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Rigid, Specific, and Discrete Gold Nanoparticle/Antibody Conjugates

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Abstract: A general method of rigid, specific labeling of proteins with gold clusters has been devised. The method relies on the conjugation of a glutathione monolayer-protected gold cluster (MPC) with a single chain Fv antibody fragment (scFv), mutated to present an exposed cysteine residue. Efficient formation of a gold−thiolate bond between the MPC and scFv depends on activation of the gold cluster by chemical oxidation. Once formed, the MPC−scFv conjugate is treated with a reductant to quench cluster reactivity. The procedure has been performed with an MPC with an average Au_{71} core and an scFv directed against a tetrameric protein, the influenza neuraminidase. A complex of the MPC−scFv conjugate with the neuraminidase was isolated, and the presence of four gold clusters was verified by cryoelectron microscopy.

Introduction

Gold nanoparticle/protein conjugates are finding increasing application as biochemical sensors,[3,4] enzyme enhancers,[5,6] nanoscale building blocks,[7] and immunohistochemical probes.[8,9] Two chemistries have been developed for forming nanoparticle/protein conjugates: the reaction of protein sulfhydryls with functionalized phosphine-protected undecagold and nanogold; and electrostatic interaction of proteins with citrate-passivated colloidal gold. Neither of these conjugation strategies can produce a specific, linker-free, bond between a nanoparticle and a protein. These limitations have prevented certain applications of gold nanoparticles[10] from being implemented and have prevented others from being fully realized.[6] We report here a conjugation strategy that overcomes these limitations, through redox control of the reactivity of organothiolate monolayer-protected gold clusters (MPCs)[13,14] toward protein sulfhydryls.

MPCs differ from the gold nanoparticles traditionally used for bioconjugation in their extraordinary stability and size-dependent optical, catalytic, and capacitance properties.[12] One- and two-phase syntheses of MPCs afford synthetic control over particle size, dispersity, and surface functionality.[12] The adaptation of MPCs for water solubility has enabled their conjugation to proteins and DNA. Derivatization of the MPC monolayer with ethidium bromide[13] and biotin[14] has been reported for binding duplex DNA and streptavidin. Thiolated single-stranded DNA[15,16] and glutathione[14] have been incorporated in the MPC monolayer for hybridization with complementary DNA and for binding glutathione-*S*-transferase. A method for coupling a physiologically compatible MPC to virtually any biological particle has been put forward,[17] as has a method for general functionalization of water-soluble MPCs.[18]

We have sought a general method for the rigid, specific binding of MPCs to proteins. To this end, we have formed conjugates between 1.5 nm-diameter, glutathione-protected MPCs[1] and single chain Fv antibody fragments (scFvs). Rigidity is assured by the direct coupling of the gold cluster to an scFv sulfhydryl group, by the structure of the scFv, which contains none of the flexibly jointed regions present in intact antibodies, and by the nature of the scFv−protein interaction, which typically entails multiple points of contact with the protein

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surface. Generality and specificity derive from the facile generation of scFvs with high affinity for practically any protein of interest.[19]

We undertook the covalent reaction of an scFv bearing a surface cysteine with the gold core of the MPC by simple mixing of the components. We expected the cysteine would substitute for a glutathione in the MPC monolayer through a "Murray place exchange" reaction.[20−22] This approach was initially disappointing, resulting in little or no reaction. We tried many variations, experimenting with both the position of the surface cysteine residue and the chemistry of the gold cluster, and finally discovered a redox method that was both successful and advantageous for our purposes. Oxidation activated the MPC[4] for reaction with the scFv, and subsequent reduction rendered the MPC essentially inert toward further reaction. Such control of MPC reactivity can be of great benefit for use in biological systems, where the MPC−scFv conjugate may be exposed to many sulfhydryl-bearing proteins, whose reaction with the MPC would be a potential source of artifact.

Results

Initial experiments on MPC−scFv conjugation entailed the synthesis of clusters with different thiolate monolayers[23] and assay of the ability of the MPCs to form conjugates with scFvs bearing exposed, reduced cysteine residues. This work showed that, in general, MPCs with monolayers of bulky thiolates would react more completely than MPCs with monolayers of less bulky thiolates (data not shown). Subsequent studies focused on glutathione-protected MPCs and entailed both the creation of scFv mutants with cysteine residues in various locations and exploration of the sulfhydryl−gold coupling chemistry.

Optimization of scFv Reactivity toward MPC: The Importance of Sulfhydryl Accessibility and of Neighboring Positively Charged Residues. The anti-influenza N9 neuraminidase NC10 scFv was employed here for two reasons, first because the X-ray crystal structure of an NC10−neuraminidase complex has been determined,[24] serving as a guide for mutagenesis, and second because the neuraminidase is tetrameric, enabling critical tests of MPC−scFv binding and rigidity by electron microscopy (see below). Initial experiments with cysteine mutants of the NC10 scFv showed that a cysteine residue at the end of a C-terminal affinity tag was much more reactive toward a gold cluster than a cysteine residue introduced in the scFv surface ("framework" region). This prompted us to engineer and express a set of nested mutants, with progressively longer peptide linkers between the cysteine residue and the ordered region of the scFv (Figure 1). The design of these mutants took into account four considerations: first, the last structured residue in the X-ray structure of the NC10 scFv is Ser113; second, bacterial expression is known to be problematic for proteins with cysteine at the C-terminus; third, the peptide Glu−Cys−Gly should occupy the same footprint on the surface

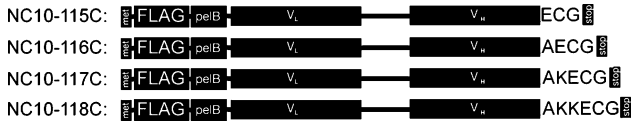

Figure 1. Nested set of NC10 scFv mutants. Motifs of the NC10 scFv gene are depicted as follows: met, the start codon; FLAG, an affinity purification tag; pelB, the leader sequence that directs delivery of the scFv into the periplasm of *E. coli*; VL, the light chain of the scFv, which is followed by a 15 amino acid $(Gly_4Ser)_3$ linker; VH, the heavy chain of the scFv, followed by a C-terminal tail of varying length, which includes a cysteine residue and, in two cases, lysine residues.

of a cluster as the glutathione molecule (γ-L-glutamylcysteinylglycine) it is replacing in a place exchange reaction; and, fourth, the incorporation of lysine residues to impart a positive charge to the C-terminal region could enhance reactivity toward the negatively charged Au_{71} MPC, as well as increasing the rigidity of the product.

Mutants NC10−S112C to NC10−118C (Figure 1) were combined with the "activated" glutathione-protected MPC described below. There was no appreciable reaction with the cysteine residue at positions 112, 113, or 114 (not shown), slight reaction at positions 115 and 116, significant reaction at position 117, and nearly quantitative reaction at position 118 (Figure 2). We subsequently found that an NC10−113K−114K−115G−116C mutant was similar in reactivity to NC10−117C (data not shown), and we settled on this mutant for the large-scale preparation of MPC−scFv conjugate, as a compromise between reactivity toward the MPC and rigidity of the linkage in the product. Our studies with NC10 mutants also revealed that the capacity of scFvs for forming MPC conjugates correlates with their capacity for forming disulfide-linked dimers, showing similar steric requirements for the two processes.

Optimization of MPC Reactivity toward scFv: The Importance of the Charge State of the Cluster. Glutathione MPCs were synthesized and separated by polyacrylamide gel electrophoresis as described.[1] MPCs recovered from band 5,[1] with a gold cluster core of 14 kDa, correspond to average composition of Au_{71}. Because the empirical assignment of Au_{71} does not correspond to a theoretical closed shell structure, the gold core of this particle may be the closed shell Au_{75}.[25] These Au_{71} particles were examined by electron microscopy, revealing particles of the expected diameter, about 1.5 nm, which were sufficiently homogeneous that hexagonally close-packed arrays were occasionally observed (see Supporting Information). Seeking ways of improving the reactivity of the MPC toward protein sulfhydryls, we investigated the effect of the charge state of the gold cluster, based on a report that a +3 charge on an Au_{140} hexanethiolate MPC increased the rate of Murray place exchange by 2-fold.[22] In the case of the Au_{140} MPC, the cluster charge was manipulated by differential pulse voltammetry in CH_2Cl_2 to discover charge inflection points, followed by bulk electrolysis in the same solvent to charge the cluster to these points.[22] This procedure is technically difficult to perform with water as solvent,[15,26] but thiolate MPCs can be used as donors and acceptors in redox reactions,[27] so we investigated the use of chemical oxidants for charging the Au_{71} glutathione MPC.

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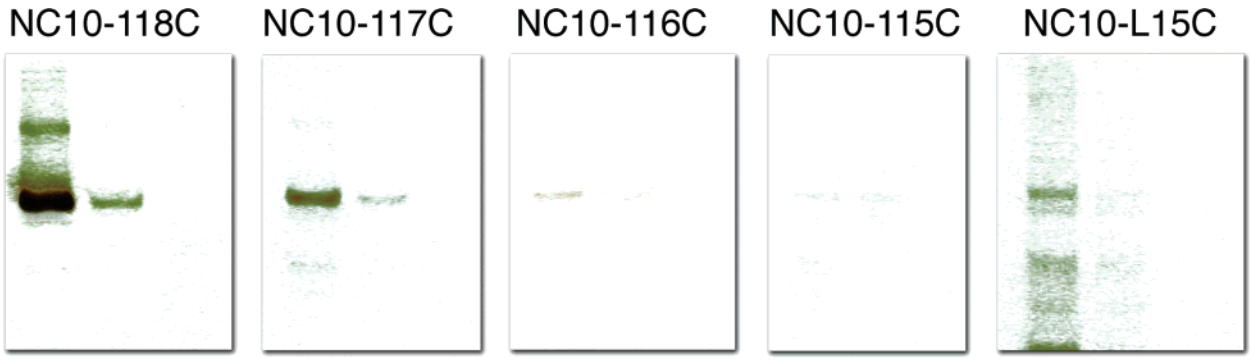

Figure 2. Reactivity of the nested set of NC10 mutants toward activated Au_{71} MPC. An SDS gel stained for gold is shown. The major band, which has an electrophoretic mobility slightly less than that of the scFv alone, corresponds to the NC10 scFv−Au_{71} MPC conjugate. Bands above the major (darkest) band correspond to a NC10 scFv−Au_{71} containing the pelB leader sequence and (NC10 scFv)$_2$−Au_{71}, respectively. Bands appearing below the major band correspond to degraded NC10 scFv−Au_{71} conjugates. NC10−L15C is a framework cysteine substitution mutant of the NC10 scFv.

The voltage required to charge an MPC to a given potential depends on the cluster size and the monolayer, but does not vary dramatically with solvent.[22,28] We expected the Au_{71} glutathione MPC, with a core similar to Au_{75} and a peptide like monolayer with a high dielectric constant, would have more charge inflection points over the same range than those reported for a virtually identical hydrocarbon protected gold cluster core, Au_{75}, in CH_2Cl_2.[25] We therefore expect that more charge states are sampled over a given potential range for this Au_{71} MPC than for other electrochemically characterized MPCs such as Au_{75}[25] and Au_{140}.[22] We screened chemical oxidants for solution potential at pH 9, because thiolates are more effective in place exchange than thiols, and most surface cysteine residues will be in thiolate form at this pH. A number of oxidants, including quinhydrone (solution potential −0.050 V vs Ag^+/AgCl at pH 9), potassium perchlorate (solution potential 0.202 V vs Ag^+/AgCl at pH 9), sodium nitrate (solution potential 0.257 V vs Ag^+/AgCl at pH 9), and potassium permanganate (solution potential 0.627 V vs Ag^+/AgCl at pH 9) gave solution potentials in the range that would charge the hexanethiol Au_{140} MPC to values between +1 and +4, and that should also produce multiple charge states of the Au_{71} MPC. The strongest of these oxidants, potassium permanganate, enhanced the reactivity of the Au_{71} glutathione MPC toward the 116C mutant of the NC10 scFv (Figure 3).

"Activation" of the glutathione MPC by oxidation with permanganate could be reversed by reduction. Tiopronin (solution potential −0.2 V vs Ag^+/AgCl at pH 9) was effective for the purpose. One equivalent of tiopronin was sufficient to quench reactivity of the glutathione MPC (Figure 4), and several equivalents could be added following reaction with the scFv without destroying the MPC−scFv conjugate.

We noted some hysteresis in the redox reactions, as the potential of a solution of Au_{71} glutathione MPC following oxidation was ∼ +0.28 V, greater than the starting potential of +0.05 V, but less than that of +0.51 V for the permanganate solution used. The potential of the MPC following reduction was −0.05, failing to attain the value of −0.20 of the tiopronin solution.

We never observe complete reaction of oxidized MPC with protein. We suspect this is because the oxidized MPC can itself oxidize surface cysteine residues of proteins, producing disulfide dimers, as well as sulfenic, sulfinic, and sulfonic acids.

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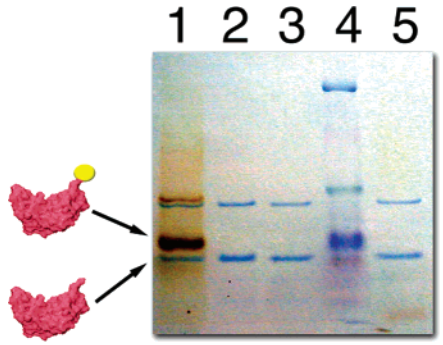

Figure 3. Reaction of NC10−116C sFv with Au_{71} MPC. An SDS gel stained with Coomassie Brilliant Blue and also stained for gold is shown. In lane 1, the MPC was oxidized potassium permanganate. The scFv appears as two bands due to some formation of a covalent (S−S bridged) dimer. In lane 2, the MPC was oxidized with quinhydrone. In lane 3, no MPC was added. Lane 4 contains Bio-Rad kaleidoscope markers. In lane 5, the MPC was unoxidized.

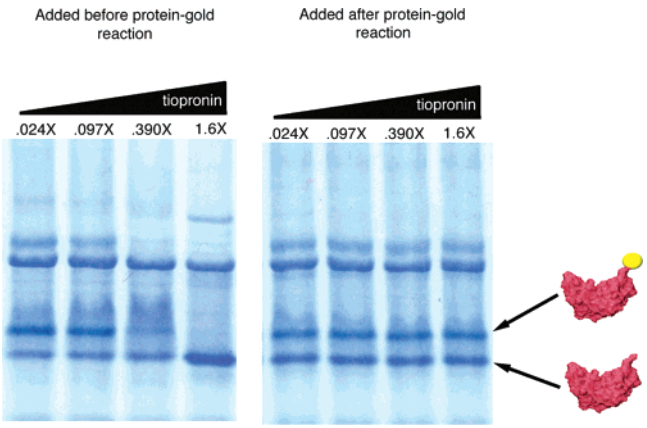

Figure 4. Quenching of cluster reactivity. Tiopronin was added in the molar ratios to gold clusters indicated above the lanes. When tiopronin is added in at least 1.6× molar excess to MPC prior to MPC reaction with protein, MPC reactivity is completely suppressed. When the same amount of tiopronin is added to an already formed complex, the complex is unperturbed.

Verification of the MPC−scFv Conjugate. The Au_{71} MPC−scFv conjugate was purified by ion exchange chromatography on Mono Q to remove unreacted scFv (Figure 5), and by gel filtration through Superdex 200 to remove unreacted Au_{71} MPC (Figure 6). The elution positions from the Superdex column corresponded well with the expected molecular weights of the scFv (apparent mol. wt. 25 kDa, based on an average retention volume of 1.68 ± 0.008 mL for 6 runs), the Au_{71}

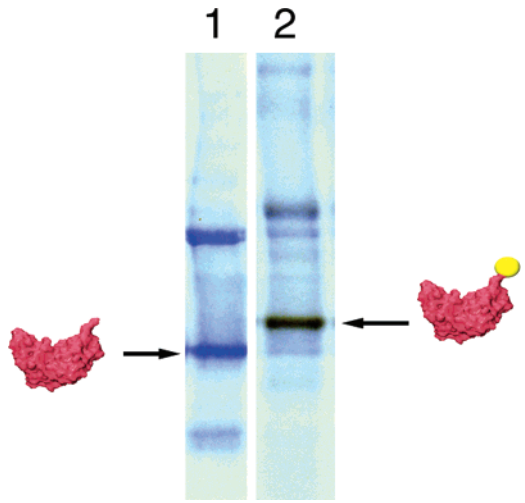

Figure 5. Separation of Au_{71} MPC−scFv conjugate from unreacted scFv on Mono Q. An SDS gel stained for protein (Coomassie) and for gold is shown. The scFv eluted at 130 mM NaCl (lane 1), while the conjugate eluted at 190 mM NaCl (lane 2).

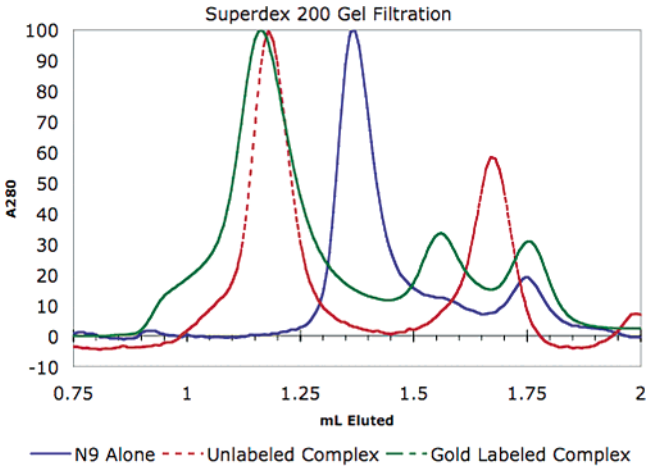

Figure 6. Separation of Au_{71} MPC, Au_{71} MPC−scFv conjugate, and Au_{71} MPC−scFv−neuraminidase complex by gel filtration. Blue trace, neuraminidase alone, showing two peaks, the neuraminidase (left) and an inert contaminant (right). Red trace, neuraminidase mixed with excess, monomeric, scFv, showing two peaks, the neuraminidase complexed with four scFvs (left) and free scFv (right). Green trace, neuraminidase mixed with excess Au_{71}−scFv conjugate, showing three peaks, the neuraminidase complexed with four conjugates (left), excess conjugate (middle), and free conjugate together with the inert contaminant in the neuraminidase preparation.

glutathione MPC (app. mol. wt. 15 kDa, based on average retention volume of 1.748 ± 0.015 mL for 12 runs), and the Au_{71} MPC−scFv conjugate (apparent mol. wt. 48 kDa, based on an average retention volume of 1.554 ± 0.008 mL for 8 runs). Gel filtration through Superdex 200 was also used to characterize and purify a complex formed by mixing the Au_{71} MPC−scFv conjugate with N9 neuraminidase, prepared as described[29] (Figure 6). The elution position of the complex corresponded to a molecular weight, of 520 kDa, consistent with the binding of four Au_{71} MPC−scFv conjugates to the tetrameric neuraminidase, based on an average retention volume of 1.156 ± .014 mL for 3 runs. This is an apparent 80 kDa more massive than the tetrameric−scFv−neuraminidase complex, which has an apparent molecular weight of 440 kDa, based upon

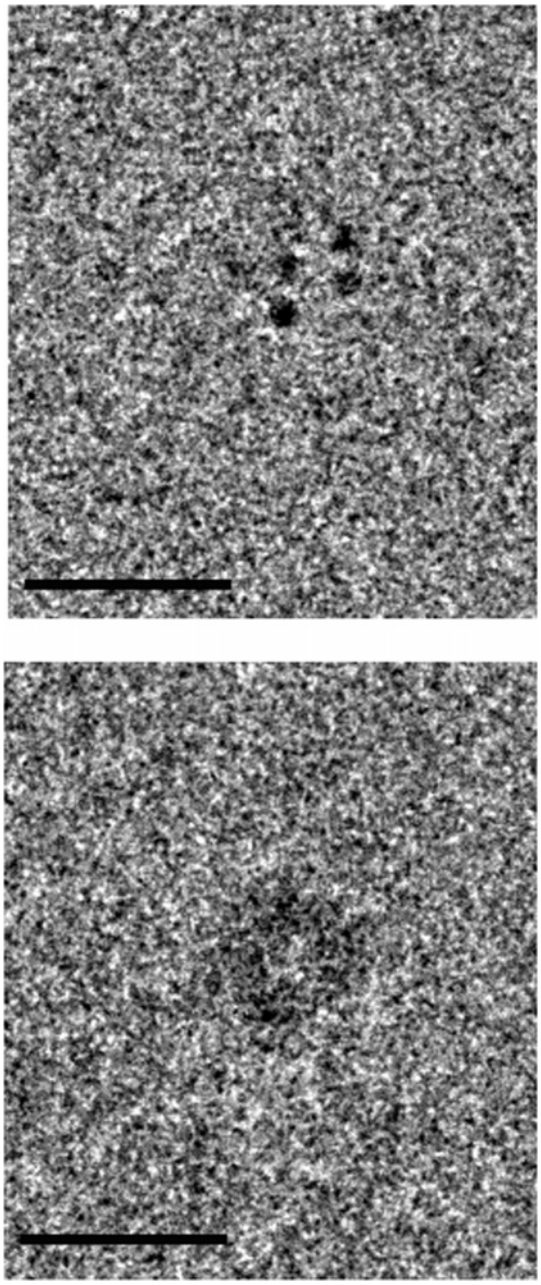

Figure 7. Cryoelectron micrographs of Au_{71} MPC−scFv−neuraminidase complex. Images were recorded at 1 μ underfocus (upper panel) and 4 μ underfocus (lower panel). The scale bar represents 5 nm.

a retention volume of 1.185 mL in a single run. The Superdex 200 column was calibrated with thyroglobulin (669 kDa, retention volume 1.058 mL), ferritin (476 kDa, retention volume 1.195 mL), catalase (232 kDa, retention volume 1.361 mL), aldolase (157 kDa, retention volume 1.382 mL), ovalbumin (43 kDa, retention volume 1.542 mL), and chymotrypsinogen (25 kDa, retention volume 1.723 mL). For final verification of the MPC−scFv−neuraminidase complex, the peak fractions from the Superdex column were examined by cryoelectron microscopy. Images recorded at a high defocus (Figure 7, lower panel) revealed the neuraminidase protein and displayed the false 5-fold symmetry of the NC10−neuraminidase complex seen in previous cryoelectron microscopy studies.[30] Images recorded at a low

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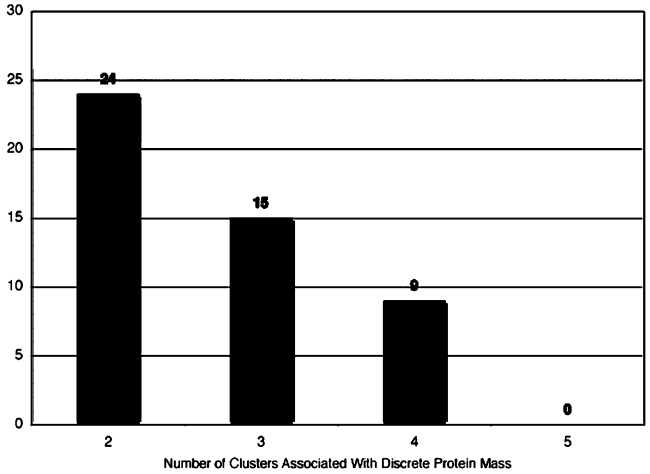

Figure 8. Histogram of the distribution of observed gold cluster conjugates by cryoelectron microscopy.

defocus (Figure 7, upper panel), where the gold clusters are most evident, revealed that many of the neuraminidase particles were decorated with four clusters. Particles decorated with fewer clusters were also observed (Figure 8). We believe the appearance of heterogeneity was exaggerated by interaction of the clusters with the surfaces of the Quantifoil grids used.

Discussion

The labeling of proteins with heavy atom clusters is of widespread importance, but its implementation is often problematic. An ideal labeling method would be general, without restriction regarding the target protein, and would attach a molecularly defined cluster rigidly to the protein surface, without the intervention of a flexible linker. Labeling would, moreover, be limited to the target protein and not permit the attachment of multiple proteins to a single cluster. We describe here a method that meets these requirements. We use an scFv as a rigid adapter between the protein and the cluster, enabling the full diversity of antibody specificity to be brought to bear on the problem. We employ a precisely defined cluster and report a novel chemistry for its rigid attachment to the scFv. The chemistry includes the termination of cluster reactivity, so only a single step of attachment can take place.

Mechanism of MPC−scFv Conjugate Formation. Reaction of equimolar amounts of MPC and scFv under thermodynamic control should give a 50% yield of conjugate, regardless of the cluster charge state. We find, however, a strong dependence on oxidation of the cluster for obtaining a significant yield. We believe this dependence is due, in part, to the competition between place exchange and oxidation of the cysteine sulfhydryl of the scFv. Oxidation of the cluster increases the rate of place exchange,[22] allowing reaction with the scFv to occur before its conversion to disulfide dimers or oxidation of the cysteine sulfhydryl to sulfenic, sulfinic, or sulfonic acids.

We have attributed the enhanced reactivity of oxidized MPCs toward protein sulfhydryls to the removal of electrons from the cluster core. An alternative is that the oxidant, potassium permanganate, damages the monolayer protecting the cluster, creating gaps through which a protein sulfydryl can penetrate to the cluster surface. If so, the quenching of reactivity by tiopronin might be explained by the ability of tiopronin as a thiol to adsorb to the cluster surface and fill the gaps created by permanganate. We cannot entirely rule out this interpretation, although we believe it is unlikely for a number of reasons, including the capacity of borohydride ion and Tris carboxyethyl phosphine (TCEP) to quench reactivity (data not shown). Borohydride ion cannot rebuild the monolayer as tiopronin may do. While TCEP may passivate gold nanoparticles such as other triply substituted phosphines, this passivation is unlikely to quench reactivity toward thiols, due to the greater affinity of thiols than phosphines for gold. The mechanism of activation of the MPC by permanganate is, in any case, of secondary importance, as the purpose of enhancing reactivity toward protein sulfhydryls in a reversible manner is well served by the use of this reagent.

Stability of MPC−Protein Conjugates. While MPC−scFv conjugates stored in the cold are quite stable, with an apparent half-life of at least 1 month when stored at 4 °C or an indefinite lifetime when frozen with 20% glycerol, it may be possible to increase their stability even further by appropriate cluster charging. Only four cluster charge values have been investigated for their effects on place exchange,[22] but as many as 15 redox states have been resolved for highly purified gold clusters.[28] If the trends so far observed extend to higher charges, the rate of place exchange may be further enhanced. Conversely, the rate may be further diminished by reduction to lower charges, rendering the MPC−protein conjugates stable for extended periods under a wide range of conditions.

Rigidity of Conjugates. Although MPC−protein conjugates are formed here by direct gold−cysteine sulfhydryl bonding, rotation about the gold−sulfur bond is still possible, and there are additional degrees of freedom within the cysteine side chain as well. This may pose a problem for some applications, and many methods of reducing cluster mobility, by variation of scFv and MPC surface chemistries, may be imagined. The most straightforward would be to shorten the linker between the last structured residue at the C-terminus of the scFv and the cysteine residue that reacts with the cluster. The NC10−117C scFv used for conjugation with the Au_{71} MPC contained a linker of three residues − two alanines and a glutamic acid expected to fill the gap left by the Glu of the glutathione replaced on the cluster surface. Indeed, one or even both alanines can be removed (in NC10−115C and NC10−116C) and reaction with Au_{71} MPC still occurs, albeit with a reduced efficiency (Figures 1, 2).

As an alternative to shortening the linker, an unpaired cysteine may be introduced in the scFv surface, by mutation of "framework" residues on the face of the scFv opposite from the antigen-combining site. We have already mutated several framework residues, and some of the mutants show reactivity comparable to or greater than that of NC10−116C (Figure 3, NC10−L15C). The reactivity and also the rigidity of the MPC conjugates obtained may be enhanced by framework mutations introducing positively charged residues in the vicinity of the reactive cysteine. Yet another example of a framework mutation likely to enhance the rigidity of the Au_{71} MPC−scFv conjugate would be a second reactive cysteine residue. This second residue must be far enough from the first to avoid disulfide formation, but close enough to contact the cluster surface and bond to it as well.

Comparison to Other Gold Nanoparticles. The conjugation chemistry reported here represents a significant improvement over the two other widely used chemistries for gold-labeling

biomolecules, the nonspecific binding of citrate-reduced colloidal gold,[31] and reaction through a linker of about 16 Å to triphenylphosphine-protected Nanogold[8] and undecagold.[32] The Au_{71} glutathione MPC is more homogeneous in size than colloidal gold and better defined than Nanogold.[11] MPCs are also more stable than citrate- or phosphine-passivated gold nanoparticles.[25,33,34] Whereas glutathione MPCs, like many thiolate MPCs, are stable to long-term storage, to boiling, and to a range of salt concentrations and other solvent conditions, the citrate- and phosphine-stabilized particles are comparatively unstable, do not tolerate high temperatures, and are extremely thiol-sensitive (limiting utility for use in vivo).

Experimental Section

MPC Synthesis and Oxidation/Reduction. MPCs were synthesized and purified by high density-polyacrylamide gel electrophoresis with a 20T/7C gel as described.[1] The identity of band 2^1 was verified by ESI-MS, and band 5 was used in this work. A Beer's law extinction coefficient of 821 500 at a wavelength of 241 nm was determined for band 5 and used to calculate MPC concentration. Oxidations were for 10 min at room temperature in 0.1 M Borate, pH 9.0. Oxidation was performed with a 5-fold molar excess of oxidant over MPC unless otherwise noted. Reductants were dissolved or diluted in distilled water and were added in either calculated molar or empirically determined amounts. Reduction was for at least 1 h at room temperature and was frequently followed by storage overnight at 4 °C. Most oxidants could be removed by methanol precipitation of the glutathione MPC. Reductants were removed by anion exchange chromatography. Oxidants soluble in methanol, such as potassium permanganate, were removed by dialysis in a 1000MWCO Spectra-Por 300 μL volume microdialysis device.

Solution potentials were measured with a Corning Ag/AgCl pH electrode with the exterior electrode isolated as the reference electrode and a platinum wire used as a working electrode. The solution potentials of several redox compounds measured in this way, including quinhydrone, sodium nitrate, and potassium ferricyanide, compared favorably with literature values. Several oxidants, including ferricyanide, iodine, and chromate, were incompatible with the glutathione MPC, causing decomposition of the nanoparticle.

scFv Mutagenesis and Expression. The NC10 scFv, encoded in the pGC−NC10 vector[35] with a 15-residue $(GGGGS)_3$ linker, was mutated to introduce a C-terminal cysteine residue. A FLAG tag was first inserted at the N-terminus, by cleaving the vector with SacI (NEB Biolabs), gel purifying the product, and religating the cleaved vector in the presence of the FLAG-tag encoding oligonucleotides 5′-cgactataaagacgatgacgataagggcatcgagct-3′ and 5′-cgatgcccttatcgtcatcgtcttatagtcgagct-3′. C-terminal cysteine mutants were created by gel purifying the SacII/EcoRI cleaved plasmid, which removes the C-terminal sequnece, and ligating the oligonucleotides 5′-ggcagaatgcggataag-3′ and 5′-aattcttatccgcattctgccgc-3′ for NC10−116C, 5′-ggcaaaagaatgcggataag-3′ and 5′-aattcttatccgcattcttttgccgc-3′ for NC10−117C, and 5′-ggcaaaaaaggaatgcggataag-3′ and 5′-aattcttatccgcattccttttttgccgc-3′ for NC10−118C. Mutants NC10−C112, −C113, −C114, and −C115 were made by deleting single amino acids from NC10−C116, using a QuickChange Site Directed Mutagenesis Kit (Stratagene). The primers used to create NC10−C112 were 5′-ggaccacggtcaccgaatgcggataaga-3′ and 5′-tcttatccgcattcggtgaccgtggtcc-3′, those for NC10−C113 were 5′-ccacggtcaccgtcgaatgcggataaga-3′ and 5′-tcttatccgcattcgacggtgaccgtgg-3′, those for NC10−114 were 5′-cggtcaccgtctccgaatgcggataaga-3′ and 5′-tcttatccgcattcggagacggtgaccg-3′, and those for NC10−115 were 5′-tcaccgtctccgcggaatgcggataaga-3′ and 5′-tcttatccgcattccgcggagacggtga-3′. To add positive charge near the gold cluster conjugation site, two lysine residues were introduced into NC10−116C with a QuickChange kit and the primers 5′-gaccacggtcaccgtctccaagaaagaatgcggataagaattcag-3′ and 5′-ctgaattcttatccgcattctttcttggagacggtgaccgtggtc-3′.

NC10 scFv and mutants were expressed and purified as described,[35] except that Top10F′ (Invitrogen) *E. coli* was cultured in 4.5 L, and the filtered osmotic shock fluid was concentrated with a Vivaflow 50 concentrator (Vivascience). The yield of NC10 cysteine mutants was 5−10-fold less than that of wild-type NC10.

MPC/scFv Conjugate Formation and Purification. The scFv (20 μM) was reduced with mercaptoethylamine (44 mM) at 37 °C for 30 min in 200 mM glycine/100 mM Tris, pH 8.0. The scFv was subsequently gel filtered with the use of a BioRad 10DG desalting column in 0.1 M Borate, pH 9.0. A volume of oxidized gold clusters in 0.1 M Borate, pH 9.0, was added to give final concentrations for the scFv of 12.5 μM and for the clusters of 375 μM, and the mixture was kept for 1 h at 42 °C. Optimal concentrations of TCEP and oxidized MPC were separately determined for each labeling experiment.

Ion exchange chromatography was performed on a 1 mL HiTrap Q column (Pharmacia) with a BioRad HR pumping station. Elution was with a gradient of 20 column vol from 20 mM Tris, pH 8, to 20 mM Tris, pH 8, 1 M NaCl. Gel filtration was performed on a Superdex 200 3.2/30 column in phosphate-buffered saline[25] with an Ettan FPLC system. A 50 μL injection loop was used with a 50 μL sample volume.

Gel Electrophoresis. SDS-PAGE was performed as described.[36] Gels were fixed in 40% methanol, 10% acetic acid, 50% water for 30 min, stained (or not) with Coomassie Brilliant Blue, equilibrated in water, washed twice with 1 mM EDTA, twice with water, and agitated with a mixture of equal volumes of silver stain solutions A and B (Sigma) (5 mL of each for a 4 × 6 cm gel) for ∼30 min. If bands did not form within 30 min, the gels were washed twice with water and fresh silver stain solution was added.

Cryoelectron Microscopy. Samples were applied to R2/2 Quantifoil grids, which had been glow discharged under vacuum, and were frozen with a vitrobot. Images were acquired on an FEI T12 transmission electron microscope, equipped with a liquid nitrogen cooled cryostage, operated at 100 kV, at magnifications between 52 000 and 67 000, with the use of a CCD detector.

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Supporting Information Available: Additional EM for the Au_{71} MPC alone. This material is available free of charge via the Internet at http://pubs.acs.org.

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