

Enhancement of immunogold labelled vinculin in cells cultured on metal implant surfaces

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INTRODUCTION: Cells adhere onto proteins adsorbed on implant materials by focal adhesions. The focal adhesion is a complex of integrin transmembrane receptors associated indirectly with the actin cytoskeleton by linker proteins. One important linker protein stabilising the entire focal adhesion structure is vinculin. The area of focal adhesions is known to correlate with adhesion strength and has been used as a method to determine the cytocompatibility of implant materials (1-2). Many techniques have been used to label the focal adhesions with the objective of measuring the total area of adhesion on different materials. Limitations to the specificity and resolution of these labels have proven to be problematic and consequently results are highly variable. A specific label, utilising ultrasmall gold particles attached to secondary antibodies, made it possible to localise individual antigens at high resolution with an electron microscope (3). Immunogold labelling with small probes has not been successful, to date, when labelling cells on metal substrates. Enlarging these probes with a silver solution is necessary to image them with a scanning electron microscope (SEM). However, heavy metal stains such as osmium tetroxide etch the enhanced particles reducing them to approximately their original size. High concentrations of osmium are used to provide sufficient electron density to the cell for imaging on the metal substrate. One method to protect the silver is by plating the silver with gold. Gold plating (toning) however replaces the outer silver shell with several gold particles therefore quantification is not possible since, stoichiometrically, the number of gold particles do not reflect the number of antigenic sites. Consequently a new Gold EnhanceTM solution has been produced by Nanoprobes. This enhancement is resistant to the etching effects of osmium. We show that immunogold labelling on metal surfaces is possible with both silver enhancement, in the absence of osmium tetroxide, and gold enhancement in the presence of osmium. We postulate a mechanism to explain the etching of silver particles in material attached to metal surfaces.

METHODS: Fibroblasts and osteoblasts were cultured on implant quality stainless steel and tissue culture plastic for 2 days at a density of 20,000/ml. The cells were immunogold labelled for vinculin following the procedure by Richards *et al.* (submitted). Two enhancement solutions were tested. Silver enhance

(British Biocell International Ltd, Cardiff, UK) and Gold enhanceTM (Nanoprobes Incorporated, Yaphank, NY 11980-9710, USA). Stainless steel was used as a test substrate since it was found to be the most problematic regarding self-nucleation and non-specific background deposition of silver from the enhancing solution. The postfixation stage was varied in osmium tetroxide concentration and duration on both substrates as seen in Table 1. All samples were then dehydrated through an ethanol series, critical point dried and coated with carbon for observation with a field emission SEM.

Table 1- Variations used in the postfixation stage of the immunogold labelling procedure. Au = gold enhance; Ag = silver enhance; M = Metal; P = Plastic; P+M = Plastic with stainless steel particles; Conc. = concentration

Enhance	Substrate	Osmium conc. (%)	Incubation (min)
Au	M	1.0	60
Ag	M	1.0	60
Ag	M	1.0	30
Ag	M	0.1	60
Ag	M	0.1	30
Ag	M	0	0
Au	P	1.0	60
Ag	P	1.0	60
Ag	P+M	1.0	60

RESULTS: SEM observations have been summarised in Table 2.

Table 2- Summary of observations from SEM images.
S =spherical enhanced gold label, *N*=non-spherical enhanced gold label See Table 1 for other abbreviations.

Enhance	Substrate	Os (%)	Incubation	Labelling	Particles
Au	M	1.0	60	Yes	S
Ag	M	1.0	60	No	
Ag	M	1.0	30	Yes	S
Ag	M	0.1	60	Yes	S
Ag	M	0.1	30	Yes	S
Ag	M	0	0	Yes	S
Au	P	1.0	60	Yes	S
Ag	P	1.0	60	Yes	N
Ag	P+MP	1.0	60	No	

DISCUSSION: Silver enhancement is a nanoscale molecular version of electroplating. Silver is plated onto the gold label in a time dependent manner. The gold label acts as a sink of electrons thereby catalysing the reduction of silver deposition onto gold. Silver is consequently plated on the gold label in a time dependent manner. GoldEnhance™ works on a principle similar to silver enhancement but its composition and formulation are not available from Nanoprobes. Silver enhanced gold particles are etched in the presence of osmium on a plastic substrate. Gold enhanced particles are not affected by osmium. Differences between silver and gold enhancing solutions can be explained by their redox potentials. The redox potential for OsO₄ to Os(0) (0.85V) is higher than that for Ag(I) to Ag(0) (0.96V), so the silver deposit will be oxidized by OsO₄, but since it has a lower potential than that for Au(I) to Au(0) (1.68V), Au(0) would not be oxidised (personal communication R. Powell, Nanoprobes). This reaction is osmium concentration and time dependent. Reducing the osmium concentration and/or time increased the observed number of labels but reducing both improved the labelling, agreeing with Bury's (4) observations.

Non-visualisation of immunogold labelling on metal substrates can be explained by the enhanced etching action of the osmium on the silver coating, catalysed by the underlying metal substrate. This is a concentration and time dependent reaction similar to the enhancing reaction.

If one is interested in immunogold labelling an antigen, in material attached to a metal surface, then the duration and concentration of osmium fixation should be scrutinised for its effect on the silver enhancement. Where optimal osmium staining is required, it is advisable to use GoldEnhance™.

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