SCIENCE SERVICES

GOLD NANOPARTICLE CONJUGATION
Adsorption or Covalent Binding?

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Introduction: some history

Introduced as an electron dense marker in 1971 by Faulk and Taylor [10] gold nanoparticles have proven to be the electron dense marker of choice for post-embedding immunolabeling. The beneficial effect of gradient centrifugation to narrow down the particle size distribution facilitated multiple labeling studies at the EM level. Conjugates prepared with conventional gold particles, particle diameter 5nm or larger, have sufficient electron density to make them visible in TEM. They are in general also the marker of choice in correlative light and electron microscopy studies. See e.g., Loussert Fonta et al. [19].

In the eighties the silver enhancement technique [6] further broadened the application of immunogold reagents to light microscopy. At the end of the eighties Leunissen et al. [17] developed a new immunogold detection system based on gold particles with a diameter <1nm. In such conjugates the overall size and thus steric hindrance are decreased resulting in higher labeling densities and improved penetration [16]. Efficient silver enhancement methods were required and developed to visualize these Ultra Small particles and to fully benefit from their characteristics as illustrated by a.o., Zeuschner et al. when detecting COPII in 3-D electron tomography [24].

Ultra Small conjugates opened the option for pre-embedding labeling and electron microscopic evaluation of the results after embedding in epoxy resin and ultra-thin sectioning (AURION Newsletter nr 5). By taking advantage of the homogenous enhancement properties of AURION R-Gent SE-EM, Yi et al [23] were able to co-localize GFAP and synaptophysin in vibratome sections of mouse brain tissue by using a sequential silver enhancement procedure. One of the latest developments using Ultra Small gold conjugated antibodies is the detection of polymerase II in the nucleus of living cells, a set-up which only works using gold nanoparticles size < 1nm [20].

Immunogold reagents used in the publications referred to in this introduction were prepared by direct adsorption of immuno reactive macromolecules to the gold particle surface, the standard approach for the preparation of the AURION Conventional and Ultra Small gold reagents. Such conjugates pair high affinity with long term stability.

Gold nanoparticle conjugation via adsorption

Our in-house developed procedures used to prepare AURION Conventional Gold Nanoparticles, sizes 6-10-15 and 25nm, are derived from the original method described by Frens [11], producing citrate stabilized gold particles in “water”. Due to surface plasmon resonance and the presence of citrate ions on the gold nanoparticle surface in a Frens goldsol, these gold nanoparticles carry a negative surface charge. Colloidal gold particles also display hydrophobic properties. Hydrophobic interactions are maximally expressed at the iso electric point of the macromolecule to be conjugated.

In general optimal conjugation pH will be an approximate 0.5 pH unit higher than the IEP of the molecule that is intended to be adsorbed on the particle surface. At this pH the overall charge of the protein is slightly negative which avoids clustering. There will be however sufficient positive charges (areas rich in lysine and tryptophan) present in the molecule to promote electrostatic interaction with the negatively charged gold particles. Most stable and strongest binding between protein and gold particle is due to chemisorption of thiol groups [22] from cysteines exposed at the surface of the protein.

Goodman et al. [13] already demonstrated in 1981 that a single layer of protein on the gold particle surface results in optimum stability. Excess amount of added protein results in the formation of additional layers and less stable conjugates.
Contemporary highly sensitive analysis techniques such as Dynamic Light Scattering [4] and Differential Centrifugal Sedimentation [7] have confirmed the presence of a so-called hard corona and soft-corona. This is reflected in two crucial steps in a “classic” gold nanoparticle conjugation protocol: the determination of the minimal protecting amount of protein which produces the hard corona followed by purification steps for removal of immunoreactive molecules from the soft corona.

The combination of chemisorption via thiol groups, hydrophobic interaction and electrostatic binding of a single layer of proteins on the surface of the gold nanoparticles ensures optimum stability and activity of the conjugate.
Such conjugates are suitable not only for research applications but also in diagnostics. Many of the over the counter tests, e.g. pregnancy tests, are based on immunogold conjugates prepared by adsorption.

A simple experiment that shows stability of Aurion Conventional Immunogold reagents is described in Figure 1. 10nm Gold particles conjugated to F(ab')2 fragments of Goat anti Rabbit IgG were first treated with 8M urea for 60 minutes. Urea was already used in the mid sixties [21] in affinity chromatography as it dissolves antibody clusters and dissociates high affinity antigen-antibody interaction. The presence of 8M urea does not only demonstrate conjugate stability, as there is no color change/flocculation of the gold reagent, its also shows that incubation in 8M urea does not affect reactivity of the gold reagent.

Other examples of retained bio-activity after conjugation using the adsorption method are enzyme-gold nanoparticle conjugates that can be used for on-section detection of their corresponding substrates as described in, a.o., a review by Bendayan in 1989 [3] as well as in a publication by Esbach et al., which describes the binding and intracellular processing of Ultra

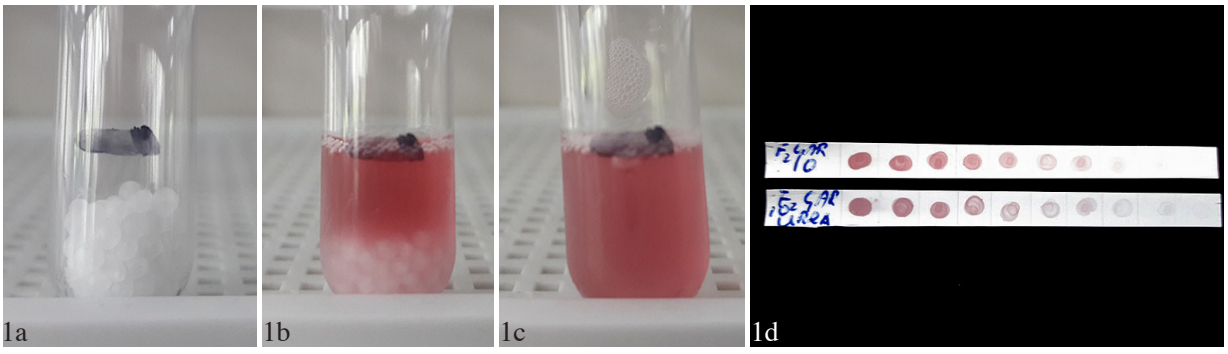

Figure 1: Effect of 8M urea on activity of a F(ab')2 GAR 10nm gold conjugate prepared via the classic adsorption technique
1a/b/c: Addition of 8M urea has no visible effect on stability of the gold conjugate
1d: Both urea treated F(ab')2 GAR 10nm and non-treated F(ab')2 GAR 10nm give the same number of spots in an activity dot-spot test

Small gold nanoparticle (MD ~ 0.8nm) conjugated acetylated low density lipoproteins (Ac-LDL-Au) in rat liver endothelial cell [9].

Gold nanoparticle conjugation via covalent binding

In search for alternative methods to prepare gold nanoparticles for high resolution electron microscopy, Hainfeld and Furuya [14] described the preparation and use of antibody conjugated organophosphine-gold atom nanoclusters. Nanogold is composed of a 67 atom gold core in an organic shell. The gold atom core has a diameter of 1.4nm whereas the overall dimensions of the organophosphine-gold complex is 2.7nm.

A reduced gold particle size is also beneficial for surface area to particle volume ratio which in turn is beneficial for their catalytic and semiconducting properties. Brust et al [5] developed a two-phase protocol to prepare 5-6nm gold nanoparticles in organic liquid (toluene) using sodium borohydride as the reducing agent and tetraoctylammonium bromide (TOAB) for phase transfer and initial stabilization. In the Brust-Schiffrin method TOAB is exchanged for dodecathiol which has a higher affinity for the gold particle surface. One of the main advantages of preparing stabilized gold nanoparticles in organic media is the option for bulk production. To make these particles suited for biological applications a phase transfer to water is needed. Ligand transfer using water soluble thiol-components with higher affinity than the ones initially used for gold nanoparticle preparation makes this possible [1] [12].

Different to citrate stabilized gold nanoparticles the surface of both organophosphine and thiol stabilized gold nanoparticles is not available for conjugation via the adsorption method. Conjugation via covalent binding is the alternative. The organophosphine shell of nanogold is functionalized with maleimide [14]. Functionalized thiolated polyethylene (PEG) polymers are in general used for gold nanoparticles prepared via the ligand exchange method. The functional groups can be, a.o., carboxyl, maleimide and azido groups.

With a strength of 47 kcal/mol (197 kJ/mol) [8] the sulphur (thiol)-gold bond is considered to ensure prolonged stability of ligand-stabilized gold nanoparticles. However Hostetler et al. already described in 1999 [15] that thiol bound ligands appear to be mobile, i.e., diffuse to some extend on/from the surface of metal particles. This is considered to be also the main reason for deactivation of monothiol-DNA functionalized gold nanoparticles at e.g., increased temperature [18].

One solution to increase the shelf life of commercially available thiol-PEG stabilized gold nanoparticles is freeze drying. To avoid particle aggregation large thiolated PEG polymers having a MW of 5kDa are being used. The effect of 5kDa thiolated PEG on the actual diameter of the complex is significant. Arnida et al. [2] described an increase from 50nm (non stabilized particle) to 89nm when using 5kDa thiolated PEG.

AURION Gold Nanoparticles - Carboxyl Functionalized -

Is it possible to use thiolated components with a shorter PEG chain length for gold nanoparticle functionalization, ones that are more compatible with resolution requirements of the electron microscope, without losing stability and decrease in shelf life? Product development at Aurion solved the issues and the result is a series of carboxyl functionalized conventional gold nanoparticles, sizes 6, 10, 15 and 25nm. The PEG chain length is well below the diameter of the gold particle. AURION Gold Nanoparticles - Carboxyl Functionalized - have a guaranteed shelf life of 12 months from the date of quality control analysis.

Biomolecules that are too small to be conjugated to gold nanoparticles via the classic adsorption method can be covalently linked to carboxyl-functionalized gold nanoparticles. via primary amines present in e.g. the N-terminal side of peptides and in the side group of the amino acid lysine.

The conjugation relies on well known and proven

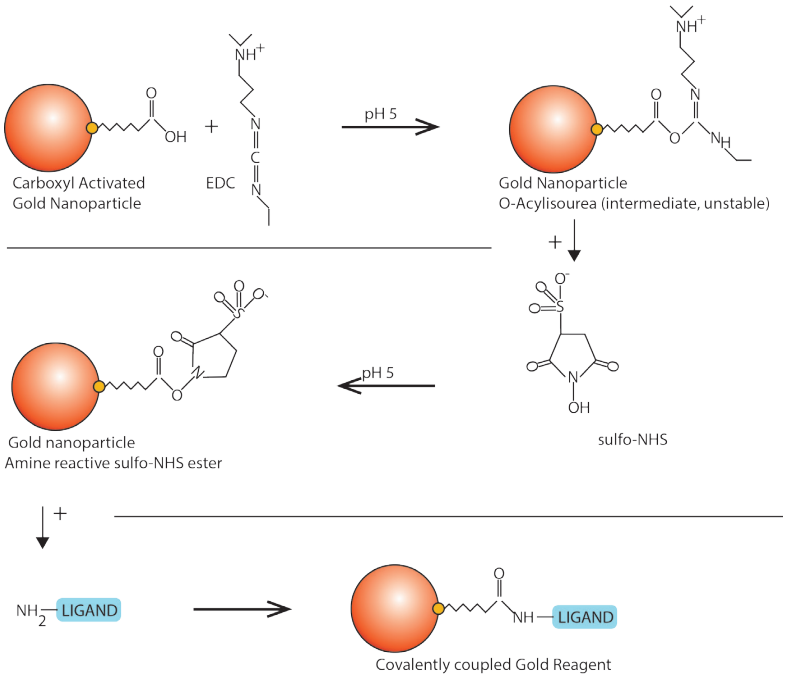

Figure 2: Covalent conjugation of Carboxyl-functionalized gold nanoparticles: reaction scheme
EDC /sulfo-NHS activation at pH 5 results in an amine reactive sulfo-NHS ester, immediately followed by binding of the sulfo-NHS ester to free amine on the target molecule (ligand).

EDC/sulfo-NHS chemistry. EDC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) is a water soluble carbodiimide which transforms the carboxyl groups on the gold to an active ester in the presence of sulfo-NHS (N-Hydroxysulfosuccinimide, sodium salt). These sulfo-NHS esters are relatively stable in acidic environment and couple rapidly to the amine(s) in the target molecules (see Figure 2).

When is covalent conjugation more favorable than adsorption conjugation?

As mentioned earlier conjugation of high molecular weight molecules (e.g., antibodies) to conventional gold particles is very well possible using the adsorption method. A shift of the maximum optical density (OD_{max}) as well as the flocculation test will illustrate whether adsorption is successful or whether a covalent conjugation protocol is to be preferred. As an alternative, Aurion can prepare a conjugate to either conventional or Ultra Small gold particles via our Custom Labeling service. We have successfully conjugated proteins with a MW down to 5 kDa (Aprotinin) to Ultra Small gold nanoparticles .

For conjugation to conventional gold nanoparticles the "gray area" are macromolecules having a MW between approx. 30 and 40kDa.

Protein A but also recombinant Protein A conventional gold conjugates can be routinely prepared using the adsorption method. Protein A is a Fc-specific antibody binding protein derived from Staphylococcus aureus with a MW of 42 kDa. The recombinant protein A used in Figure 3 has a molecular weight of 34 kDa. Figure 3 shows no difference in labeling density between a recombinant protein A conjugate prepared with the adsorption method and a similar conjugate prepared using carboxyl functionalized gold nanoparticles. Also, with respect to stability and activity we can not detect any difference. Binding activity of both types of recombinant Protein A gold conjugates to rabbit IgG is comparable and remains comparable over a time span of minimally 1 year.

A different approach is required for a stable conjugation of Apolipoprotein-E (ApoE) to 6nm gold particles. ApoE and recombinant protein A have similar molecular weight. ApoE does adsorb to the gold particle surface as can be inferred from the shift of OD_{max}. The stability is however not sufficient to protect the conjugate from flocculation when the salt concentration in the ApoE nanoparticle mixture is raised to 1%. Consequently a covalent binding needs to be the method of choice.

AURION Gold Nanoparticles - Carboxyl Functionalized -

Example: conjugation of Biotin Hydrazide (MW = 258.34Da) to Aurion Carboxyl-functionalized 10 nm Gold Nanoparticles

Materials
1. Aurion Gold Nanoparticles - Carboxyl Functionalized -, 20ml, product 410.133
2. Amicon Ultra-4 30K filter units, Millipore
3. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)
4. N-Hydroxysulfosuccinimide, sodium salt (sulfo-NHS)
5. 2-Morpholinoethanesulfonic acid Monohydrate (MES)
6. Biotin Hydrazide (e.g., 21339 Thermo Scientific) in Dimethyl Sulfoxide

All chemicals are reagent grade

Method
- Prepare a 10mM MES buffer and adjust pH to 5.0 with NaOH
- Prepare a stock solution of 100mg/ml of Biotin Hydrazide in Dimethyl Sulfoxide
- Wash 2 Ultra-4 filter units with deionized water
- Bring 2ml of Aurion carboxyl-functionalized 10nm Gold Nanoparticles (Cat. nr 410.133) in each of the 2 Ultra-4 units
- Centrifuge at 1500xgav for 10 minutes
- Add 3ml of 10mM MES buffer, pH 5.0 per filter unit, mix and centrifuge at 1500xgav for 15 minutes
- Remove the concentrated Aurion carboxyl-functionalized Gold Nanoparticles from the filter units and adjust the collected volume to 4ml with 10mM MES buffer, pH 5.0
- Immediately before use, prepare a 100mM EDC solution in 10mM MES buffer, pH 5.0
- Add 80 μl of 100mM EDC in MES buffer to 4ml of Aurion carboxyl-functionalized Gold Nanoparticles and mix well
- Incubate for 5 minutes
- Immediately before use, prepare a 100mM sulfo-NHS solution in MES buffer, pH 5.0
- Add 80 μl and incubate for 30 minutes
- Add 20 μl Biotin Hydrazide stock solution, incubate for 2 hrs
-Centrifuge at 1500xgav for 10 minutes using the Ultra-4 filter units, repeat this step 2x
- Collect the concentrated biotinylated conjugate. Dilute to OD_{520nm} = 1.0 in PBS, 0.1% BSA, 15mM NaN_3, pH 7.4

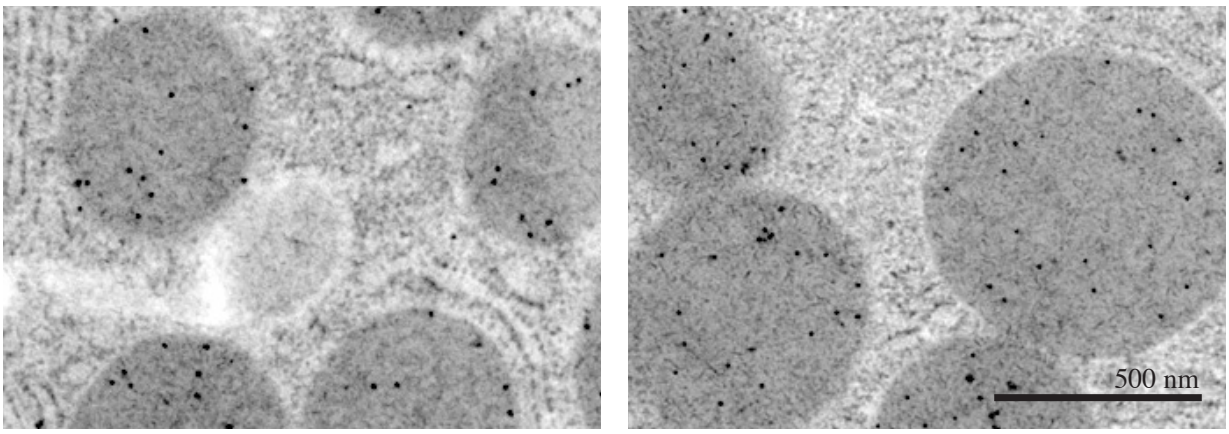

Figure 3A: Recombinant protein A gold conjugates prepared via the adsorption- (left) or covalent conjugation method (right hand panel) give comparable labeling density in post-embedding immuno labeling of alpha-amylase in Lowicryl HM20 embededded rat pancreas tissue

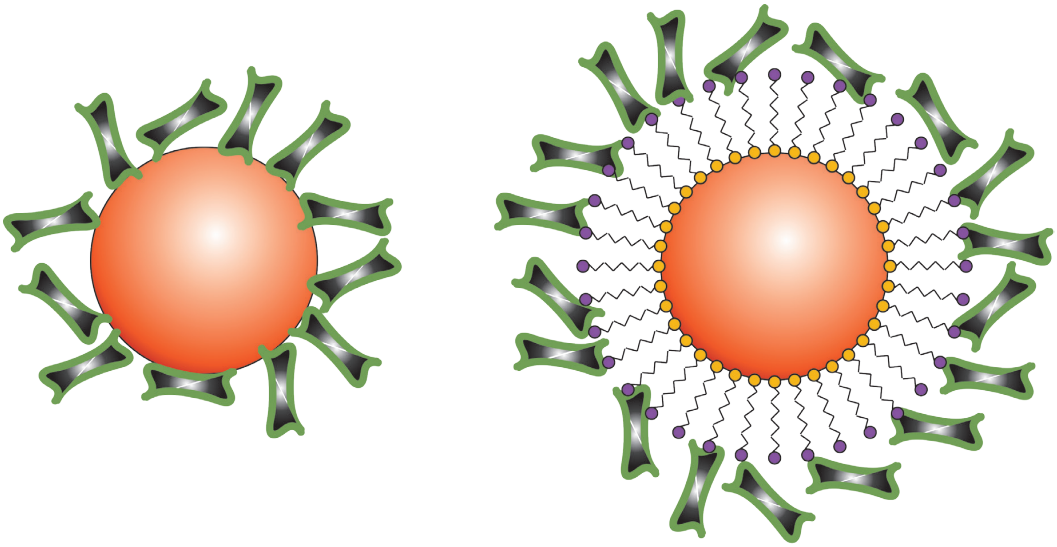

Figure 3B: Schematic representation of a Protein A gold nanoparticle conjugate prepared via “classic” adsorption and via covalent conjugation. Distance between ligand and gold nanoparticle is smallest in the “classic” conjugate. This is beneficial for resolution. The PEG chain in the covalent conjugate with a length smaller than the particle, allows for more steric freedom of the ligand which may have a positive effect on labeling density.

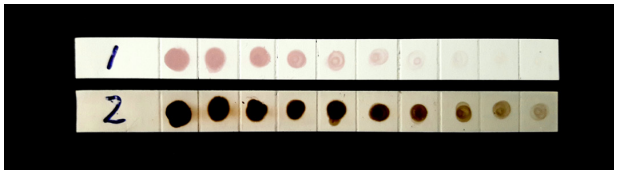

Figure 4:
Activity dot-spot test of 10 nm gold conjugated biotin prepared with Aurion Gold Nanoparticles - Carboxyl Functionalized -

The gold signal clearly visualizes the 1 ng spot (spot nr 7)
Sensitivity of detection is increased to 30 pg using silver enhancement

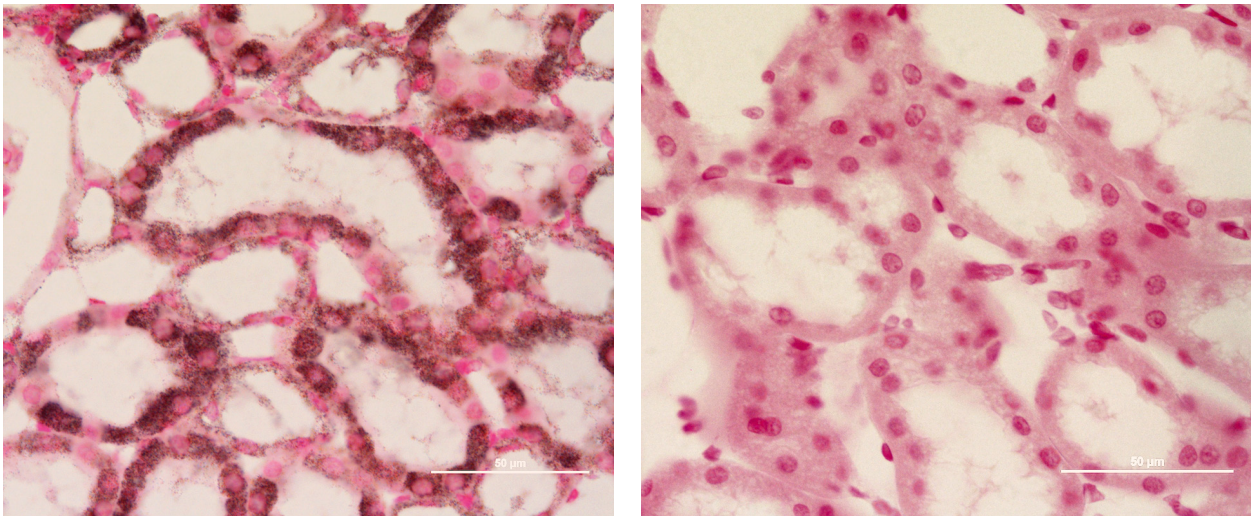

Figure 5:
Detection of endogenous biotin on formalin fixed rat kidney tissue. After antigen retrieval in 10mM citrate buffer, pH 6, biotin was detected with streptavidin and visualized with 10nm gold conjugated biotin and silver enhancement. Positive signal (5a) is mainly present in epithelial cells of proximal tubules. 5b Negative control.

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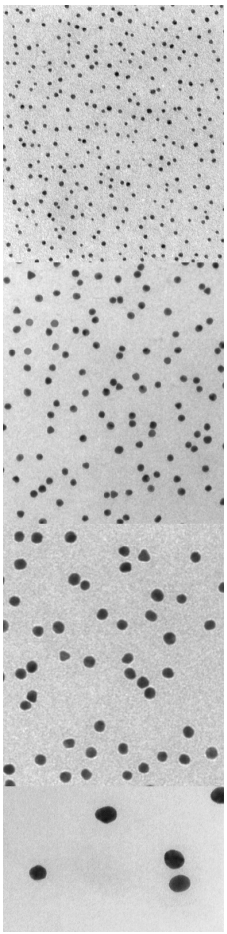
• Product description



Aurion Gold Nanoparticles - Carboxyl Functionalized - are prepared according to a unique protocol, warranting narrow size distribution, long term stability and optimal conjugation properties
They are available in size ranges 6, 10, 15 and 25 nm. The particle population is monodisperse and thus shows minimal size variation and overlap. Typically, the coefficient of variance for the 6 and 25 nm particle size sols is less than 12%, whereas the 10 and 15 nm size sols show less then 10% variation. Actual lot specifications (size, variation and expiry date) are indicated on the accompanying package insert.

Aurion Gold Nanoparticles - Carboxyl Functionalized - are supplied in 10mM MES buffer, pH 5.0
Package size: 4 x 5 ml of high quality Carboxyl-functionalized Gold Nanoparticles at an OD520nm = 1.0

• Storage

Aurion Gold Nanoparticles - Carboxyl Functionalized - have a guaranteed shelf life of 12 months from the date of quality control analysis.
The products should be stored at 4-8°C.
Freezing is not recommended.

• Application Instructions

Detailed instructions for covalent conjugation, purification and evaluation are described in the package insert.

• Ordering Information Gold Nanoparticles	**Product code**
Aurion Gold Nanoparticles 6 nm Carboxyl Functionalized	406.133
Aurion Gold Nanoparticles 10 nm Carboxyl Functionalized	410.133
Aurion Gold Nanoparticles 15 nm Carboxyl Functionalized	415.133
Aurion Gold Nanoparticles 25 nm Carboxyl Functionalized	425.133

Aurion Gold Nanoparticles - Carboxyl functionalized
Four vials each containing 5 ml of functionalized product. Separate vials eliminate the risk of contamination when several syntheses are done over time. See back cover for product codes and ordering info.

The AURION NEWSLETTER aims to be a platform for immunogold users presenting and discussing practical aspects in immunogold labeling (e.g. technical tips and tricks suited for a wider application). We like to encourage users to send in contributions for this NEWSLETTER to:

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