Application Note

Comparative Analysis of Different EDC and Sulfo-NHS Activation Reagents for Covalent Coupling of Antibodies to Estapor® Carboxyl-Modified Dyed Microspheres

Introduction

Proteins are a rich source of primary amine groups which represent the most common target for bio-conjugation. They are frequently covalently coupled to the surface of carboxylated microspheres by activating the carboxyl groups with a zero-length crosslinker like the water-soluble 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC). The reaction forms an active intermediate (O-acylisourea) that reacts quickly with primary amines (nucleophile) to form a stable amide bond (Hermanson, 2013).

The active intermediate, however, is unstable and readily hydrolyses in water, regenerating the original carboxyl group. To prevent rapid hydrolysis of the active intermediate, N-hydroxysulfosuccinimide (Sulfo-NHS) can be added to the reaction in a first step to form a more stable Sulfo-NHS ester intermediate that reacts slowly with primary amines in a second step to form a stable amide bond. The two-step method also facilitates buffer exchange and the removal of excess EDC before adding protein, eliminating the chance of activating carboxyl groups on the protein and the possibility of protein cross-linking (Grabarek and Gergely, 1990; Staros, Wright and Swingle, 1986).

A two-step covalent coupling protocol using EDC and Sulfo-NHS activation reagents was previously published under MilliporeSigma application note MS_AN1260EN00. In this subsequent application note we demonstrate the equivalence of using activation reagents from different suppliers in the same coupling procedure. Activation was carried out in MES (2-(N-morpholino) ethanesulfonic acid) buffer (carboxyl and primary amine group-free) at pH 6 (Nakajima and Ikada, 1995). MES was also used as the coupling buffer but can be exchanged for an alternate buffer at a different pH if required for coating optimisation. The conjugation procedure outlined in application note MS_AN1260EN00 which conjugates protein to carboxyl-modified microspheres is a general two-step EDC/Sulfo-NHS covalent coupling protocol. Optimisation may be required, depending on the type and molecular weight of the protein and the carboxyl charge density on the microspheres.

A lateral flow assay (LFA) to detect the Hepatitis B surface antigen (HBsAg) was selected as a model to illustrate the performance of two different lots of MilliporeSigma EDC and Sulfo-NHS activation reagents in comparison to one reference lot from Competitor A. The detector particle used for the three conjugations was Estapor® Red Intense carboxylated microspheres (K1-030 Red Intense), to which an anti-HBsAg detection antibody was conjugated. The increase in diameter size of the conjugated microspheres due to the covalent binding of the antibody was first confirmed on a Zetasizer (Malvern Ultra Red) instrument. Then after drying and assembly into a finished lateral flow strip, the performance of the conjugated detector particles was compared by qualitatively assessing a sensitivity detection limit of 8 ng/mL HBsAg, absence of non-specific binding at the test line, full clearance of the conjugate from the conjugate pad as well as negligible background on the nitrocellulose membrane. Quantification of signals at the test line over a range of analyte concentrations was also measured on an Axxin AX-2X-S reader to assess assay range and curve shape.

Experimental Section

Table 1: Product Description

Cat. No.	Product Information
E7750	1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC)
56485	N-hydroxysulfosuccinimide (Sulfo-NHS)
398136	Ethanolamine
FR180380637	Estapor® K1-030 Red Intense COOH Microspheres
M8890	Goat Anti-Mouse IgG
H4522	Human Serum

Table 2: Recommended Buffers

Buffer	Solution
Activation/Coupling buffer	50 mM MES pH 6.0
EDC Solution	200 mM EDC in Milli-Q® water
Sulfo-NHS Solution	200 mM Sulfo-NHS in activation/coupling buffer
Blocking buffer	50 mM Tris, 0.5% Casein, pH 8.0
Conjugate Pad diluent buffer	50 mM Tris, 0.5% Casein, 0.5% PVP10, 10% Sucrose, 2.5% Trehalose, pH 8.0
Sample Pad buffer	10 mM Tris, 1% Tween20, pH 8.2

Table 3: Lateral Flow Strip Materials

Cat. No.	Product Information
CFSP223000	Surewick® C083 Cellulose Fiber Sample Pad
GFDX203000	Surewick® GFDX Glass Fiber Conjugate Pad
SHF1350225	Hi-Flow™ Plus 135 Membrane, 2-mil backing
CFSP223000	Surewick® C083 Cellulose Fiber Absorbent Pad
HF000MC100	Adhesive Backing Card

Comparative Results for 'MilliporeSigma' and Competitor A EDC and Sulfo-NHS Activation Reagents

Particle size measurements were carried out using the Malvern Zetasizer Ultra Red instrument. The results of all liquid conjugated detector particles indicated a diameter size increase from the raw microspheres which is proportional to that expected from the addition of an antibody on both sides of the sphere. In addition, a low Polydispersity Index (PDI) value of less than 0.1 for all conjugates indicated a similar narrow distribution around the mean particle size as seen with the raw microspheres (**Table 4**).

The Qualitative lateral flow strip results (**Figure 1**) together with the quantification measurements performed on the Axxin AX-2X-S reader (**Figure 2**) indicate that the two covalent conjugations carried out with MilliporeSigma EDC and Sulfo-NHS activation reagents are equivalent to the one carried out using Competitor A products. No non-specific reactions were identified at the test lines in the absence of analyte. All three conjugates were fully released from their conjugate pads and no background colour was evident on any of the nitrocellulose membranes. Sensitivity was detected at the target level of 8 ng/mL HBsAg and signal intensity increased similarly for all three lateral flow test strip types throughout the tested range of 0 to 240 ng/mL.

Table 4: Zetasizer Measurements of Estapor® Stock microspheres versus microspheres conjugated with anti-HBsAg Antibody (covalently bound with either Competitor A or MilliporeSigma EDC and Sulfo-NHS activation). A 5% stock of Red-intense microspheres was diluted in Milli-Q® to a final concentration of 0.005%, while 0.2% anti-HBsAg antibody conjugates (in 50mM Tris; 0.5% Casein) were also diluted in Milli-Q® water to the same final concentration of 0.005%.

Zetasizer Measurements	Z average Diameter Size nm	PDI
Raw microspheres	324.2	0.1016
Competitor A EDC/Sulfo-NHS Covalent Conjugation	378.2	0.0995
MilliporeSigma lot 1 EDC/Sulfo-NHS Covalent Conjugation	374.3	0.0645
MilliporeSigma lot 2 EDC/Sulfo-NHS Covalent Conjugation	370.0	0.0616

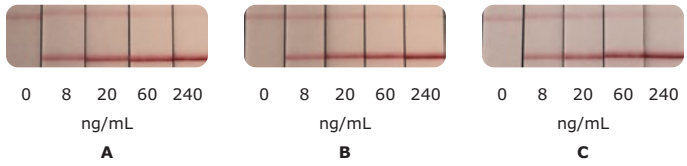

Figure 1: Qualitative images of HBsAg lateral flow test strips (0.005% Conjugates). Reference Competitor A (A) EDC and Sulfo-NHS activation reagents were compared to MilliporeSigma lot 1 (B) and MilliporeSigma lot 2 (C) EDC and Sulfo-NHS activation reagents in a covalent conjugation of anti-HBsAg monoclonal antibody to Estapor® Red Intense carboxylated microspheres.

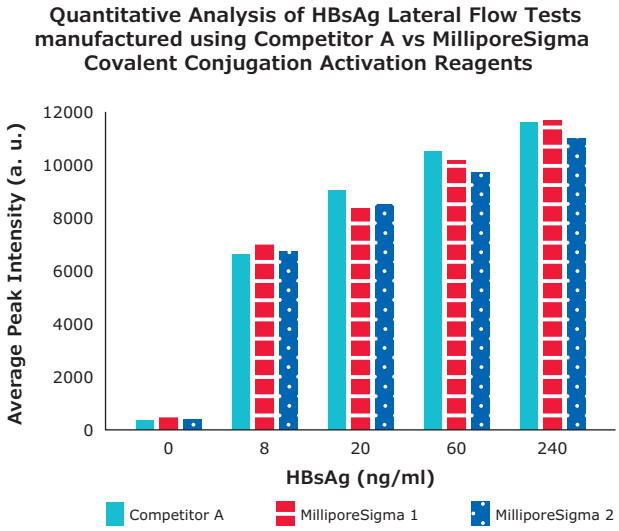

Figure 2: Quantitative Test line results (Peak Intensity-average of 3 strips x 2 replicates=6 reads per HBsAg concentration) from Axxin AX-2X-S Reader for HBsAg lateral flow tests constructed with anti-HBsAg antibody/Estapor® Red Intense carboxylated microsphere conjugates at 0.005% concentration. The conjugates were manufactured with different EDC and Sulfo-NHS activation reagents (a reference Competitor A lot and two test lots from MilliporeSigma).

Conclusion:

This study was carried out to demonstrate that MilliporeSigma EDC and Sulfo-NHS activation reagents (E7750 and 56485) are equivalent to the same Competitor A reagents when used in a two-step covalent coupling bioconjugation procedure. The performance of the covalent conjugations of anti-HBsAg antibody to Estapor® Red Intense carboxylated microspheres using two lots of MilliporeSigma reagents and one lot of reference reagents from Competitor A were assessed in a subsequent HBsAg lateral flow assay.

All three conjugates were dried down at the same concentration of microspheres (0.005%), assembled into lateral flow test strips and a HBsAg sample range of 0 to 240 ng/mL was applied. All three types of conjugates produced LFA strips which were sensitive down to at least 8 ng/mL. All three conjugates fully released from their respective conjugate pads upon resolubilisation and no background colour was evident on the nitrocellulose membrane. In addition, no non-specific binding was identified at the test line in the absence of HBsAg analyte. Quantitative measurements on the Axxin reader also showed that similar signal intensities across the range of the HBsAg assay were achieved between the reference Competitor A lot and the two lots of MilliporeSigma activation reagents, indicating equivalence between the two types of products.

Hence MilliporeSigma EDC and Sulfo-NHS activation reagents are suitable to be used in a standard two-step covalent conjugation of protein to carboxylated polymeric microspheres.

To find the products used in this paper please visit:
https://www.sigmaaldrich.com/US/en

References:

1. Application Note (**MS_AN1260EN00**). Microsphere Coupling-Two-step EDC/Sulfo NHS Covalent Coupling Procedure for Estapor® Carboxyl-modified Dyed Microspheres.
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3. Hermanson, Greg T. 2013. Bioconjugate Techniques, **Academic Press, London**.
4. Nakajima N, Ikada Y. 1995. Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. **Bioconjugate Chemistry**, 6(1), pp. 123-130.
5. Staros, J. V., Wright, R. W. and Swingle, D. M., 1986. Enhancement by N-Hydroxysulfosuccinimide of Water-Soluble Carbodiimide-Mediated Coupling Reactions. **Analytical Biochemistry**, 156(1), pp. 220-222.

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