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Dean A. Handley
Sandoz Research Institute

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RECEPTOR-MEDIATED BINDING, ENDOCYTOSIS AND CELLULAR PROCESSING OF MACROMOLECULES CONJUGATED WITH COLLOIDAL GOLD

Dean A. Handley

Platelet Department
Sandoz Research Institute
East Hanover, New Jersey USA 07936

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Abstract

Receptor-mediated expression of cellular functions assures biological specificity, regulation, and control of nutritive and metabolic requirements. The study of receptor-ligand interactions is a central theme in many published reports employing colloidal gold labeling. With the ligand directly conjugated with colloidal gold, the investigator is afforded the opportunity to observe all phases of cellular binding, endocytosis, lysosomal delivery and catobolism. Reviewed are published studies employing direct ligand conjugation with colloidal gold, the relative merits and disadvantages of this type of procedure and data from recent studies investigating endothelial receptor binding of proteins in the coagulation and fibrinolysis cascades.

Key words: Colloidal gold, receptors, endocytosis, plasma proteins, coagulation factors, humoral factors, membranes, lysosomes, electron microscopy, clotting

Address for correspondence:
Dean A. Handley
Platelet Department
Sandoz Research Institute
E. Hanover, N.J. 07936 USA
Phone no.: (201) 386-8753

Introduction

First introduced over 25 years ago [58], colloidal gold labeling is now enjoying exceptional popularity among cell biologists and electron microscopists. It is a readily available, inexpensive, reproducible and versatile research method that is being applied to a variety of cell structure-function studies. While the immunogold methods are still the most frequently employed labeling techniques, a considerable number of studies have been reported that utilize direct ligand or macromolecule conjugation with colloidal gold. This type of direct ligand conjugation is one of the very few approaches that permits evaluation of ligand receptor binding, endocytosis and cellular processing. Reviewed will be the status of direct conjugation studies, several of the inherent advantages and limitations, and cited references that employ this method. Specific areas of direct ligand conjugation will include cellular interactions with metabolites, humoral factors, coagulation proteins and general aspects of pinocytosis.

Methodology For Colloidal Gold-Ligand Labeling

The methodology of colloidal gold conjugation with ligands is well established and has been applied to a variety of proteins, lectins, enzymes, antibodies and hormones [27]. Ligand conjugation is complete within seconds after addition to a colloidal gold solution and apparently does not involve chemical changes to the ligand [38, 79]. There is a critical relationship between optimal conjugation of ligands with colloidal gold and the pH of the reactants. Generally, to insure optimal adsorption, the pH of both the colloidal gold solution and the ligand should be adjusted to the isoelectric point of the ligand [19] before labeling [27, 38]. Failure to conjugate in this manner may result in ligand-induced flocculation or limited adsorption due to charge repulsion between the ligand and the gold colloid [17, 19]. Proteins should be ideally solvated in water or a weak (0.001 M) nonionic buffer, so as to minimize the interference of ions with the adsorption process [19, 38]. One must observe the relationships as well as limitations between the ability of a ligand to adsorb to and stabilize a colloidal preparation

(against electrolyte-induced flocculation), the ligand molecular weight, the diameter of the gold particle and the number of ligand molecules bound per gold colloid [27, 38, 70]. For example, low molecular weight ligands (insulin, 5700 MW) may adsorb to a colloidal gold preparation (25-50 nm diameter) but be unable to stabilize such large colloidal particles against electrolyte-induced flocculation [27, 38, 70]. The number of gold particles per ml [2, 16] and ligand molecules per gold colloid should be calculated and compared to published reports of ligand-gold ratios, using published reports of ligands with comparable molecular weight [1, 2, 12, 16, 29, 39, 46, 47, 73, 79]. Non-specific stabilizers (polyethylene glycol, 20,000 MW or albumin, 67,000 MW) are often used to minimize possible aggregation of the ligand-gold probes [2, 39, 69]. Before such stabilizers are introduced, the stability of the ligand-gold preparation should be evaluated in the presence of electrolytes [38, 78, 79] and when necessary, separation of labeled from unlabeled ligand should be performed [78, 79].

Evaluation of Colloidal Gold Conjugates

The use of ligands conjugated with colloidal gold in studies of receptors, endocytosis or surface binding requires conventional controls (false positives, false negatives, [70]). In all direct gold conjugation studies, the multimeric nature of the gold-conjugated ligand should be reflected in the amount of probe to be used in a receptor study, [28, 29]. A gold colloid stabilized with a large number of molecules of a particular ligand will be less effective as a probe than the same amount of unlabeled, dispersed ligand [1, 28, 29, 47]. The loss of biological activity of the ligand after gold conjugation may reflect immobilization on the gold surface, restriction of active sites (in the case of enzymes) or other forms of steric hindrance. The loss of biological activity for any given amount of ligand conjugated with gold should be quantitated using the equivalent amount of unlabeled ligand for comparison. Loss of thrombin clotting activity [28, 47] or the reduced mitogenic potential of platelet-derived growth factor after gold conjugation [29] indicates that adsorption to gold can reduce biological activity of a ligand to 10-20% of that seen in the unlabeled state. Concentration of the probe after labeling may be required.

Negative staining may be used after conjugation to confirm the integrity of the adsorbed ligand [26, 28, 32, 51, 67]. Parameters to be considered are the morphology of the free ligand, thickness and uniformity of the adsorbed ligand, the degree of dispersion and the extent of unreacted material.

Cellular Pinocytosis

Pinocytosis is non-receptor mediated uptake of extracellular ligands. The rate of pinocytosis is influenced by physical conditions, such as temperature, ligand concentration, and ligand charge. Investigations of pinocytosis using gold-conjugated albumin have been examined in stimulated leukocytes [8], endothelium [18, 21], platelets [16], sinus endothelium [41, 73], hepatocytes [11, 32, 46], macrophages [65], and neuronal pathways [72]. However, transcytosis of gold-albumin conjugates has been shown to be receptor-mediated in capillary endothelium [22], as has been suggested by a previous study [18]. The effects of particle size [5, 58, 82], pressure [5] and influence of surface charge [21] on endothelial permeability have been studied using colloidal gold as a tracer to evaluate receptor-independent pinocytosis. Colloidal gold conjugated-fibrinogen, fibronectin or polyethylene glycol have been used as tracers for fluid endocytosis [22]. Lactosylated [43, 45], galactosylated [11, 41, 46] or mannosylated [31, 41] forms of modified albumin have been used in conjunction with gold conjugation to study the influence of chemical modification on endocytosis of galactose-terminating proteins. As apposed to native albumin, a small percentage (10%) of galactosylated albumin was retroendocytosed [11]. The use of radioactive gold [9, 60, 61, 68, 82] provides for an effective method to quantitate pinocytotic rates and the eventual lysosomal fate of endocytosed colloidal particles [10, 61]. The intracellular diffusion of exogenous karyophilic proteins has been examined using colloidal gold conjugated with nucleoplasmin [15].

Cellular Endocytosis

Transport Proteins and Metabolites

Receptor-mediated uptake and the metabolic fate of low-density lipoproteins (LDL) conjugated with colloidal gold have been extensively studied (Table 1). The development of colloidal gold-LDL conjugation represents one of the first applications involving large macromolecule adsorption to gold [26]. Unlike soluble proteins, LDL can be considered as colloidal particles. Consequently, the negatively-stained image of the gold-LDL will present different geometric patterns depending upon the axis at which the conjugate is viewed (Fig. 1). The gold labeling of LDL has been used to visualize the events of endocytosis and lysosomal delivery [24, 25, 27]. Following the development and application of gold-LDL conjugation to study receptor-mediated endocytosis [24-26], this technique has been widely used to evaluate the effects of chemical modification of LDL on endocytosis [51, 52, 64-66, 71], effects of membrane perturbation on LDL receptor topography [63, 64], the surface distribution of LDL receptors [36, 62, 64-66], effects of lysomotrophic amines on uptake [27, 34], and endocytosis of LDL [3, 23, 50, 52, 57, 67] and high-density lipoproteins (HDL) [67, 71]. Similar to modified albumin, about 10% of the HDL was retroendocytosed [71]. The HDL receptor domain has been observed as morphologically distinct from regions which bind gold conjugated with transferrin, acetylated LDL or BSA [65]. With use of 12 nm colloidal gold particles, a stable HDL-gold conjugate (prepared with a 100-fold excess of HDL) reveals that about 4-5 HDL bind around each gold particle (Fig. 2A). By reducing the amount of HDL used for conjugation, it is possible to

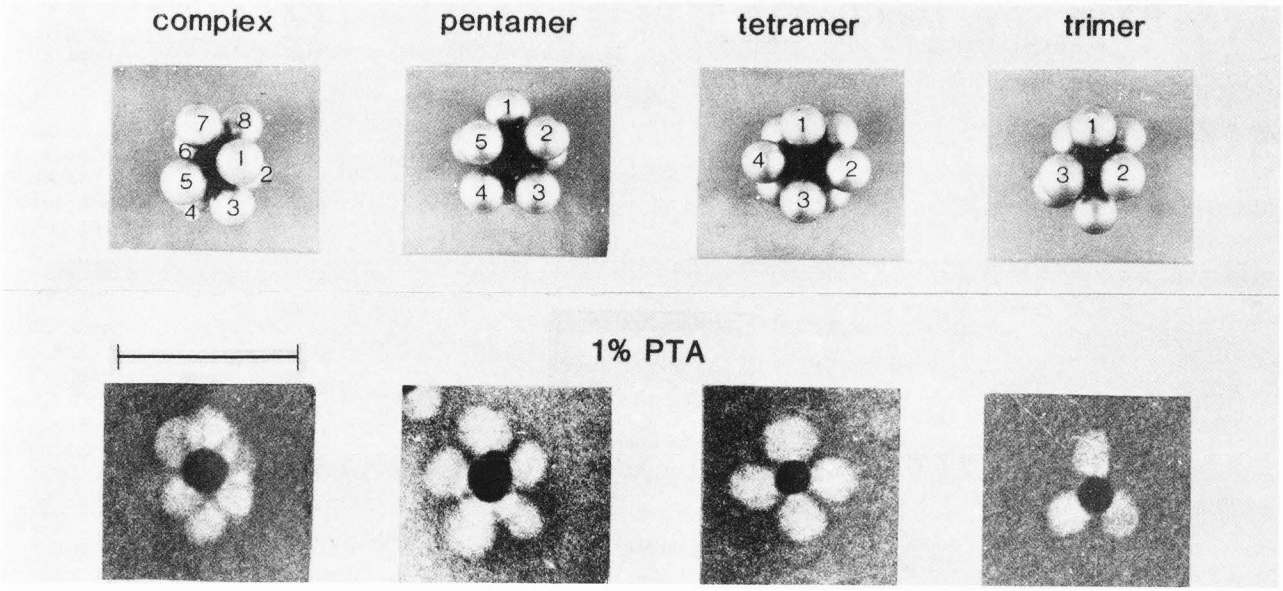

Fig. 1: Shown is a three-dimensional model of the gold-LDL conjugate (upper panel) and corresponding negatively-stained images using phosphotungstate (lower panel). The LDL-gold conjugate was prepared as previously described [25,26]. The optimal labeling ratio is about 8 LDL to each 22 nm centrally-positioned gold particle (complex image). However, the conjugate has several different axes, which when viewed by negative staining present trimer, tetramer, pentamer and complex images. The negatively-stained images can be reproduced with the model by appropriate positioning (upper panel). Bar=0.1 μm.

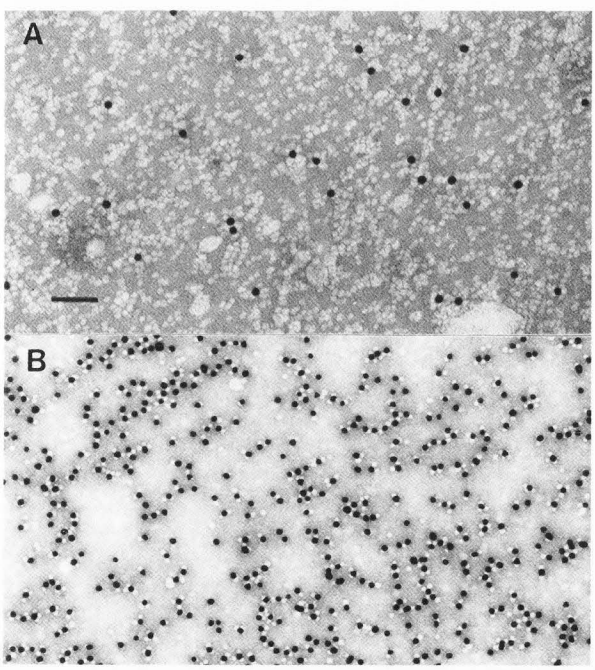

Fig. 2A-B Negatively-stained images of HDL-gold, formed with a 100-fold excess of HDL (A) or after sequential reduction of the HDL to a concentration (prior to labeling) that yields the same conjugate but virtually devoid of unlabeled material (B). With the use of 12 nm gold particles, the resulting conjugate consists of 4-5 HDL per gold colloid. Bar=0.1 μm.

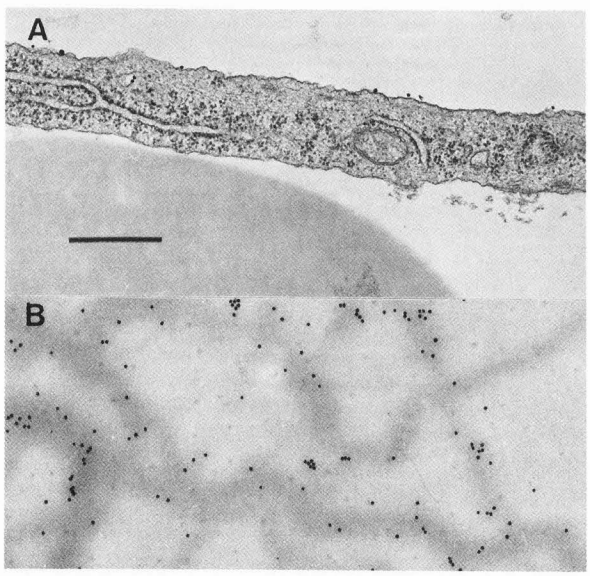

Fig. 3A-B Tissue plasminogen activator was conjugated to 18 nm colloidal gold as described for other proteins [28, 29]. The probe was reacted with confluent bovine aortic endothelial cells for 1 hr at 4°C at 5 μg/ml in cell culture medium containing 1% BSA [28]. Cells were prepared for electron microscopy [24]. The probe was observed randomly distributed on the cell surface and in close association with the outer membrane bilayer (A). The probe also interacts with formed fibrin strands (viewed directly without negative staining), exhibiting random distribution along the fibrin (B). Bar=0.5 μm.

TABLE 1

APPLICATIONS OF COLLOIDAL GOLD CONJUGATED MACROMOLECULES

PLASMA PROTEIN	MOL. WT.	CELL TYPE	REFERENCE
carrier proteins			
albumin	67,000	endothelial	18,21,22,41
		hepatocyte	32
		Kupffer	46
		neuron	72
asialoglycoproteins	—	hepatocyte	43,46,74,77
		hepatoma	20,56
		macrophage	43,74
ceruloplasmin	132,000	endothelial	42
		hepatocyte	40
		leukocyte	8
fibrinogen	340,000	endothelial	22
		platelets	49
galactosylated albumin	67,000	endothelial	41
		hepatocyte	11,46
		macrophage	42
high density lipoproteins	400,000	fibroblast	65, 66
		macrophage	71
low density lipoproteins	3,500,000	endothelial	23-25,50,51
		fibroblast	26,36,62-67
		granulosa	3,57
		hepatocyte	51
		macrophage	52,65,66
α_2-macroglobulin	725,000	epithelial	13,14
		Swiss 3T3	4,12,81
retinol-binding protein	21,000	epithelial	37
transferrin	80,000	endothelial	73
		epithelial	13
		hepatoma	55
		macrophage	31,65
		reticulocyte	30,31
coagulation			
factor VIII	1,100,000	platelet	16,17,59
factor IX	55,000	endothelial	54
factor X, Xa	55,000	endothelial	55
thrombin	37,500	endothelial	28
		fibrin	27,47
		platelet	27,28
tiss. plasminogen activator	70,000	endothelial	48
tumor necrosis factor	45,000	endothelial	53
humoral factors			
glucagon	3,500	hepatocyte	80
		leukocyte	2
immunoglobulins	160,000	macrophage	33,34,44,75
insulin	5,700	lymphocyte	6
		neutrophil	1
		monocyte, platelet	1
interferon		kidney	83
platelet derived-growth factor	33,000	fibroblast	29,69
thyroglobulin	669,000	epithelial	35

In all studies the ligand is conjugated directly with the colloidal gold.

achieve a stable HDL-gold conjugate that is virtually devoid of unlabeled HDL when viewed by negative staining (Fig. 2B). As with gold labeling of LDL, HDL is a colloidal particle and when viewed by negative staining after labeling will present several images reflecting the apparent number and arrangement of HDL adsorbed to each gold particle.

The pathway of receptor-mediated endocytosis of asialoglycoproteins has also been examined using the direct conjugation with colloidal gold [20, 43, 46, 56, 74, 77]. Receptor-binding and the endocytotic fate of several plasma carrier proteins, including α_2-macroglobulin [4, 12-14, 81], proteoglycans [76], transferrin [13, 30, 31, 65, 72, 73], retinol-binding protein [37] and transcytosis of ceruloplasmin [40, 42] have been studied as gold-conjugated probes.

Conjugation Factors

Thrombin conjugated with colloidal gold has recently been developed as an ultrastructural probe suitable for studying interactions of thrombin with platelets [28], fibrin [47] and endothelial cells [28]. Platelet receptor binding of gold-factor VIII [16, 17, 59] and fibrinogen [49] has also been studied. The procoagulation and thrombo-resistance potentials of the endothelium have been examined using gold conjugated with factors IX [54], X and Xa [55] and tumor necrosis factor [53]. Tissue plasminogen activator is a protein that is involved in fibrinolysis and is reported to bind to endothelial cells and fibrin. These interactions have been visualized by the development and use of a tPA-gold probe (Figs. 3A-B). In these studies it has also been shown that binding of tPA to the endothelial surface is not accompanied by endocytotic removal.

Humoral Factors

Cellular processing of insulin [1, 6] glucagon [2, 80] and immunoglobulins [33, 34, 44, 75] have been studied after direct conjugation with colloidal gold. Cellular receptor binding of platelet-derived growth factor conjugated with colloidal gold has been examined [29, 69] as well as receptor-mediated endocytosis of interferon [83]. The visualization of transcytosis of thyroglobulin, using a conjugated colloidal gold form [35], has provided a possible mechanism for the plasma occurrence of this protein in man.

Advances in Colloidal Gold Applications

The methodology, controls and varied applications of colloidal gold conjugation have now matured to a level as a routine and accepted approach to investigate cell structure-function relationships. Accordingly, the literature now reflects an increasing number and variety of ligands that are being conjugated with colloidal gold. New methodologies that have enhanced detection and quantitation of colloidal gold probes include backscattered electron imaging and silver intensification [7]. The application of freeze fracture [65] and surface replication [67] to visualize cell binding of gold probes offer unique approaches towards understanding cell membrane function related to receptors. The use of gold probes of different sizes for simultaneous labeling [36, 65, 67] or transport studies [5] are controllable methods that permit good resolution and greater efficiency toward quantitation of specific cell functions.

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Discussion with Reviewers

M. Tavassoli: How reliable is the use of colloidal gold in studies of endocytosis? Is the pathway followed by proteins conjugated to gold the same as protein alone? Is is possible that gold conjugation may alter the pathway that otherwise may be followed by the protein that is iodinated?

Author: Qualitatively, many proteins conjugated to colloidal gold are reported to behave in a manner nearly identical to the unlabeled protein. Proteins conjugated to gold bind to their specific receptors, can be competed from the receptor with free protein, and are internalized by coated vesicles, delivered to lysosomes and degraded in fashion similar to the native protein. However, there are reports that gold conjugation alters cell sorting of ligands, compared to the unlabeled ligand (van Deurs et al., J. Cell Biol. 102, 37-47, 1986). In any receptor study, controls for specific binding (conjugated vs. native protein) and non-specific uptake (a protein of similar molecular weight and charge conjugated to colloidal gold) should always be included. Proteins conjugated to colloidal gold are quantitatively different than the free protein. There is a loss of activity of thrombin (in terms of clot formation) and of platelet-derived growth factor (in terms of stimulation of cell proliferation) following conjugation to colloidal gold, reflecting immobilization to the gold surface, restriction of active sites or other forms of steric hinderance. Alternatively, as the conjugate is multimer in nature (many macromolecules bound to a single gold particle), the actual amount of any macromolecule that is internalized by a receptor-mediated process is enhanced. In the case of the gold-LDL conjugate (which consists of about 8 LDL absorbed to a single gold colloid), receptor binding of just one of the LDL molecules is accompanied by internalization of all eight. Therefore, the protein:gold ratio of the conjugate should be calculated and used to normalize the observed amount of a particular protein that is internalized.