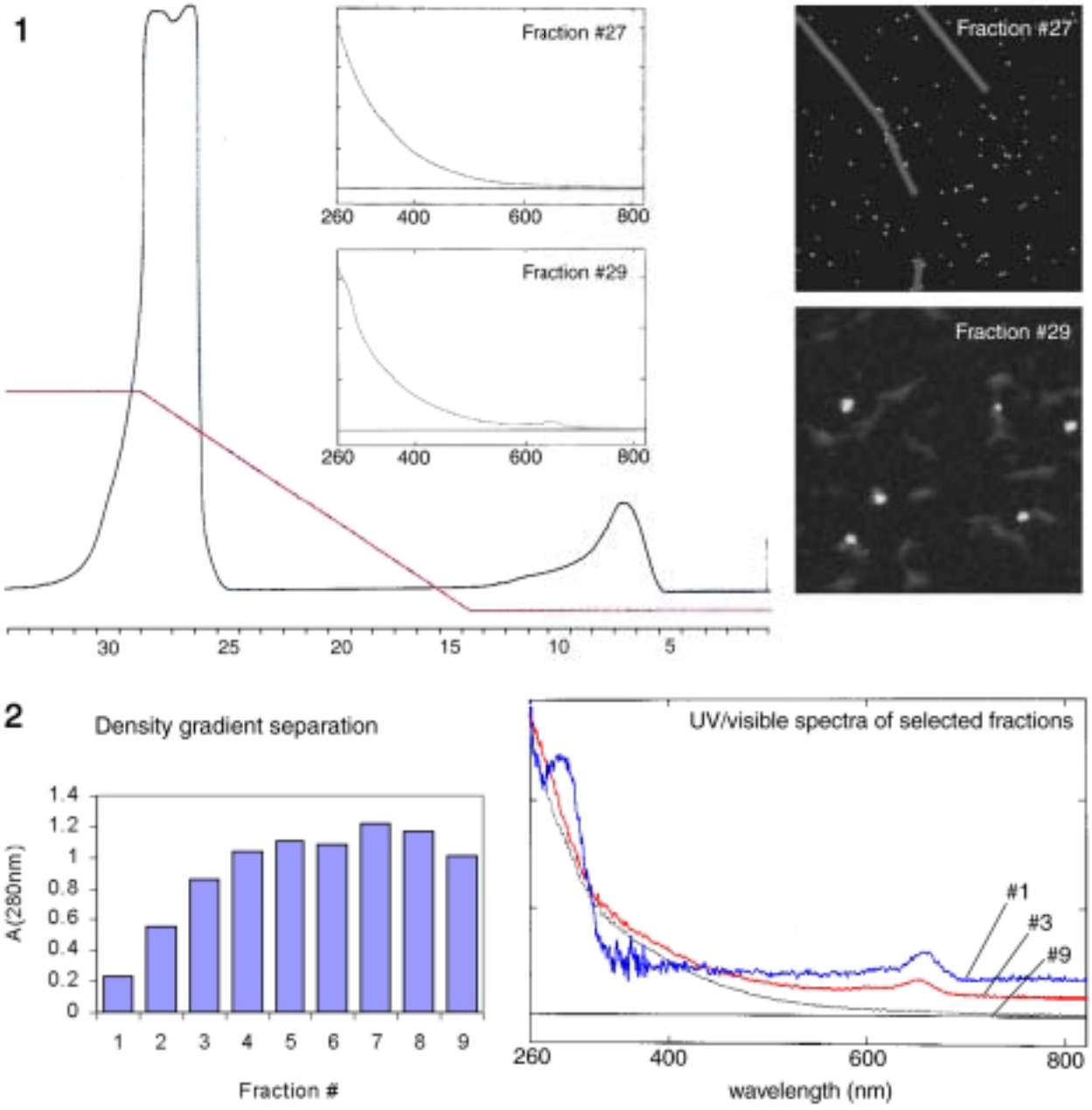

Figure 1: Separation of [Au_{3nm}]-Fab'-Cy5 by liquid chromatography over hydroxyapatite type 1, with UV/visible spectra and STEM micrographs for selected fractions. (red line shows gradient; scale shows fraction. Image width for STEM micrographs: 512 nm for #27, 128 nm for #29).

Figure 2: Separation by density gradient ultracentrifugation from top (fraction #1) to bottom (fraction #9). Selected UV/visible spectra indicate Cy5-labeled antibody and gold in fraction #3.

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