Novel dPEG® Reagent for Eliminating Aggregation Problems in Protein Complexes

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ABSTRACT

Protein instability is a key problem during the industrial production of active substances for pharmaceutical and biomedical applications. As a bioprocess dilemma, instability and subsequent aggregation are affected by a number of factors including, sheer stress during cell culture, temperature, co-solvents, pH, hydrophobic interactions, and irregular inter-molecular disulfide bond formation. One often overlooked cause of protein instability is due to directed, covalent coupling of small molecules to proteins, for example antibodies biotinylated with the cross linker sulfo-LC-NHS-biotin are well known to aggregate.

Biotin modification (biotinylation), results in covalent attachment of one or more bicyclic biotin rings to a protein target, (e.g. antibodies). Such biotinylated proteins are capable of binding avidin or streptavidin with the specificity and nearly the same avidity of free biotin in solution. Biotinylated monoclonals are used for a variety of purposes such as in vivo tumor targeting with/or without cytotoxic cargo or creating traceable complexes for use in imaging techniques.

Sulfo-NHS-LC-biotin is widely used to add a biotin tag to monoclonal antibodies. It contains a (amine-reactive) sulfo-NHS ester with a 6-aminocaproic acid spacer arm which provides about 22.4 Å separation between a covalently modified antibody and the bicyclic biotin rings. Although sulfo-NHS-LC-biotin and NHS-LC-biotin are very popular for biotinylation, they introduce hydrophobic aliphatic chains on to the surface of antibodies. This amplifies the tendency of biotinylated antibodies to aggregate in aqueous solution and may cause protein precipitation or loss of activity over time. For this reason, the use of more hydrophilic PEG-based biotin compounds of more optimal spacer length is a better alternative for maintaining water solubility of modified proteins. In this report we demonstrate the superior performance of NHS-dPEG®$_{12}$-Biotin in eliminating streptavidin-accelerated aggregation of biotinylated antibodies. The dPEG®$_{12}$ spacer is a single compound 12 unit discrete PEG spacer designed and produced by Quanta BioDesign.

INTRODUCTION

The term dPEG® is an acronym for "discrete poly(ethylene glycol)" or "discrete PEG" and refers to a unique class of PEG®s, having a unique, specific, single molecular weight, synthesized de novo, from pure, small units (e.g., triethylene glycol or tetraethylene glycol). dPEG®s differ dramatically from long chain PEG polymers which are made through a polymer-based process and have variable chain lengths and subsequently, variable polydispersity values.

dPEG®s can be used as multifunctional protein crosslinkers and biotinylation reagents. Historically bioconjugation procedures have used aliphatic chains to provide a short spacer between a modified molecule and the bicyclic ring of biotin. This allows for enough molecular distance for streptavidin binding during detection or targeting applications. The drawback of these long chain (LC) linkers is that there hydrophobic chains can be detrimental to protein solubility. In particular, antibodies modified with long hydrophobic biotin compounds often aggregate and lose activity, especially at high modification levels. Replacing the LC units with discrete PEG arms can dramatically increase water solubility and prevent antibody aggregation as well as significantly boost long-term stability.

In this preliminary study, we compared some of the overall properties of an antibody (goat anti-rabbit IgG) that was modified by NHS-LC-Biotin and by NHS-dPEG®$_{12}$-Biotin. In particular we have examined how these antibodies affect the solubility properties of Streptavidin-biotinylated IgG complex. Our preliminary results clearly demonstrate that dPEG® reagents can provide benefit for nearly any crosslinking compound or modification reagent designed for use in aqueous environments. This benefit is most likely due to increased hydrophilic character for modified biomolecules or surfaces.

METHODS

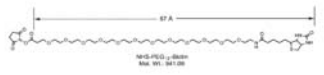
Purified goat-anti-rabbit antibodies were obtained from American Qualex while NHS-dPEG®$_{12}$-Biotin and sulfo-NHS-LC-Biotin were from Quanta BioDesign. The antibodies were modified with a ten-fold molar excess of each cross linker for two hours, then purified from unreacted cross linker over PD-10 columns (GE Healthcare). The final modified proteins were stored at 4ºC in 0.1 M sodium phosphate pH 7.4. Antibodies were characterized for biotin incorporation by a competitive binding assay using 4'-hydroxyazobenzene-2-carboxylic acid (HABA; Pierce Biotechnology).

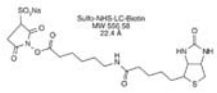
Spectral changes and turbidity measurements were carried out on a Cary-300 dual beam spectrophotometer having a thermostated 6 x 6 cell changer block. Aggregation of antibodies was monitored by turbidity at 400 nm and spectral scans were recorded from 200 nm to 800 nm.

IgG heterogeneity was analysed by capillary electrophoresis using a Beckman-Coulter P/ACE MDQ instrument. An IgG heterogeneity assay kit was purchased from Beckman-Coulter and 2-mercaptoethanol reduced IgGs were prepared exactly as described by the manufacturer. Separation was conducted on a 50 um ID fused-silica capillary, 30 cm in length, using buffers and matrix components provided in the kit.

A ProteoStat™ protein aggregation assay kit (Enzo Life Sciences) was used to test for protein aggregation. Fluorescence measurements from a 96-well plate format were recorded on a Tecan 96-well plate reader.

Figure 1: Structures and General Approach





Modify IgG with NHS-dPEG®$_{12}$-Biotin or SulfoNHS-LC-Biotin

↓

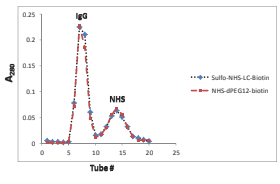
Purify over PD-10 & confirm biotin incorporation

↓

Compare conjugates for aggregation/stability

Figure 2. Elution Data, SDS PAGE and Biotin Binding Assays

2a.



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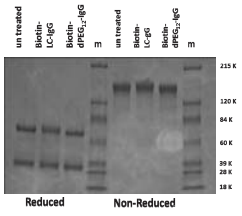


Fig 2c. Biotin Incorporation Into IgGs As Determined By HABA Assay.

Biotin, IgG Coupling Reaction Conditions	Biotin dPEG®$_{12}$-IgG	Biotin-LC-IgG
30 min; 10x molar excess	3.9 mol Biotin/mol IgG	3.7 mol Biotin/mol IgG
2 hr; 10x molar excess	4.3 mol Biotin/mol IgG	4.3 mol Biotin/mol IgG
30 min; 25x molar excess	9.0 mol Biotin/mol IgG	5.4 mol Biotin/mol IgG
30 min; 50x molar excess	10.4 mol Biotin/mol IgG	----*

HABA - dye (4'-hydroxyazobenzene-2-carboxylic acid)
* significant precipitation occurred during measurements

2d.

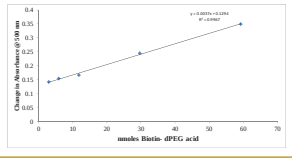


Figure 2. Elution data, SDS PAGE and biotin binding assays. This figure shows characterization of Biotin-LC-IgG and Biotin-dPEG®$_{12}$-IgG. Figure 2a shows an elution profile for the modified IgGs on PD-10 columns (Sephadex G-25) following a 2 hour incubation with sulfoNHS-LC-biotin or NHS dPEG®$_2$-biotin. "NHS" represents N-hydroxysuccinimide which elutes after the protein peak. Figure 2b shows SDS-PAGE gels for the proteins (2.5 ug) and demonstrates that the modified proteins have no significant molecular weight differences under reduced or non-reduced conditions. Figure 2c shows calculated biotin incorporation for IgGs at different reaction conditions. Figure 2d shows the difference between absorbance of the HABA only cuvette and the HABA biotin dPEG® acid cuvette plotted as a function of nanomoles of biotin dPEG® acid.

Figure 3. IgG Heterogeneity Assay by Capillary Electrophoresis

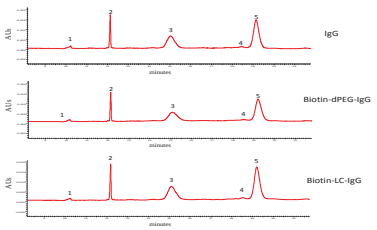


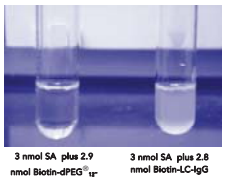
Figure 3b. Summary of CE Electrophoretograms of modified and unmodified IgGs

		Reduced IgG		Reduced Biotin dPEG®$_{12}$-IgG		Reduced Biotin-LC-IgG	
Peak #	Identity¶	Migration Time (min)	Relative Peak Area	Migration Time (min)	Relative Peak Area	Migration Time (min)	Relative Peak Area
1	2-mercaptoethanol	10.23	10,039	10.16	7,838	10.26	11,099
2	10kD peptide‡	12.13	58,061	12.13	46,944	12.12	63,807
3	IgG Light Chain	15.03	148,949	15.10	113,921	15.10	176,330
4	Non-glycosylated IgG heavy chain	18.43	3,343	18.51	1,319	18.53	6,284
5	IgG heavy chain	19.14	264,138	19.2	214,743	19.21	301,495

¶ Identity was assigned based on comparision of electrophoretograms to a validated IgG sample from Beckman Coulter as part of their IgG purity/heterogeneity assay.
‡ a validated 10 kD Beckman Coulter peptide as included in all samples as an internal reference

Figure 3. Shown are electrophoretograms for modified and unmodified goat anti-rabbit IgGs. The numbers above each peak of Panel A are identified in Panel B.

Figure 4. Aggregation of Biotin-LC- IgG and Streptavidin but not Biotin dPEG®-IgG and Streptavidin



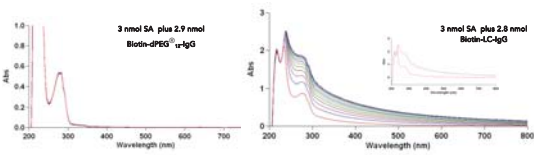


Figure 4. Figure 4a shows visible turbidity changes that occurs within minutes after mixing Biotin-LC- IgG with Streptavidin at a 1:1 mole ratio. The tube containing similar amounts of Biotin dPEG®-IgG and streptavidin remains clear. This change in turbidity is corroborated by spectrophotometric scans done over a 30 minute time period. Note that the scans for Biotin dPEG-IgG mixed with streptavidin are unchanged.

Figure 5. Characterization of Aggregates

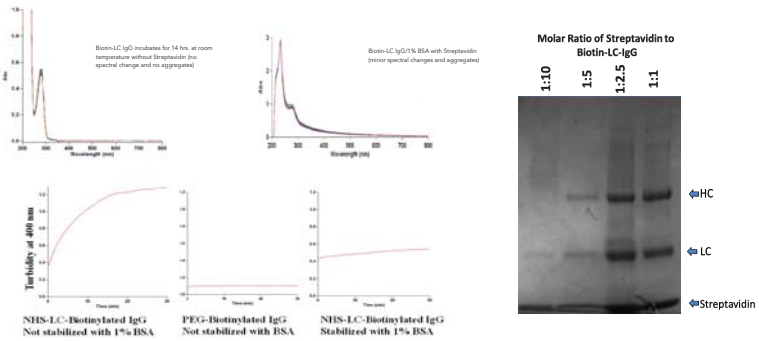


Figure 5. Analysis of the aggregation process. The top and bottom figures of Panel A shows that spectrophotometric changes in Biotin-LC- IgG (approximately 2.8 nmole) does not occur in the absence of Streptavidin, and that aggregation can be reduced by the addition of 1% BSA into the reaction. Panel B shows the absorbance curve at 400 nm that was taken from the scan data for the biotinylated proteins plus or minus BSA. Panel C shows SDS-PAGE gel (4-15% Mini-PROTEAN TGX gel, Bio-Rad) analysis of the Biotin-LC- IgG-Streptavidin aggregates. Lanes are identified and the positions of streptavidin, IgG light chain and IgG heavy chain are indicated.

Figure 6. Effect of IgG: Streptavidin Ratios on Turbidity/Aggregation

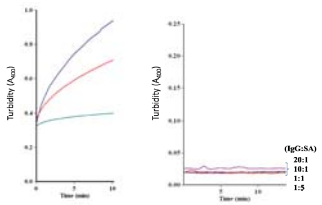


Figure 6. Panel A shows results from time course studies for the change in turbidity at 400 nm after increasing amounts of biotin-LC IgG are mixed with Streptavidin to give molar ratios (IgG:SA) of 1:5, 1:2.5 and 1:1. Panel B B shows corresponding kinetic data for mixing increasing amounts of Biotin-dPEG®$_{12}$ IgG with Streptavidin. Note that no turbidity changes were observed even at molar ratios (IgG:SA) of 10:1 or 20:1.

Figure 7. ELISA test for biotinylated Goat anti-rabbit IgG.

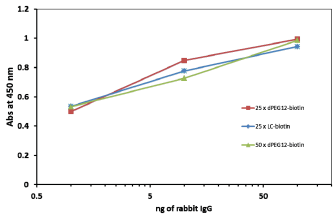


Figure 7 shows a standard ELISA and demonstrates that biotinylated dPEG antibodies can still recognize the antigen. Plates were coated overnight with variable amounts of normal rabbit antibody then blocked with 0.1% BSA in phosphate buffered saline containing Tween-20 (0.05%). Biotinylated antibodies (biotin-LC-IgG or Biotin-dPEG®$_{12}$-IgG) were diluted 1:10,000 in PBST, added to appropriate wells, and incubated 2 hrs at room temperature. Wells were then rinsed 3x with PBST, then incubated for 30 minutes with a 1:1,1000 dilution of Streptavidin-Horse Radish Peroxidase conjugate. Following another 3 rinses, the pl ate was developed with a Sureblue Peroxidase substrate kit and stopped with 0.1 M HCL. Optical densities were quantified at 450 nm on a Tecan GENios microplate reader.

CONCLUSIONS

The high affinity interaction between biotin and streptavidin, is used extensively in molecular biology and bionanotechnology, commonly, with the biotin moiety conjugated to an antibody. A significant problem associated with this technology is protein aggregation which manifests itself as background noise. In this study we have compared a traditional IgG-LC-biotin conjugate to a novel-IgG-dPEG®-biotin conjugate.

- Both conjugates appear identical by CE, SDS PAGE, elution profile on PD-10 and moles biotin per mole IgG by HABA binding (Figures 2 and 3).
- Their behavior differs sharply in the presence of the biotin-binding protein, Streptavidin (SA). The IgG-LC-biotin – SA aggregates within 30 minutes, as can be observed both visually and spectrophotometrically, while the IgG-dPEG®-biotin - SA shows no sign of aggregation (Figure 4).
- The aggregation is dependent on the presence of Streptavidin, and coprecipitates with the IgG as shown by SDS gels (Figure 5). Studies with a commercial IgG-LC-biotin preparation stabilized with 1% BSA indicates that aggregation can be reduced with carrier proteins (BSA), however carrier-free IgG-dPEG®-biotin was nonetheless still more stable. Even at 14 hours at RT, the IgG-dPEG®-biotin showed no signs of aggregation, while the stabilized IgG-LC-biotin begins to exhibit a tendency for aggregation (Figure 5). Even after 14 days, (data not shown) the IgG-dPEG®-biotin shows no signs of aggregation.
- The IgG-dPEG®-biotin shows no signs of aggregation with SA up to molar ratios of 20:1 (IgG:SA), while IgG-LC-biotin, exhibits aggregation with SA at molar ratios (IgG:SA) as low as 0.4: 1, and the rate and degree of aggregation increases significantly when the molar ratio reaches 1: 1 (Figure 6).
- A standard sandwich ELISA assay was performed to assess whether modifications with dPEG®-biotin led to any loss of antigen binding by the antibody (Figure 7). As can be seen, there was hardly any loss in antigen binding for IgGs treated with either 25 or 50 molar fold excess of the NHS-dPEG®$_{12}$-biotin. Additional studies have demonstrated that there is little difference in the antigen binding affinity between unmodified IgG and IgG-dPEG®-biotin.
- The procedure for the biotinylation of antibodies with NHS-dPEG®-biotin is identical to that used with sulfo-NHS-LC-biotin / NHS-LC-biotin. The product can be purified under identical chromatographic conditions, yielding a product indistinguishable under standard analytical techniques and with an equivalent degree of biotinylation. The Ab-dPEG®-biotin product distinguishes itself from traditional Ab-biotin products in its ability to eliminate protein aggregation without the need of additives based on blood proteins or other specialized formulations. The dPEG®-biotinylation products are commercially available from Quanta BioDesign Ltd.

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