The Conjugation of an Anti-HER2 Affibody to a 1.4nm Nanogold Monomaleimide

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Background

Increased expression of HER2 has been detected in carcinomas of breast and is considered to be a part of the malignant phenotype. Blocking HER2 signaling in breast carcinomas using the clinically approved monoclonal antibody trastuzumab results in an increase of survival. However, since the HER2 expression levels in distant metastases may be quite distinct from levels in the primary tumor, an assessment of the global expression of these receptors is essential for adequate diagnosis and selection of appropriate therapy.[1] HER2 specific Affibody® molecules are molecular probes developed to address this problem. Affibody molecules are a novel class of small (~7 kDa) phage display-selected affinity proteins, based on the B-domain scaffold of staphylococcal protein A. The small size of the Affibody molecules provides rapid tumor localization and fast clearance from nonspecific compartments.[2] The anti-HER2 Affibody molecule His_6-$Z_{HER2:324}$ containing a single c-terminal cysteine thiol group was obtained from Affibody AB (Bromma, Sweden). This Affibody molecule binds a different epitope of HER2 than do the currently used therapeutic antibodies, which may allow concurrent monitoring of HER2 expression during treatment.

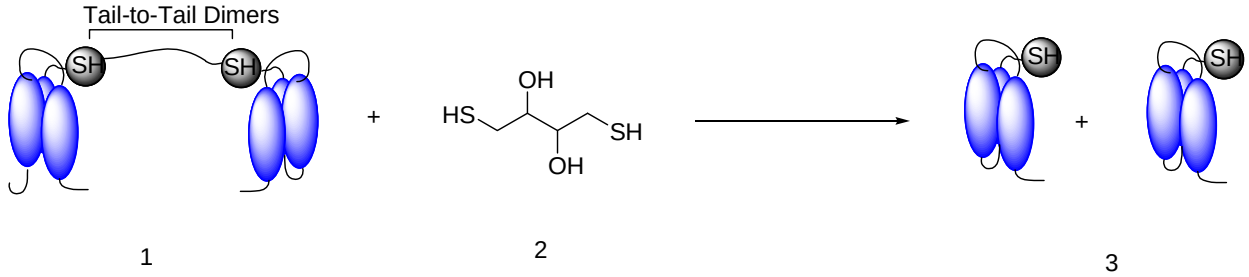
The compound being conjugated to the HER2 Affibody is a 1.4nm Nanogold® monomaleimide particle (Molecular Probes, Eugene, Oregon). This gold particle contains ~67 gold atoms in its core and has a maleimide moiety attached to its surface. The Nanogold cluster is directly visible by transmission electron microscopy (TEM) at high magnification and is stable in the beam. The Nanogold-Affibody conjugate has proven more stable than colloidal gold due to its covalent linkage, leads to a more quantitative labeling, and does not aggregate over time.[3] These ultra small gold markers also accumulate in larger numbers at targeted antigenic sites because of their

small size.[4] Taken together, these properties make a Nanogold probe useful for *in vivo* optical imaging with the Affibody molecule.

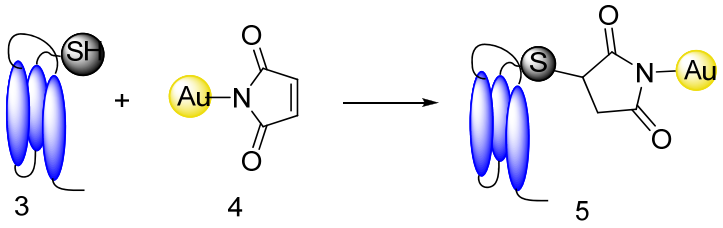
Chemistry

The synthesis of the Nanogold-Affibody conjugate is shown below (Schemes 1, 2). There were two distinct steps involved in the synthesis of the Affibody conjugates including the reduction of the Affibody and the conjugation of the Nanogold to the Affibody. The Affibody molecule contains a unique C-terminal cysteine ideal for directed chemical modifications. However, tail-to-tail dimers are spontaneously generated via a disulfide bridge between the C-terminal cysteines. Therefore immediately prior to coupling via the C-terminal cysteine, the Affibody molecule was reduced to expose the reactive cysteine residue (Scheme 1). In order to obtain the reduced Affibody molecule (**3**), dithiothreitol (DTT) was added and the solution was incubated at room temperature for 2 hours. The excess DTT was promptly removed from the solution by passage through an Illustra™ NAP™-5 column (Pierce Biotechnology Inc., Rockford, IL) to prevent rapid reverse redimerization. The Nanogold monomaleimide was reconstituted in a mixture of dimethylsulfoxide (DMSO) and water and added to the Affibody solution at a 5-to-1 molar ratio of gold to Affibody and gently mixed to reduce any chance of oxidizing the free sulfhydryls (Scheme 2). The excess Nanogold was removed from the solution by gel filtration. Sodium azide and bovine serum albumin (BSA) were added to the Nanogold-Affibody conjugate solution to prevent bacterial contamination and to prevent the protein from adhering to the surfaces of the storage container.

Scheme 1



Scheme 2



Experimental

General. All reagents were obtained from commercial sources and used as received. The Affibody molecule was received from Affibody AB (Bromma, Sweden). The Nanogold monomaleimide was purchased from Molecular Probes (Eugene, Oregon, USA). High performance liquid chromatography was performed at 25°C using an Agilent 1200 Series instrument equipped with a multi-wavelength detector. A Zorbax GF-250 gel filtration column, 9.4 x 250mm, with an isocratic gradient of 20mM phosphate buffer, 150mM sodium chloride and 1mM ethylenediaminetetraacetic acid (EDTA) was run at a flow rate of 1mL/min over 15 minutes. The eluate was measured at wavelengths 280nm and 420nm for the Affibody and Nanogold respectively. MALDI-TOF mass spectra were recorded on a Shimadzu Biotech Axima-CFP Plus spectrometer. A supersaturated solution of dihydroxybenzoic acid (DHB) (50% CH_3CN, 50% water with 0.1% trifluoroacetic acid) was used as a matrix for MALDI-TOF analysis of the reduced Affibody. UV-VIS spectrophotometry was carried out on a Perkin Elmer Lambda 25 Spectrometer.

Reduced Affibody (3). The Affibody molecule (**1**) (1mg) was obtained as a solution (255µL) in phosphate buffered saline (PBS) at a concentration of 3.91mg/mL. 129µL (72nmol, 0.503mg) of the solution was aliquoted for use. To this solution was added 375.4µL of PBS to make the final protein concentration 1mg/mL. A solution of 500mM DTT (**2**) (10.5µmol, 21µL) was added to the Affibody solution and mixed well. The mixture was incubated at room temperature for 2 hours, and excess DTT was then removed by passage through an Illustra NAP-5 column. This afforded a clear liquid that was used in the next step without further purification. *m/z* (MALDI TOF) found 6989.18 .

Nanogold-Affibody Conjugate (5). The 1.4nm Nanogold-monomaleimide was obtained as a solid, lyophilized from a 0.02M sodium phosphate buffer, pH 6.5 containing 150mM sodium chloride and 1mM EDTA. The Nanogold-monomaleimide (270nmol) was reconstituted with 900µL DMSO and 8.1mL water. The entire Nanogold solution was then added to the reduced Affibody solution immediately following its elution from the NAP-5 column. The mixture was thoroughly mixed by inverting the vessel several times and then incubated at 4°C overnight. The mixture was purified using size-exclusion HPLC, affording a clear brown liquid. The purified Nanogold-Affibody on size-exclusion HPLC showed a single peak at 11 minutes with an absorbance at 280nm and 420nm. The UV-VIS spectrum of the conjugate showed two peaks with maxima at 280 and 420nm. The peak at 280nm corresponds to the absorbance of the Affibody and the Nanogold while the peak at 420nm is specific to the Nanogold. Based on the UV-VIS spectrum, the number of Nanogold particles covalently bonded to the Affibody molecule was calculated using mathematical equations provided by Molecular Probes (Figure 2). From these equations it was determined that there is approximately one Nanogold particle covalently attached to each Affibody molecule .

$$\text{protein concentration } (M) = \frac{\left[Abs_{280} - \left(2.7 \times Abs_{420}\right)\right] \times \text{dilution factor}}{\varepsilon_{protein\,(280\,nm)}}$$

$$\text{degree of labeling DOL} = \frac{A_{420} \times \text{dilution factor}}{110{,}000 \times \text{protein concentration (M)}}$$

Figure 1 Equations provided by Molecular Probes.

The mathematical equations shown above (Figure 1) are derived from the Beer-Lambert law. When calculating the concentration of protein a correction factor of 2.7 was used to account for the absorption of the Nanogold at 280nm. The molar extinction coefficient was determined for the Affibody. It was assumed that when calculating the degree of labeling, the UV-VIS spectrum of the Nanogold or protein remained unchanged by the conjugation.

References

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