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A Method for the Quadruple Labeling of Platelet Surface Epitopes for Transmission Electron Microscopy

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Monodisperse gold colloids prepared over a broad range of particle diameters have proven useful for the simultaneous, multiple labeling of biological specimens observed by electron microscopy (EM). Antibodies and other ligands which specifically bind to the epitopes of interest are stably conjugated by means of hydrophobic interactions to the surface of each colloidal gold (cAu) particle, and the electron-dense particle in turn provides the requisite contrast that reveals the positions of the epitopes upon or within the specimen. Each set of labels is distinguished from another by conjugation of the ligand to cAu particles of different sizes. However, the number of labels which may be employed at any given time using such an approach is rather limited. The smallest particle size is dictated by the resolution of the instrument, and, at the other extreme, the maximum size must be constrained in order to diminish steric interference between individual particles as well as to optimize the spatial resolution of the labeling. Owing to the Gaussian size distribution of the particles constituting every colloidal sol, the average diameter chosen for each set of labels between these two bounds must be sufficiently offset such that they do not overlap, thus ensuring that each label is unequivocally discerned.

In practical terms, therefore, other parameters beyond differences in particle size need to be explored in order to label more than two or three epitopes simultaneously. The utilization of similarly sized particles, even for double labeling, may be advantageous by enabling the direct comparison between their respective labeling densities since the issues of steric hindrance and spatial resolution would thus be minimized. One approach we are studying is to use colloidal particles composed of other metals or combinations of metals in addition to Au. These consist most notably of the noble metals Ag, Pt, Pd, Rh, and Ru which, like Au, readily form stable, negatively-charged hydrophobe sols in aqueous solution. Such particles may be distinguished from each other using analytical techniques such as electron energy loss spectroscopy [1-3] or Z-contrast microscopy [4]. A second approach we have developed makes use of colloidal metal particles with shapes other than the smooth spheres or ovals characteristic of typical cAu preparations. These particles offer the advantage that they may be identified using conventional transmission EM, and, in some cases, scanning EM and atomic force microscopy as well [5].

As a means to demonstrate the efficacy of this latter approach, we are studying the co-localization of a number of factors and receptors on human platelet surfaces using four labels simultaneously. These include two sizes of round cAu particles together with umbonate or popcorn-shaped cPd particles and faceted cPd particles which appear in the TEM as mixtures of triangles, cubes, pentagons, and hexagons. We've developed modifications to Brintzinger's ascorbic acid reduction procedure [6] which enable the preparation of both the umbonate and faceted cPd particles with average sizes of 18nm and 15nm, respectively. In a series of papers, Gutbier [7] demonstrated that a number of proteins could be stably conjugated to other noble metal particles and protect them against electrolytic flocculation, much as Zsigmondy [8] had

demonstrated earlier for cAu sols. The process of and conditions necessary for the conjugation of biologically relevant proteins, namely antibodies, are similar for both gold and palladium sols. The process happens rapidly by hydrophobic interactions between the protein and metal surface and is most stable when electrostatic interactions are minimized. Since the charge on the metal surface is negative, this occurs when the pH of the sol is adjusted slightly basic to the pI of the protein. As with cAu conjugates, the cPd conjugates are then sedimented by ultracentrifugation and resuspended in buffer to the appropriate concentration necessary for optimal labeling as determined by spectrophotometry at the peak absorbance wavelength.

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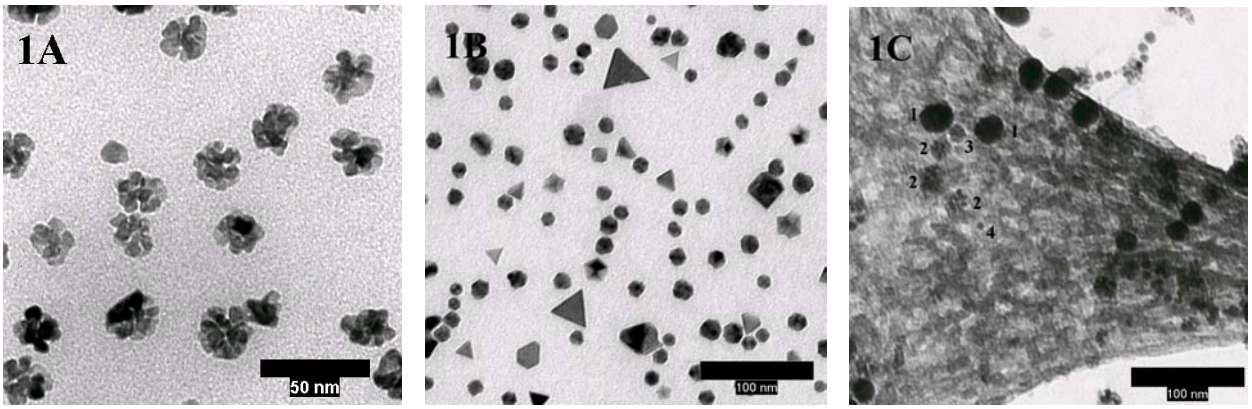

Figure 1A. Transmission electron micrograph showing umbonate cPd particles prepared by reduction of 250μM K_2PdCl_4 with 10% ascorbic acid at 0°C. Bar = 50nm. 1B. Transmission electron micrograph showing faceted cPd particles prepared by reduction of 250μM K_2PdCl_4 with 0.32% ascorbic acid at 100°C. Bar = 100nm. 1C. Transmission electron micrograph demonstrating quadruple labeling on a pseudopodial extension of an activated human platelet. (1) 18nm cAu particle conjugated to mouse anti-human P-selectin, a marker of activation. (2) Umbonate 18nm cPd particle conjugated to human factor X, a subunit of the thrombinase complex. (3) Hexagonal 15nm cPd particle conjugated to sheep anti-human factor V IgG, demonstrating location of endogenous human factor V, another subunit of the thrombinase complex. (4) 5nm cAu particle conjugated to exogenous human factor V. Bar = 100nm.

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