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LABELING WITH NANOGOLD AND UNDECAGOLD: TECHNIQUES AND RESULTS

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Abstract

A significant new development in gold labeling for microscopy has been achieved through the use of gold cluster compounds that are *covalently* attached to antibodies or other probe molecules. These unique gold probes are smaller than most colloidal gold conjugates and exhibit improved penetration into tissues, higher labeling densities, and allow many new probes to be made with peptides, nucleic acids, lipids, drugs, and other molecules. A new fluorescent-gold conjugate is useful for examining localization by fluorescence microscopy, then visualizing the same label at the ultrastructural level in the electron microscope.

Key Words: Gold, Nanogold, Undecagold, colloidal gold, immunocytochemistry, labeling, silver enhancement, fluorescence labeling, FluoroNanogold, gold lipids, electron microscopy

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Introduction

The purpose of this review is to provide an introduction to the gold clusters (Nanogold, Undecagold, and FluoroNanogold), covering their properties and coupling chemistry. Next, examples of their use in labeling of specific sites on biomolecules for high resolution structural studies will be given; results using gold clusters in immunolabeling will also be given. As a practical guide, specific protocols will be discussed or referenced for using the clusters, both as labeling reagents, and as immunolabels.

Colloidal gold

Traditionally, colloidal gold has been used as the favored label for electron microscopy. The technology for making colloidal golds of fairly precise sizes, and adsorbing antibodies and lectins, has led to many useful applications. There are, however, some limitations and shortcomings of this technology, such as: [1] The adsorbed macromolecule (e.g., antibody), is not covalently attached, and is found to desorb to some extent (Kramarcy and Sealock, 1990), [2] Many molecules (e.g., many small ones) do not make stable conjugates with colloidal golds, [3] Because the gold is "sticky", the protein conjugates are sometimes aggregated, especially for small colloidal gold sizes (Hainfeld, 1990), and [4] penetration into tissues is frequently a problem (Takizawa and Robinson, 1994), due either to the large size of the gold, or for the smaller sizes, due to the aggregation.

Gold clusters

Gold clusters are a different approach that circumvents these problems. These are gold compounds, with defined structures, with organic linking groups for chemically attaching other molecules, as opposed to colloidal gold, which is a sphere of gold atoms with an ionic/hydrophobic surface. An example is Undecagold, which has a core of eleven gold atoms, and covalently attached phosphorus atoms, which, in turn, have organic moieties attached. The structure of Undecagold has been solved by x-ray diffraction (McPartlin *et al.*, 1969), and is shown diagramatically in Fig. 1 and in an electron micrograph in Fig. 2. A larger gold cluster that

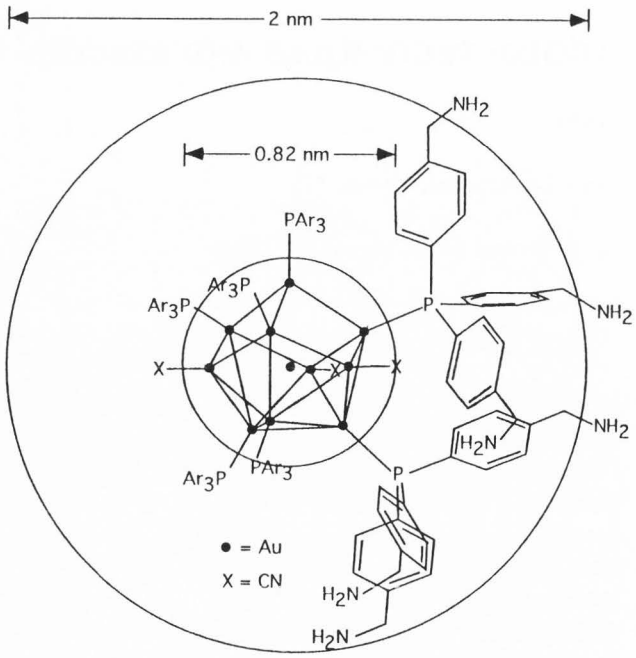

Figure 1. Diagram of the undecagold gold structure.

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is 1.4 nm in gold core diameter has been made (Fig. 3), and presumably contains 67 gold atoms (Hainfeld and Furuya, 1992). This cluster has been termed "Nanogold", and its atomic structure has not yet been solved by crystallography.

The organic moieties may be changed so that different end groups are present, e.g., amines, carboxyls, or reactive linking groups, such as the maleimido group that reacts with thiols. Although there are multiple organic groups surrounding the cluster, it is possible to synthesize and purify a product that contains only one of the desired groups on the cluster's surface (Reardon and Frey 1984; Safer *et al.*, 1986). An example of using the monomaleimido-undecagold to covalently link to the hinge sulfhydryl of an Fab' antibody fragment is shown in Fig. 4 (Hainfeld, 1987, 1989).

The ability to synthesize different groups on the outer shell of the cluster gives a tremendous flexibility and control over the desired properties of the particles. For example, multiple amino groups give it a high positive charge, or one amino group might be used to link to one specific site. The organic groups may also be changed to affect solubility properties; a cluster can be made with all phenyl groups, for example, which would make it soluble in organic solvents or membranes.

Covalent reactivity

A useful group of clusters has been those with single, preformed, single reactive groups on the surface,

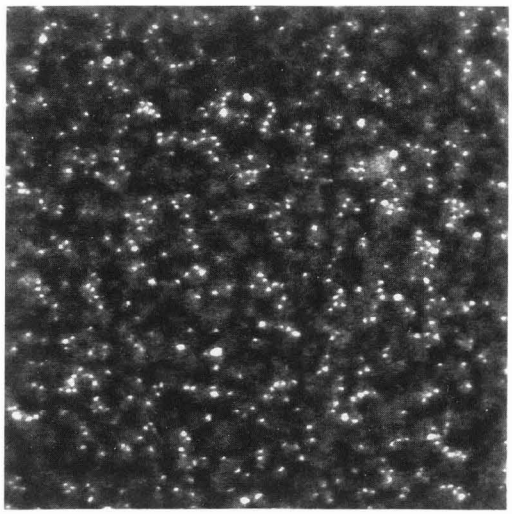

Figure 2. Darkfield field emission scanning transmission electron micrograph (STEM) of undecagold clusters on a thin 2 nm carbon film. Each bright dot is an undecagold core. Full width 128 nm.

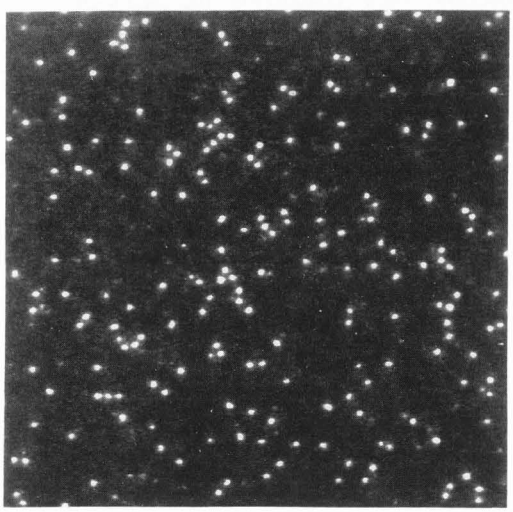

Figure 3. Darkfield STEM of 1.4 nm Nanogold clusters. Full width 128 nm.

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so that the gold particle can be attached to a specific site on another molecule. The maleimide group is one example, shown above (Fig. 4) for the Fab', where the gold then reacts very specifically with free thiols, typically with a cysteine of a protein. Another specific linker is the N-hydroxysuccinimide ester, shown in Fig. 5, which reacts with free amino groups, typically lysines and the α-amino terminus of proteins. An amino gold can be reacted with carbohydrates (Fig. 6), such as on glycoproteins (Lipka *et al.*, 1983), or with the 2',3' diol

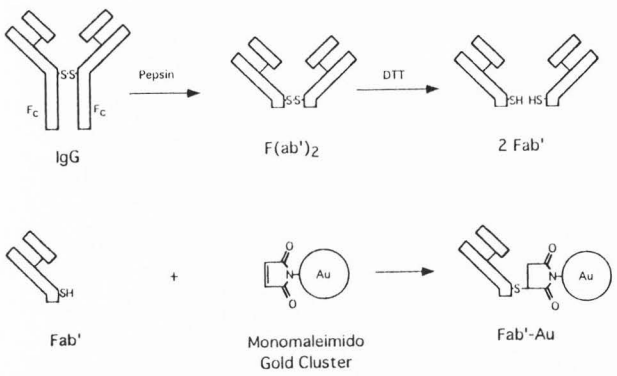

Figure 4. Coupling scheme for attaching gold clusters to the hinge sulfhydryl of Fab' fragments.

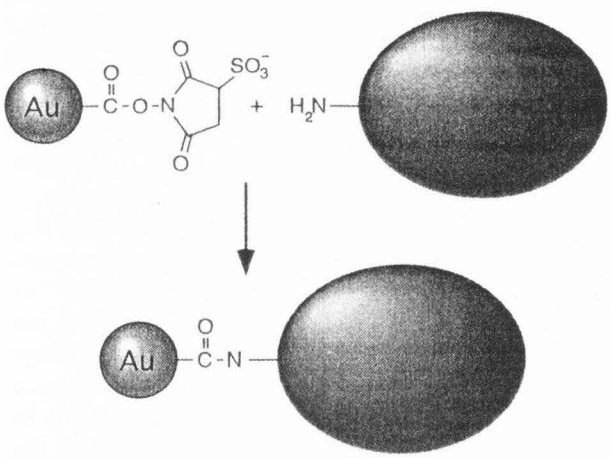

Figure 5. Reaction coupling amino groups onto N-hydroxysuccinimide (NHS) gold cluster.

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of RNA (Skripkin *et al.*, 1993). Gold clusters can also be coupled to lipids, to make membrane labels (Fig. 7). The variety of stable covalent conjugates that can be designed certainly extends the usefulness of gold as a label.

Gold cluster immunoprobes

Gold clusters may be covalently attached to the hinge sulfhydryls of IgG or Fab' fragments using maleimido-gold. Attachment to antibody amino groups (using N-hydroxysuccinimide ester-gold) has also been done and the antibodies also show good immunoreactivity. It is also possible to couple the gold to the carbohydrate moiety. There are a number of advantages of using gold cluster immunoprobes over those made with colloidal gold: [1] They are smaller; one reason is the small size of the gold, another is that Fab' fragments are about 1/3 of the size of IgG, making the whole probe substantially smaller. This has several important

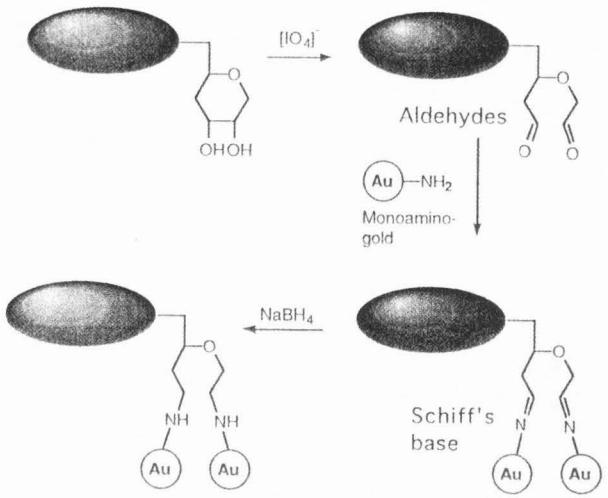

Figure 6. Reaction for covalently coupling gold clusters to carbohydrates.

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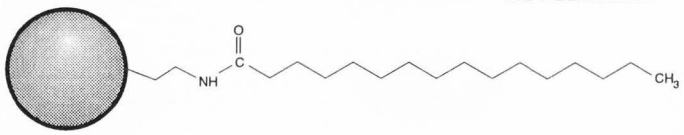
benefits: [a] better penetration into tissues (40 μm has been reported (Sun *et al.*, 1995), whereas colloidal gold is typically < 0.5 μm) (Takizawa and Robinson, 1994), [b] more quantitative staining of antigens, since there is less steric hindrance, and [c] the small size gives higher resolution. [2] They are more stable conjugates, being covalent, whereas antibodies that "fall off" colloidal gold then compete for antigens, reducing gold labeling (Kramarcy and Sealock, 1990). [3] They are not aggregated, as some fraction of colloidal gold conjugates are, since colloidal gold is "sticky". They may be purified by gel filtration column chromatography to ensure only single Fab' fragments with gold are present, for example. [4] The gold size is very uniform, since they are compounds, whereas small colloidal gold preparations show high variability (Hainfeld, 1990); this can be important in high resolution work. [5] They actually give a better signal at low electron microscopy magnifications (with silver enhancement) than large colloidal gold, due to the higher staining density (Takizawa and Robinson, 1994).

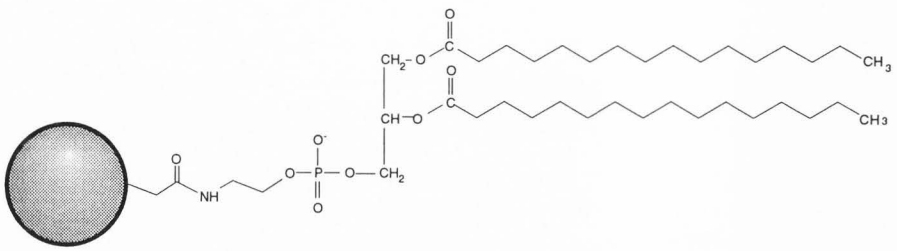
Except for high resolution, molecular level electron microscopy (EM) work, the small size of the clusters (0.8 nm and 1.4 nm) in general leads to a signal that is too weak for good visibility, so for most general EM work and light microscopy, silver enhancement is a necessity. This is a simple procedure, and is described later.

Materials

Undecagold, Nanogold, and FluoroNanogold labeling reagents and conjugates are available commercially from Nanoprobes, Inc. (25 E. Loop Rd., Ste.



Fatty Acid - gold



Phospholipid - gold

Figure 7. Gold-lipid conjugates, showing fatty acid and phospholipid derivatives.

124, Stony Brook, NY 11790, Telephone Number: 516-444-8815). A suitable gel filtration column for separating free gold clusters from labeled proteins (of higher molecular weight) is Superose 12 (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ 08854, Telephone Number 800-526-3593). For separating gold clusters from smaller molecules, GH25 column material is recommended (Amicon Inc., 72 Cherry Hill Dr., Beverly, MA 01915, Telephone Number: 800-426-4266).

Method of gold cluster labeling a protein

Making gold clusters for labeling requires organic synthesis of phosphines and ligands that are not commercially available; after these are made, the clusters must be formed, then purified (Safer *et al.*, 1986; Hainfeld, 1989). For monofunctional clusters, further purification, such as an ion exchange gradient is required. Next, to make the gold reactive, its shell must be activated with the appropriate crosslinker, such as a maleimide or N-hydroxysuccinimide ester. This must again be purified (by column chromatography) from the activating linker. Since most reactive groups hydrolyze in aqueous solution, and become inactive, the cluster must be used within a few hours (half lives are typically 4-16 hours). Fortunately, these clusters are now available commercially in activated form; these have been lyophilized so that they are stable and their hydrolysis only starts after they are reconstituted with water.

The steps for labeling a protein are therefore greatly simplified, and are reduced to the following:

[1] Dissolve lyophilized activated gold clusters (e.g., monomaleimido-Nanogold) by adding 1 ml water. Buffer is already in the gold and this reconstitutes to phosphate buffered saline (PBS).

[2] Mix with about 0.2 mg of protein to be labeled (the molar ratio of gold reagent to protein is typically 5:1). If reacting with amines, no protein pretreatment is necessary. For reacting with cysteine, proteins may have free -SH groups natively available, in which case no treatment is necessary. However, if these are oxidized into disulfides, they must first be reduced (with typically 50 mM mercaptoethylamine (MEA) for 1 hour), then separated by gel filtration from the reducing agent [MEA and dithiothreitol (DTT) contain thiols which would react with the gold]. If making Fab' or IgG conjugates, these must be prepared (For IgG, just reduce with mercaptoethanolamine, to expose hinge sulfhydryls, and column purify; for Fab', digest with pepsin to get $F(ab')_2$, then reduce and column purify to isolate the Fab' fragment (note: the Fab fragment is not used since it does not contain the hinge sulfhydryl).

[3] After 1-16 hrs, gel filter product (on e.g., Pharmacia Superose 12, in phosphate buffered saline, PBS), to separate labeled protein from unreacted gold.

Purification of the conjugate and quantitation

Step 3 above may be illustrated for Fab'-Au: after reacting Fab' with maleimido-Undecagold overnight at 4°C (although 1 hour is adequate for the maleimide-thiol reaction), the mixture was applied to a 1 x 30 cm Pharmacia Superose 12, in PBS, pH 7.4, running at 0.5 ml/min. The elution profile is shown in Fig. 8a. The first peak (here the tallest) is the Fab'-Au_{11}, and the second peak the unreacted, excess Au_{11}. Fig. 8b shows the spectrum of the first peak. Most proteins, including Fab', have a spectral peak at 280 nm, with no absorbtion above about 300 nm (they are colorless). Gold clusters have a yellow or brown color, and the spectrum of the second peak (excess Au_{11}) is shown in Fig. 8c. When gold is on the protein (first peak), the spectrum is (to first approximation) a sum of the two spectra. Here, an increase of absorbance in the 280 nm range from the protein is evident.

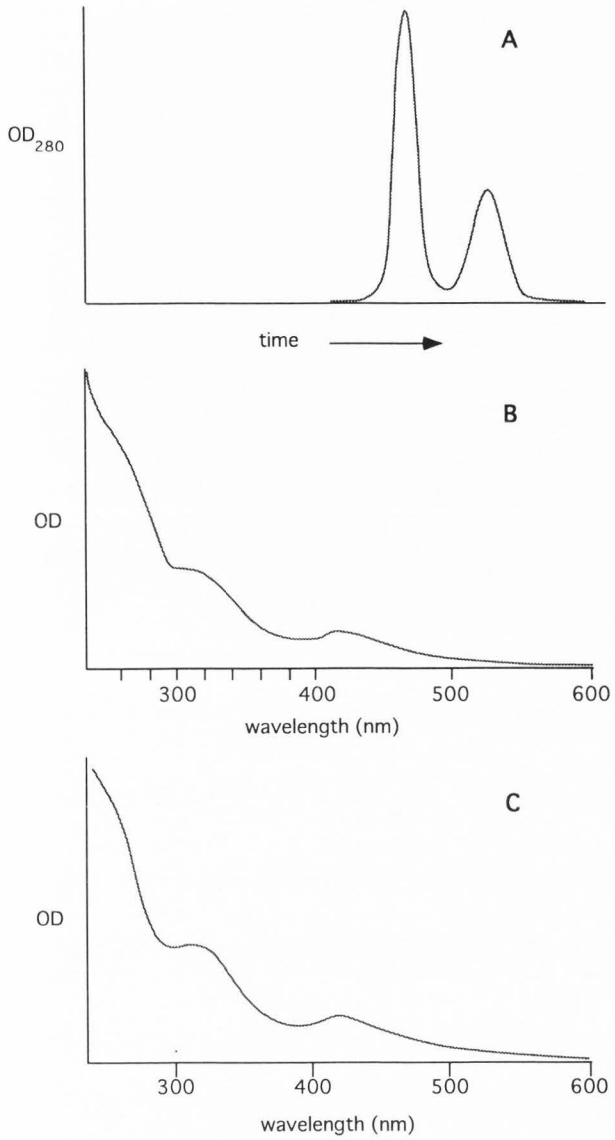


Figure 8. Gel filtration liquid chromatography of maleimide gold- Fab' reaction to isolate products. (**A**) shows the time course of products off a Superose 12 column (Pharmacia), the first and largest peak being the Fab'-Au_{11} conjugate. The second peak is unreacted gold cluster. (**B**) Spectrum of Fab'-Au_{11} conjugate peak, showing additive optical density of the two components. (**C**) Spectrum of the second peak, excess Au_{11}.

Carrying this a step further, quantitation of the labeling may be done from the spectrum; i.e., how many gold clusters are attached to each protein molecule, or what is the percentage of labeling. By knowing the spectra of the two components (and assuming that they don't alter each other when mixed), one can calculate the ratio (and amount) of each component given the composite spectrum. If the extinction coefficients are known for the two materials (gold and antibody) at two wavelengths, then a simple simultaneous equation is solved. For Fab' labeling, the equation is:

$$\text{Au/Fab ratio} = (7.5 \times OD_{420})/(4.7 \times OD_{280} - 16.8 \times OD_{420}) \quad (1)$$

This is more clearly explained in Hainfeld (1989). This is a very useful way of assaying the success of the labeling reaction without further work such as microscopy or other tests.

High resolution labeling of isolated molecules and complexes

One application of gold cluster labeling is to react the gold at particular sites on a macromolecule, then to look with high resolution electron microscopy to map the site directly, alternatively, a substrate or Fab' fragment may be labeled, then bound to the target molecule, and its position visualized. For the highest resolution, it is advantageous to see the gold particle directly, without silver enhancement. For this work, the electron microscope must be operated at 30,000 x or higher magnification, and samples must be relatively thin, without the usual high density stains, all which would obscure visibility of the small gold clusters.

STEM Molecular Applications

The high resolution scanning transmission electron microscope (STEM) has been shown to be useful at this level, due to the high visibility of the gold clusters in darkfield on freeze dried isolated molecules adsorbed to a thin carbon film. With this microscope, undecagold clusters are clearly visible (as are even single heavy atoms), whereas with a commercial TEM, undecagold is not usually clearly seen, except by image analysis of ordered arrays. An example is the labeling of a Fab' fragment to the C-terminus of the Aα chain, shown in Fig. 9. Since this is a review, the reader is referred to published articles concerning:

* labeling Fab' with undecagold and Nanogold (Hainfeld, 1987, 1989; Hainfeld and Furuya, 1992).

* Fab'-Au_{11} localization on the phosphorylase kinase molecule to map a specific subunit (Wilkinson *et al.*, 1994),

* gold labeling of the mobile substrate-carrying arm in the pyruvate dehydrogenase complex in order to determine its position and range of motion (Yang *et al.*, 1994).

* gold labeling the substrate, dihydrofolate reductase (dhfr), of GroEL in order to determine the location where ATP-dependent folding occurs (Braig *et al.*, 1993).

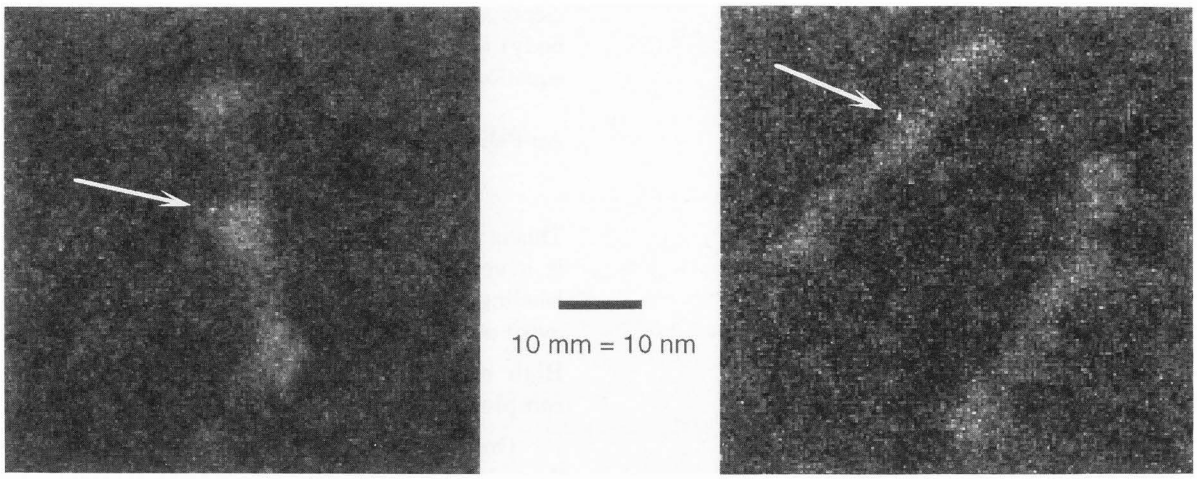


Figure 9. Darkfield field emission STEM micrographs of Fab'-Au_{11} bound to C-terminus of Aα chain of fibrinogen showing its putative molecular location (done in collaboration with G. Matsueda).

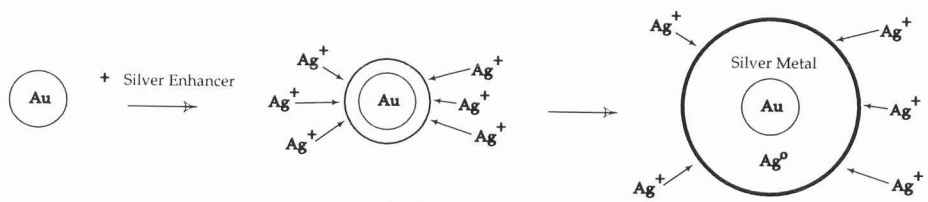


Figure 10. Schematic of the silver enhancement of gold process.

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* direct labeling of tRNA and its use in mapping the ribosome binding site (Hainfeld *et al.*, 1991, 1993; Blechschmidt *et al.*, 1993)

The Brookhaven STEM is an NIH Biotechnology Resource, and is freely available to scientists with suitable molecular labeling studies. Inquiries should be directed to the author at: Brookhaven National Laboratory, Biology Dept., Upton, NY 11973, Telephone Number 516-344-3372.

TEM Molecular Applications

Although the Undecagold cluster is more difficult to see directly in conventional TEMs, the 1.4 nm Nanogold cluster is easily visualized in thin samples (< about 80 nm), and in frozen hydrated samples (Boisset *et al.*, 1992). The clusters give their best signal with low values of defocus, about 0.5 μm, and can be seen at approximately 10 electrons/Å^2. Undecagold damages with beam dose (Wall *et al.*, 1982), and its signal becomes weaker, whereas Nanogold is very beam resistant (Hainfeld and Furuya, 1992) and can be clearly seen on a thin carbon film, by going to a very high magnification (100,000 x) with a high beam current. For high resolution biological applications, the beam must be kept low to prevent specimen damage, so the usual method has been to use ordered arrays or many single particles that are later computer aligned and averaged to improve the signal-to-noise ratio. A number of interesting projects have now been completed using these gold clusters to identify important biological sites or functions. The reader is referred to some of these works:

* Cys-374 of F-actin was labeled with undecagold

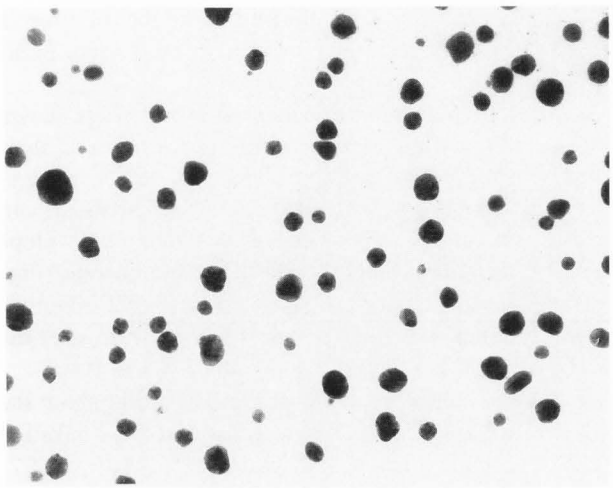

Figure 11. Electron micrograph of silver enhanced Nanogold. Particles are in the 20 nm range. Full width 530 nm.

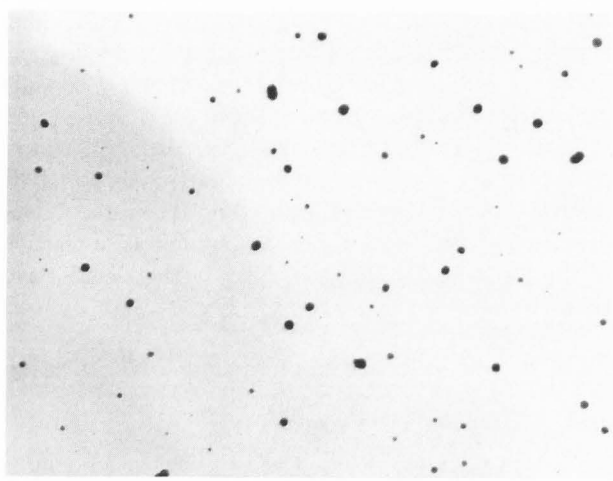

Figure 12. Electron micrograph of silver enhanced Undecagold. Particles are in the 10 nm range. Full width 530 nm.

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and its position visualized by image processing fibers in ice (Milligan *et al.*, 1990).

* A thiol on calmodulin was labeled with Nanogold, then it was bound to the calcium release channel to determine the calmodulin binding site. This was done by image processing isolated calcium release channel complexes by cryo-EM (Wagenknecht *et al.*, 1994).

* The γ subunit of the F1 ATPase complex was localized by labeling with Nanogold and image processing of cryo-EM molecules (Wilkens and Capaldi, 1992).

* The method of insulin blocking the proteosome in protein unfolding was studied by labeling a thiol on the insulin B-chain (Wenzel and Baumeister, 1995).

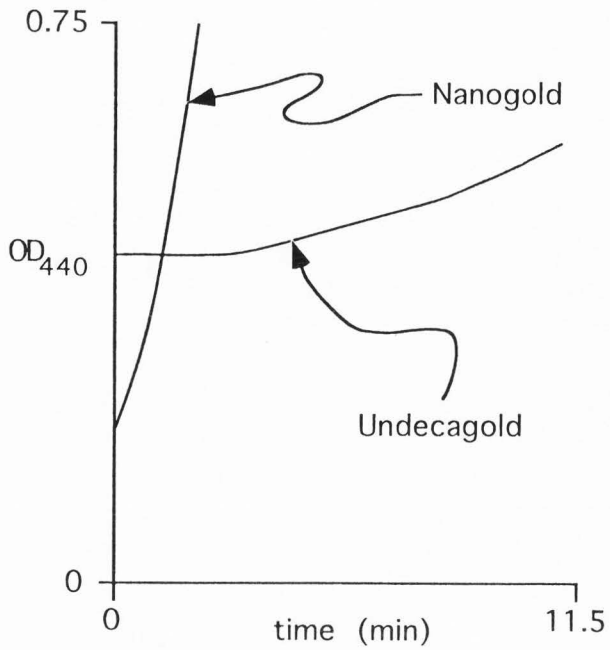

Figure 13. Graph showing the development progression with time of silver enhancement of Nanogold and undecagold. Absorbance measurements were taken of a solution in a cuvette in a spectrophotometer. Nanogold concentration was 2.2 x 10^{-6} M, and that of undecagold, 1.2 x 10^{-5} M. The undecagold, even though more concentrated, shows less development with a clear delay in starting development of about 5 min.

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* Active site thiols in α-2 macroglobulin were visualized by Nanogold labeling, cryo-EM and image processing (Boisset *et al.*, 1992, 1994).

* Undecagold labeled cytochrome oxidase crystals were analyzed by glucose/uranyl acetate embedding and image processing to identify the subunit III site (Crum *et al.*, 1994).

* Nanogold was visualized in unstained Lowicryl sections of immunolabeled red blood cells (Hainfeld and Furuya, 1992).

Silver enhancement

An important discovery was that the silver metal deposition process, similar to photography, could be applied to gold particles. In photography, silver halide crystals are in the emulsion, and lead to silver grains after light activation and chemical reduction. Silver enhancement of gold particles is slightly different in that the source of silver is supplied in the developer solution along with the reducing agent. Silver ions are reduced to insoluble silver metal on interaction with the gold metal surface (which then becomes a silver metal surface, Fig. 10). This is a specific interaction nucleated by the gold particle, and can amplify the signal substantially.

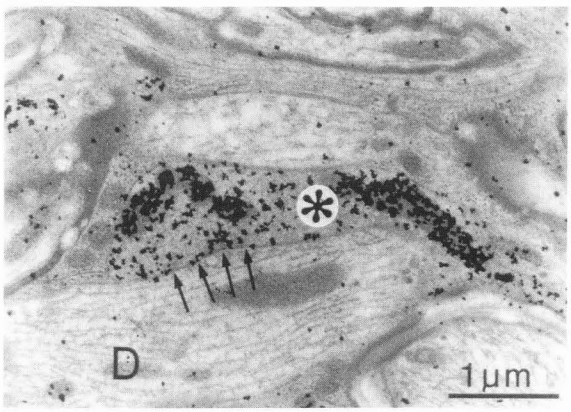

Figure 14. Transmission electron micrograph showing GABA-containing terminals (asterisks) forming symmetrical synaptic specializations (arrows) with dendrites (D) in the thoracic spinal cord of the rat. After embedding in Durcupan (Fluka) and sectioning, immunolocalization was done by incubating with GABA antiserum (Incstar, 1:2000, 4°C, 18 hours), Nanogold (goat anti-rabbit, 1:40, room temperature, 90 min), and intensified with HQ Silver (Nanoprobes) for 6 min. Counterstaining was with lead citrate. There is excellent structural preservation and the silver enhanced gold particles are about 20 nm in size. Besides the main GABA-containing region heavily stained in the center (arrows), smaller GABA-positive structures are also seen where there are multiple gold/silver particles, e.g., above and to the left of the central region. This work was done by Dr. Sarah Bacon, Oxford University, Department of Pharmacology, U.K.

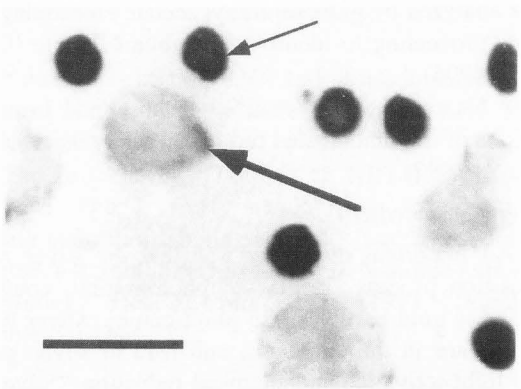

Figure 15. Light micrograph of human red blood cells (RBCs, small arrow) and HeLa cells (large arrow) incubated with anti-RBC IgG-Nanogold, then silver enhanced. The red blood cells turned totally black. Bar = 18 μm.

Developers can autonucleate after a period of time (a much slower process) and may contribute some background.

Micrographs of silver enhanced Nanogold is shown in Fig. 11 and Undecagold in Fig. 12. Even with their organic shell, they develop; however, undecagold is substantially slower, and has a delay of about 5 min before appreciably starting, whereas Nanogold develops rapidly, and immediately starts off at a high rate (Fig. 13). Gold clusters may be grown to sizes of 2-80 nm or more, but their size distribution is less uniform after the process.

Several studies report quantitative results about the silver development of Nanogold and its optimization (Hainfeld and Furuya, 1995a; Burry *et al.*, 1992; Takizawa and Robinson 1994, Stierhof *et al.*, 1995; Burry, 1995).

Osmium

Osmium tetroxide is commonly used as a counterstain. However the osmium can oxidize the silver back into solution, thus removing the silver grain either partially or completely. Burry studied this process and found a moderate level of osmium (0.1% for 30 min) gave good staining in tissues, and did not appreciably affect the silver size of the developed Nanogold (Burry *et al.*, 1992). An alternative solution was found by Sawada and coworkers, who gold toned the silver particles, which leaves a gold coating that is unreactive with osmium (e.g., 1% OsO_4 for 2 hours; Sawada and Esaki, 1994).

TEM Results in Tissue Studies: Immunolocalization with Nanogold

An excellent application of gold cluster labeling is immunohistological studies, for the reasons given above under "Gold Cluster Immunoprobes". In short, they generally give better staining than with colloidal gold probes due to their small size, high immunoreactivity and stability, and superior penetration. Several comparative studies have been done at this point to demonstrate these differences (Vandré and Burry, 1992; Takizawa and Robinson, 1994). Due to the thickness of tissue sections and counterstaining with OsO_4, lead citrate, uranyl acetate, or other stains, it is a requirement that the gold is silver enhanced. Because Nanogold develops more quickly and robustly than Undecagold, Nanogold is used in most of these applications. An example of the excellent localization, heavy antigen staining, and structural preservation is shown in Fig. 14. The use of Nanogold immunoconjugates in virtually all methods of tissue preparation (pre- and post-embedding, frozen sections and resin embedded) has now been documented.

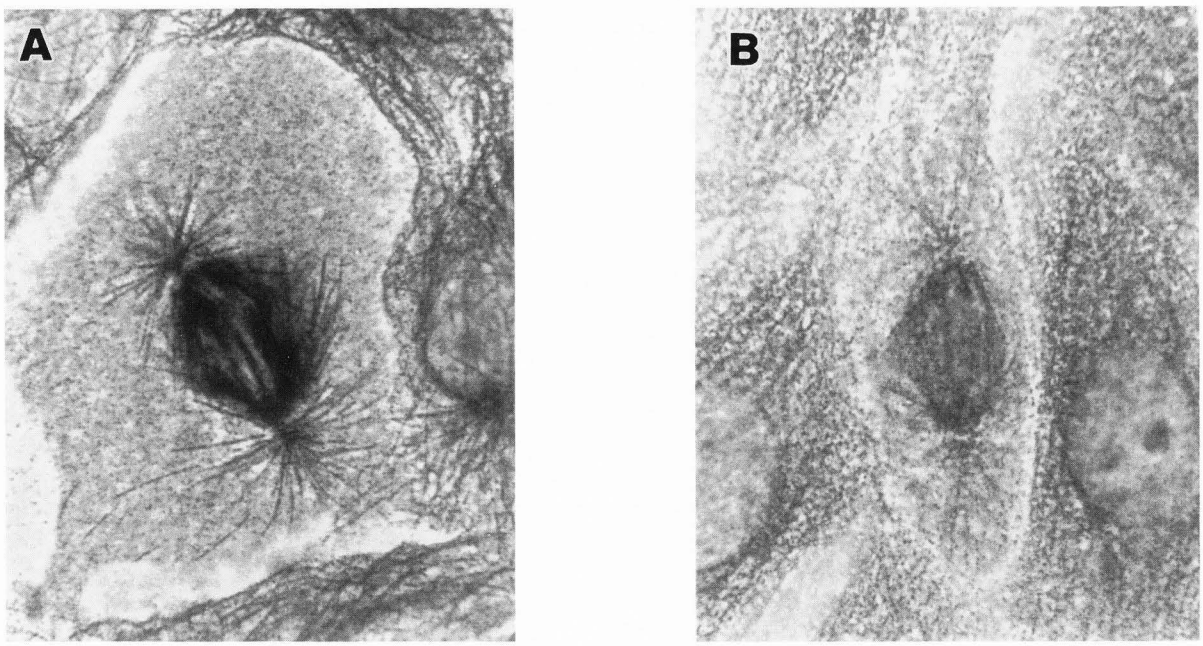

Figure 16. (A) Light micrograph of spindle microtubules labeled with a monoclonal anti-tubulin antibody, followed by Nanogold-Fab', and silver enhanced. LLC-PK cells were grown in monolayer culture, fixed with glutaraldehyde (0.7%, 15 min), permeablized with 0.1% saponin, incubated with anti-tubulin antibodies (1:250, Amersham) for 1 hour at 37°C, rinsed, then incubated with anti-mouse Nanogold-Fab' (1:50, Nanoprobes) for 1 hr at 37°C. Samples were post-fixed, silver enhanced with a N-propyl gallate enhancer for about 9 min. Cells were mounted in Mawwol and examined by bright field microscopy. Intense staining of microtubules was observed. Full width 95 μm. (B). Same preparation as described in A, except that AuroProbe One goat anti-mouse was used (1:50, Amersham). Only weak staining was observed. Full width 95 μm. This work was done by Drs. Dale Vandré and Richard Burry, Department of Cell Biology, Neurobiology, and Anatomy, The Ohio State University.

The reader is referred to the following original papers that use Nanogold for immunoelectron microscopy for detailed experimental protocols and results:

Preembedding

* Phosphoproteins associated with the mitotic spindle were localized; embedding in Epon (Vandré and Burry, 1992).

* Metabotropic glutamate receptor localization in neurons; freeze substitution and Lowicryl embedding (Baude *et al.*, 1993)

* Improved methods for *in vitro* cells; localization of synaptophysin in rat PC12 cells; embedding in Epon (Tao-Cheng and Tanner, 1994).

* Study of silver enhancement in pre-embedding immunocytochemistry; embedding in Epon (Burry *et al.*, 1992).

Localization of glutamic acid decarboxylase in the cerebellum; embedding in Spurr's resin (Gilerovitch *et al.*, 1995).

* Immunocytochemical localization of the α1 and β2/3 subunits of the $GABA_A$ receptor; freeze substitution and Lowicryl embedding (Nusser *et al.*, 1995a).

* Study of neural connections with neurobiotin and biocytin; embedding in Epon/Araldite (Sun *et al.*, 1995).

* Immunolocalization of *Drosophila* phosphatidylinositol transfer protein; embedding in Epon (Suzuki and Hirosawa, 1994).

* Quantitative immunogold method to determine densities of $GABA_A$ receptors on cerebellar cells; embedding in epoxy resin (Nusser *et al.*, 1995b).

* Anti-laminin and anti-fibronectin in rat testis localization using Nanogold, silver enhancement, gold toning, and osmium fixing; embedding in Epon 812 (Sawada and Esaki, 1994)

* Study of developers, buffers, and osmium effects; introduction of buffered n-propyl gallate as a better developer (Burry, 1995).

Postembedding

* Labeling calcium-ATPase in sarcoplasmic reticulum; embedding in LR White (Krenács and Dux, 1994).

* Quantitative immunogold method to determine densities of $GABA_A$ receptors on cerebellar cells; slam-

freezing, freeze-substitution, and Lowicryl embedding (Nusser *et al.*, 1995b)

* Metabotropic glutamate receptor localization in neurons; freeze substitution and Lowicryl embedding (Baude *et al.*, 1993)

* Immunocytochemical localization of the α_1 and $\beta_{2/3}$ subunits of the $GABA_A$ receptor; freeze substitution and Lowicryl embedding (Nusser *et al.*, 1995a).

* Comparison of embedding media (Epon 812, Durcupan ACM, Lowicryl K4M, LR-White and LR-gold; discussion of microwave treatment (Krenács and Krenács, 1995).

Ultra-thin cryosections

* Localization of lactoferrin and myeloperoxidase in neutrophils; 90 nm cryosections were labeled then embedded in Epon and thin-sectioned (Takizawa and Robinson, 1994).

Low magnification TEM and double labeling

Another interesting finding was that the silver enhanced Nanogold immunoprobe was more easily visible at low magnifications (about 10-20,000 x) than other larger colloidal golds, due to the much higher labeling density followed by the very visible silver size (approximately 20 nm) (Takizawa and Robinson, 1994). These authors also demonstrated that silver enhanced Nanogold (approximately 25 nm) could be clearly used as a double immunolabel with 10 nm colloidal gold.

Light microscopy

Immunolabeling with Nanogold-Fab' conjugates gives well labeled antigens and leads to clear visibility of the structures in the light microscope after silver enhancement (Fig. 15). Better penetration into tissues than with colloidal gold immunoprobes (even 1 nm colloidal gold) produces better visibility at the light microscope level (Fig. 16) and also at the EM level (Vandré and Burry, 1992).

An advantage of silver enhanced gold labeling at the light microscope level is the brown color produced that does not interfere with most other stains typically used. Many cells autofluoresce, and since the silver enhanced gold has equal or better sensitivity, it may be used in such cases, or as a dual label with fluorescent probes.

Staining blots

The high sensitivity of silver enhanced Nanogold makes it useful for immunoblot detection (Fig. 17). Sensitivity goes to the 0.1 pg range (7×10^{-19} moles), making it one of the most sensitive methods available (Hainfeld and Furuya, 1995a); it is in the same range as chemiluminescence, but requires about 40 min development and produces a permanent record, whereas chemiluminescence generally takes 24 hrs for this level, and requires film and film processing/printing. An example of its use in a blot of a gel was described by Wilkens and Capaldi (1992).

Staining gels

The use of gold to detect bands on gels was described for colloidal gold, but necessitated making a blot first, due to the limited penetration of colloidal gold into the gel matrix. Nanogold or undecagold labeled proteins run on gels similar to the native proteins, or in some cases, shifted up by the weight of the gold (15,000 or 5,000, respectively, Weinstein *et al.*, 1989, Hainfeld and Furuya, 1995a). When the gel is exposed to the silver enhancing solution (a different formulation than the standard silver stain for gels), a black-brown band develops in < 5 min, giving more rapid detection than with other gel stains (Coomassie blue, etc.); only the gold labeled proteins are stained.

New Developments

In situ hybridization (ISH)

Testing of Nanogold-streptavidin has recently shown it to give a better signal than alkaline phosphatase or avidin-biotin (S-ABC)-peroxidase/DAB for ISH, with sensitivity down to the single copy or very low copy level (G. Hacker, personal communication). The increased sensitivity without background is probably due to the higher binding constant of streptavidin as compared to antibodies, and the efficient and high amplification silver enhancement yields with Nanogold.

Gold Lipids

Since the gold clusters can be reacted with most molecules, one can imagine a number of novel conjugates. One is to link them to fatty acids or phospholipids, thus producing a gold on one end, and a long alkyl chain on the other (Fig. 7). Since the gold is water soluble (hydrophilic), this yields an ampipathic molecule that should behave similar to the original lipids, namely, they should insert into membranes, for example. It should be possible to use them in synthetic liposomes to follow these structures microscopically.

FluoroNanogold

Another recently developed conjugate is the covalent combination of a fluorophore with a gold cluster (Powell *et al.*, 1994), also attaching a Fab' antibody fragment (Fig. 18). This has the interesting property that the fluorescence is not appreciably quenched by the gold particle; earlier work attempting dual gold-fluorescent probes with colloidal gold showed extensive quenching by the colloidal gold particle, and so further use was halted. By combining two probes into one, live cells, for example, can be viewed in the confocal microscope and a label visualized. When the distribution is optimal, these same cells can be processed for EM to determine

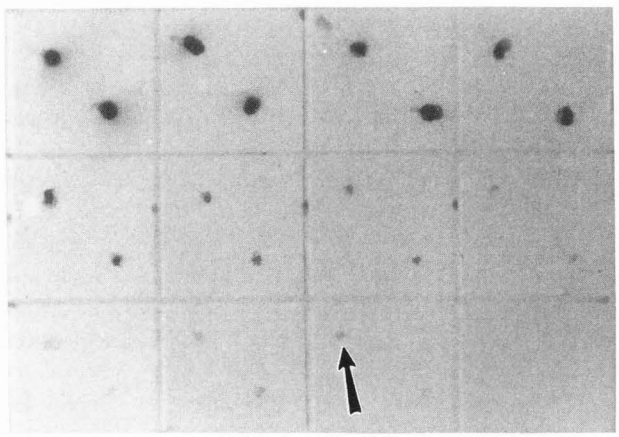

Figure 17. Sensitive immunodot blot obtained with Nanogold anti-mouse Fab' to a mouse IgG target showing 0.1 pg detection (arrow), corresponding to 6.7 x 10^{-19} moles of target. 1 μl of mouse IgG (antigen target) was spotted onto nitrocellulose containing the following amounts (each box contains a duplicate spot): top row (of spots): 10 ng, 2.5 ng, 1 ng, 0.25 ng; middle row: 100 pg, 25 pg, 10 pg, 2.5 pg; bottom row: 1 pg, 0.25 pg, 0.1 pg, buffer blank. Membrane was blocked with 4% BSA. Goat anti-mouse Fab'-Nanogold was incubated 2 hr, washed and developed 2 x 15 min with LI Silver. Buffer and non-specific antibody/antigen controls were blank.

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the ultrastructural organization.

Polyaldehyde gold

It is possible to synthesize gold clusters with multiple reactive groups, which might be useful for attaching multiple peptides, antibody fragments, DNA strands. Undecagold has 21 organic "arms" on its surface (Fig. 1), which can then be made reactive for linking. This has been done using the aldehyde functionality (Fig. 19), which can then lead to singly labeled or oligomeric products (Hainfeld and Furuya, 1995b).

Radioactive gold clusters

Natural gold (^{197}Au) can be made radioactive (^{198}Au and ^{199}Au) by neutron bombardment in a nuclear reactor. Only a small percentage of the gold is converted (about 0.03%), and if all gold atoms are to be radioactive, pure ^{199}Au can be made by irradiating ^{198}Pt. These golds have approximately a 3-day half life, emit an intermediate β, as well as a an imageable γ. The β can kill cells, and has an average range of 100-460 μm. One possible application is to attach gold clusters to anti-tumor antibodies and thus target the dose for selective cell killing (Hainfeld *et al.*, 1990; Hainfeld, 1995). Radio-

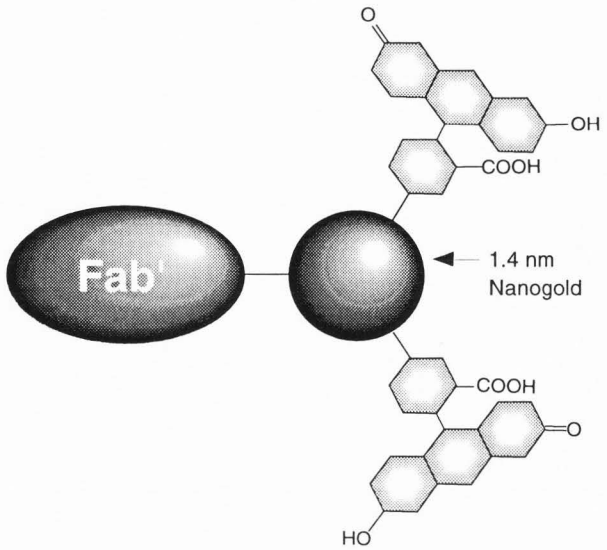

Figure 18. Diagram of FluoroNanogold.

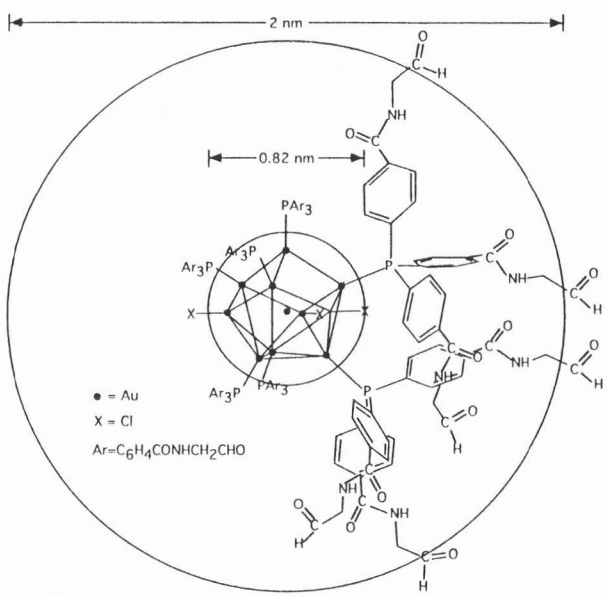

Figure 19. Diagram of polyaldehyde Undecagold.

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active gold is one of about 10 radionuclides deemed to be best suited for this type of work. The major problems seem to be that intravenous injection of these conjugates have uptake in other tissues which limits the dose to tumor to unsatisfactory levels. A more tractable application, for the moment, is a topical application, such as treatment of superficial bladder carcinoma, where the conjugate can be introduced into the bladder for about 0.5 hr, then lavaged, leaving the specifically targeted gold to do its work. A graph showing the

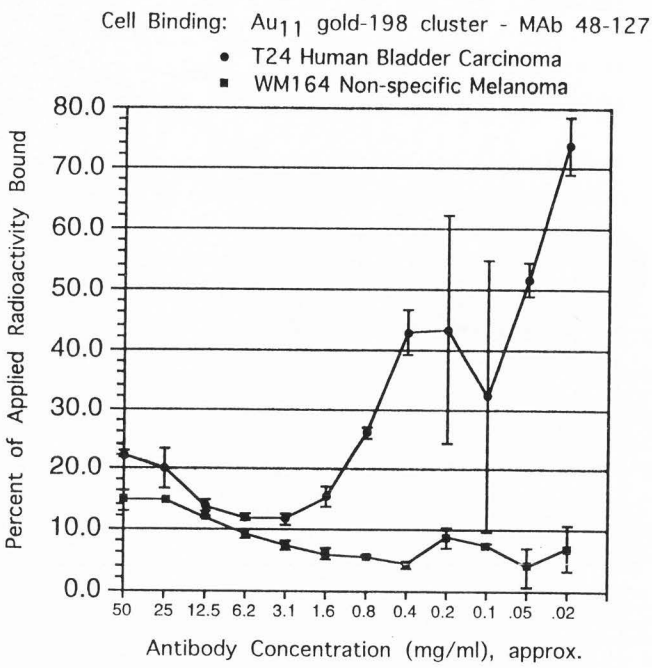

Figure 20. Graph showing the targeting of $^{198}Au_{11}$ clusters conjugated to a monoclonal antibody to human bladder carcinoma cells (MAb 48-127, gift from Y. Fradet), with minimal uptake by a non-specific cell line, WM164. Maximal tumor to non-tumor ratio was 13.6.

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targeting of $^{198}Au_{11}$ clusters conjugated to a monoclonal antibody to human bladder carcinoma cells, with minimal uptake by a non-specific cell line is shown in Fig. 20.

Conclusion

Gold cluster chemistry has opened new areas of applications in cell biology, medicine, and material science. Many novel conjugates can be formed with stable covalent links and well defined products. The properties of the gold clusters often give them important advantages over colloidal gold counterparts. The gold provides a versatile and highly sensitive reporter group that can be seen by electron microscopy, or with silver enhancement, light microscopy, and the unaided eye.

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The author would like to thank N.I. Feng for excellent technical assistance, J.S. Wall, M. Simon, B. Lin, and F. Kito for STEM microscopy, F.R. Furuya and R.D. Powell for significant contributions, and R. Burry and S. Bacon for use of their micrographs. Support of this work was through NIH #RR017777 and US DOE, OHER.

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Discussion with Reviewers

R.M. Albrecht: Do non-covalent interactions between the gold-organic clusters and antibodies, ligands, etc. occur and is this ever a consideration when conjugating them to molecular species or when labeling with the conjugates?

Author: For the most part the answer is no. One can take gold clusters that do not have the activated linking arm and incubate them with the antibody, or other target, then separate the products by column chromatography or polyacrylamide gel electrophoresis. Peaks from columns can easily be quantitated for gold cluster by the UV-visible absorption, e.g., at 420 nm, where most proteins don't absorb. Gold in gels may be detected by silver enhancement. Typically, virtually no gold is found with the protein, unless it is specifically covalently linked. The linkers used, e.g., maleimide and N-hydroxysuccinimide ester are very specific for thiols and amines, respectively, and this can be similarly demonstrated. In a few unusual cases, gold clusters have been found to bind to certain macromolecules. However, this may usually be reversed by using a higher ionic strength. Another alternative is to make the gold with different surface groups, e.g., with a sugar- or PEG-like coating. The converse is also true: gold clusters can be made to intentionally non-covalently bind to certain substrates, e.g., by making them highly charged (plus or minus) or hydrophobic. The usual clusters are made with N-methyl benzamide groups which confer high water solubility, but have no charge.

R.M. Albrecht: What is the overall size, gold plus organic shell, of the nanoprobes?

Author: For Undecagold, the core contains 11 gold atoms in a 0.82 nm sphere, and the covalently attached phosphine ligand shell makes the total diameter 2.0 nm. This structure has been solved by x-ray crystallography. For Nanogold, the core contains approximately 67 gold atoms in a 1.4 nm sphere, and with the ligand shell, it is 2.7 nm in diameter. This has not been solved by x-ray, but in the EM we have observed small crystals. When labeling a target molecule, the distance measured would be from the linking site to the center of the gold cluster, giving about 1.4 nm resolution.

R.M. Albrecht: In our experience the conjugation of 3 nm colloidal gold to Fab fragments is useful as it produces a 1:1 gold:Fab ratio; the probe has a valence of 1 and is readily detectable. However, not all Fab fragments can be successfully conjugated to 3 nm gold via the non-covalent, hydrophobic route. Do the undecagold-Fab' or nanogold-Fab' conjugates (via the sulfhydryl link) generally work well with most Fab' without a drop in antibody affinity?

Author: Yes. The hinge sulfhydryl on Fab' fragments is very accessible, and labels very well across all species. The maleimide linking group is very specific and reactive with free thiols, and forms a stable covalent bond. Coupling is done under mild conditions (e.g., pH 7, phosphate buffer, 150 mM NaCl, 4°C), so that the antibody is not denatured. Since the hinge site is at the opposite end of the molecule to the antigen binding hypervariable region, this is an ideal place for the gold, so as not to compromise immunological activity. Some quantitative measurements of antibody activity after gold cluster labeling were made using a radioimmunoassay with mouse monoclonals and showed immunoreactivities of 76-83% (Hainfeld, 1995).

R.M. Albrecht: Probes synthesized to contain one undecagold or one nanogold cluster per Fab' or per

other molecule or active molecular fragment are desirable for quantitative studies. In cases where silver enhancement is necessary to detect the undecagold or nanogold is the enhancement procedure sufficiently uniform and controllable to permit accurate quantitative analysis?

Author: For quantitation, one would like to have no particles so underdeveloped that they are below the detection limit, or so overdeveloped that they coalesce. Nanogold may be developed to produce ~15 nm particles which are not generally overlapping, but their size varies more than a well prepared 15 nm colloidal gold. Frequently a higher density of labeling is seen with Nanogold than in a parallel experiment with colloidal gold (e.g., Vandré and Burry, 1992), which raises some questions about quantitating antigens with colloidal gold probes (which may not penetrate as well, and may have antibody that has dissociated from the gold and competes for sites). In high resolution STEM or TEM of silver enhanced Nanogold and undecagold, there are still some gold clusters that would be below the usual detection level in tissue work, so quantitation will be only approximate. A well controlled study is needed to thoroughly answer this question. Undecagold develops more slowly than Nanogold, and with more variability, and with less final product for the same concentration of gold particles (Hainfeld and Furuya, 1995). It would appear that undecagold is less suitable for quantitative work when using silver enhancement.

R.M. Albrecht: Where the gold particles are sufficiently smaller than the antibody or ligand or the active fragments of antibody or ligand, we have seen, via correlative HR-SEM and SFM, that the colloidal gold particles intercalate themselves within the molecule rather than sticking to the "outside" of the molecule. What is the structure of Fab'-undecagold and Fab'-nanogold conjugates? Does attaching undecagold or nanogold via the free amino route ever tend to "blanket" the molecule and reduce activity or penetration?

Author: Contrary to your results with colloidal gold, undecagold and Nanogold appear at the periphery of many molecules, and specifically for Fab', which has a ellipsoid shape (5.0 x 4.0 x 3.0 nm), it appears at one end (see Fig. 4 in Hainfeld, 1987). Another interesting result demonstrating the site specific attachment of the gold clusters was shown for Nanogold, where it was reacted with the hinge sulfhydryls in IgG, and high resolution images showed it to be at exactly the expected position at the vertex of the "Y" shaped molecules (Fig. 7a in Hainfeld and Furuya, 1992). Nanogold synthesized with two reactive arms reacted with 2 Fab's and the gold was found in the center of this construct, as expected (Fig 7b, same paper).

Your second question pertains to amino labeling; since proteins can have many lysines, do gold clusters that react with primary amines (e.g., NHS-Nanogold) overly coat the molecule and reduce activity/penetration? This is not an actual problem, since the microenvironment (pK_a) of the amines varies and some are more reactive. One limits the amount of NHS-Nanogold, and the reaction time, so that typically one gold cluster is attached (if that is what's desired). From the column purification and UV-visible spectrum, the stoichiometry can easily be determined (of gold to protein). In practice, overloading proteins with gold is not a problem.

R.M. Albrecht: It is suggested that penetration of undecagold probes (2 nm diameter gold+organic) and nanoprobes may be better than probes attached to 1-3 nm colloidal gold particles. Given that in both cases the marker, undecagold or colloidal gold, is considerably smaller that the molecule to which it is conjugated, what do you feel is the most likely explanation for the increased penetration? In the Vandré paper whole antibody molecules were used for the primary antibody. Increased labeling seen with the second antibody-nanoprobe conjugate as compared to second antibody-1 nm colloidal gold conjugate was attributed to the presence of un(gold)labeled 2nd antibody in the prep. The Takizawa and Robinson (1994) study showed that nanoprobes attached to Fab' gave greater intensity of labeling, than 1 nm colloidal gold conjugated to whole antibody. Not surprising since the total probe size (antibody fragment + gold) of the Fab'-nanoprobe is very much smaller than the whole antibody-1 nm colloidal gold. Again this study employed gold labeled second antibody and in both cases the primary antibody was whole antibody. So in any case the presence of specific second antibody (either as whole ab or as Fab' antibody fragments) is limited to wherever the primary antibody, a whole antibody, can access. Have there been any comparative studies where the primary antibody prepared as an Fab' conjugated to 1-3 nm colloidal gold was compared to primary antibody prepared as an Fab' conjugated to nanogold and/or undecagold -- with care taken to insure that neither prep had free un(gold)conjugated Fab'?

Author: Fab'-3 nm colloidal gold conjugates (goat anti-mouse) were freshly prepared and chromatographed, and compared with Fab'-Nanogold (using the same antibody) on immunoblots targeting mouse IgG, followed by silver intensification. The Nanogold probe gave a factor of 10 greater sensitivity; this result was consistently found with other antibodies. Since accessibility was not a problem with this assay, one simple explanation is that the Nanogold conjugates retain better immunoreactivity.

When a 1 nm colloidal gold antibody conjugate was

analyzed by high resolution STEM microscopy, substantial aggregation was observed (Hainfeld, 1990). The STEM is useful since both antibody and gold are clearly visible, and mass measurement may be used to identify one antibody. Since Nanogold conjugates have no tendency to aggregate, and are column purified as monomers, it appears that typical colloidal gold conjugates may have limited penetration due to the fraction of the material that is aggregated. The STEM study also revealed a much wider gold size distribution for the "1 nm" colloidal gold; it varied from 1 to 3 nm.

The Takizawa and Robinson (1994) study compared penetration of 1.4 nm (Nanogold)-Fab', 1.4 nm (Nanogold)-IgG, and 1 nm colloidal gold IgG in 1-2 μm cryosections that were then cross-sectioned. They found 1.4 nm (Nanogold)-Fab' > 1.4 nm (Nanogold)-IgG > 1 nm colloidal gold-IgG (their Fig. 8). In other words, better penetration was not only due to the use of Fab' rather than IgG.

A last point concerns the actual size of the gold conjugates. Nanogold has a 0.6 nm organic shell around it and needs no further stabilization. It has a single Fab' covalently attached. Colloidal gold needs to be stabilized with antibody, BSA, PEG, or other substance. BSA is standardly used, which is 68 kD, larger than an Fab' (50 kD). Hence a colloidal gold may have other molecules bound that are 4-5 nm in size. This is about 7 times the size of the Nanogold shell, and could easily double the size of a Fab'-3 nm colloidal gold probe, thus making it an overall larger probe. Baschong and Wrigley (1990) discuss in detail experience in making 1.5-2.6 nm colloidal gold-Fab conjugates. They found that 2.6 nm colloidal gold adsorbed from 1 to 6 active Fab molecules per gold particle (their Fig. 4). By titrating BSA, they could prepare conjugates that contained predominantly one active Fab molecule (but also had adsorbed BSA molecules).

J. Beesley: In Figs. 11 and 12, especially 11, there appears to be a considerable number of small particles (approximately 1/10 size of the silver-enhanced particles) in the background. Would the author please comment on what these are and how to reduce them?
Author: My interpretation is that these are gold particles that have developed less than the larger particles. The silver enhancement of gold is already known to produce some variability in size, but in many examples, the development of Nanogold has produced strikingly uniform particle sizes. See, for example, Krenács and Krenács (1995), Fig. 4 on page 65. They found that more uniform particles developed with post-embedding immunostaining using Lowicryl K4M and LR-White than with Epon. Development for 3 min on a Lowicryl K4M section using a silver acetate developer (without gum arabic) gave extremely uniform 8-10 nm particles with no small (or large) particles (their Fig. 4). One might well use their methods to also achieve very uniform development.

Silver growth characteristics will also depend on the developer used. R. Burry and colleagues compared the growth time course and particle size distribution of silver enhancement using his N-propyl gallate developer formulation, and found that Nanogold gave a tighter size distribution than 1 nm colloidal gold (Burry *et al.*, 1992, see Fig. 5).

J. Wroblewski: There is a great need to obtain highly specific probes and good detection systems for *in situ* hybridization on the ultrastructural level. Are there any other Au-based, sensitive detection systems than Nanogold-streptavidin that can be used in electron microscopical studies?
Author: Colloidal gold-streptavidin conjugates have been used, but are somewhat unstable and give poorer results (lower signal/more background) than Nanogold-streptavidin (Hacker *et al.*, 1996). A recent improvement in the sensitivity to routine single copy detection has been achieved by combining the CARD system (catalyzed reporter deposition, where biotin is enzymatically amplified by peroxidase acting on the substrate biotinyl-tyramide) followed by Nanogold-streptavidin (Hacker *et al.*, 1996). Another alternative to streptavidin is to use a Nanogold-anti-biotin conjugate, but in our tests this does not perform as well as Nanogold-streptavidin. Also, DNA/RNA probes with digoxigenin or fluorescein (or other antigens) can be probed with gold-antibody conjugates to digoxigenin or fluorecein.

J. Wroblewski: Can Nanogold-streptavidin be used on resin-embedded material, and which resins are most suitable for this technique?
Author: No exact study of this question has been done, to my knowledge. However, rather extensive work was done evaluating Nanogold-antibody conjugates on resin-embedded material (i.e., incubation of gold with embedded material that had been sectioned) (Krenács and Krenács, 1995). Nanogold was successfully used with Epon 812, Araldite (Durcupan ACM), LR-White, LR-Gold, and Lowicryls. Epoxides needed to be treated with sodium (m)ethoxide, and acrylate (Lowicryl and LR-white/gold) sections were microwaved for best results. Further technical details may be gleaned from other articles referred to above under the heading "Pre-embedding" and "Postembedding".

Additional References

Baschong W, Wrigley NG (1990) Small colloidal

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