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Technical Note

# Evaluation of chemically modified carrier proteins for developing monoclonal antibodies against a clinically relevant mutation of cKIT

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ABSTRACT

In this report we show that combining double-chemically modified carrier proteins and hetero-functional cross-linkers allows preparing tailor-made hapten-protein carrier conjugates. Accordingly, a new carrier protein has been designed where carboxylic groups were transformed into highly reactive primary amino groups by reaction with ethylendiamine after activation with EDCI. The aminated protein carrier is then modified by different cross-linkers (hyper-activated proteins) at different conditions in order to control the conjugation ratio from 1 to >12 molecules of hapten per carrier protein. Finally, this novel strategy has been successfully used to develop antibodies against a short specific peptide corresponding to a point mutation (D816V) of cKIT, which is a clinically relevant mutation related to mastocytosis and gastrointestinal stroma tumor.



## 1. Introduction

Most protein antigens or haptens of molecular weight under 1000–3000 Da are not immunogenic by themselves when are used alone as immunogens (Rajewsky et al., 1969) because of they need to be chemically conjugated to macromolecules such as highly immunogenic foreign proteins (carrier), peptides or synthetic polymers, in order to stimulate a potent immune response (Basta et al., 2004; Clementi et al., 1991; Partidos et al., 1992; Pincus et al., 1973.).

Ideally, a conjugation procedure should: *i.*-Provide a high yield of well-defined and reproducible composition without inactivation of hapten molecule. *ii.*-Produce a stable link between the hapten and the carrier protein. *iii.*-Require a minimum amount of the hapten. *iv.*-Supply a simple and practical protocol.

One commonly method, considered as a standard methodology used for chemical conjugations is based on agents such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), SMCC, glutaraldehyde, BS3, SPDP,... which involve the primary amino groups (Hermanson, 2008; Muckerheide et al., 1987b). However, the final product is not always reproducible, resulting in large variations in the generation of antigen (for example: ratio molecules of protein carrier/molecules of hapten) and naturally in the quality of specific recognition capacity of the antibody.

The degree and yield of the chemical conjugation may be very important in many instances, for example when hapten availability is not very high or if its immunogenicity is poor (Milich, 1990). In fact, with the objective of increasing the reactivity of the carrier proteins, some proteins for instance bovine serum albumin (BSA), mouse serum albumin (MSA, or hemocyanin from *Megathura crenatula* (KLH)) are commercialized already modified with succinic anhydride (Muckerheide et al., 1987a). This treatment increases the specific immune

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response against the antigen, thanks to several reasons (according to some authors) such as a faster cleared from the circulation than native carrier proteins (Border et al., 1982) or because the cationized proteins tend to adhere better to the weak anionic cell membrane, as shown by other authors (Schalkwijk et al., 1985); although the most obvious explanation is increase of probabilities to linking the hapten per carrier molecule (Fuentes et al., 2005). Furthermore, the lack of primary amino groups in the protein prevents the possibilities of covalent aggregation, thus cross-linking between two carrier proteins may not be possible.

In general, this chemical modification approach has been successfully used in the development of antibodies (Trier et al., 2012). However, this strategy has not been widely employed because insuficient results have been obtained when the hapten is a short peptide corresponding with point mutations. These unsatisfactory results are mainly due to problems related to hapten-protein carrier conjugation: *i.*- conjugation chemistry might not preserve the epitopes, *ii.*- over-conjugation of the antigen could limit processing and presentation of the antigen, *iii.*- some homofunctional cross-linkers ( i.e. glutaraldehyde,…) could cause immunogenic epitope destruction and inhibition of proteolytic processing of antigen because of its promiscuous reactivity.

In this work, a new strategy is proposed to modify and characterize different carrier proteins with the short specific peptide (Sequence "N"-CDFGLARVIKNDSN-"C") called D816V-cKIT TKII. This characteristic peptide, used as an example, corresponds to a fragment of cKIT protein, whose point mutations are clinically relevant in the diagnosis of mastocytosis and gastrointestinal stromal tumor (GIST).

## 2. Materials and methods

### 2.1. Materials

Bovine serum albumin (BSA),N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 4-(N-Maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (Sulfo-SMCC) were purchased by SIGMA (Illinois, USA). Peptides ( D816V-cKIT TKII) for this experiment was obtained by solid phase peptide synthesis (SPPS) and supplied by SBS Genetech ( Beijing, China), Goat Anti-Rabbit IgG ( whole molecule) -Peroxidase SIGMA and the Liquid Substrate System for ELISA 3,3′,5,5′-Tetramethylbenzidine (TMB) were purchased by SIGMA (Illinois, USA).

### 2.2. Methods

#### 2.2.1. Chemical modification of BSA with primary amino groups

The whole superficial carboxyl residues of the bovine serum surface were converted to primary amino groups by reaction with 1 M ethylendiamine at pH 4.75 and 10 mM EDC. After 2 h (h) at RT, amino-modified BSA was split into two different aliquots which were separately dialyzed each one against distilled water and using a desalting column by spin in order to eliminate excess of ethylendiamine and EDC. The amino-modified carrier protein (active-BSA) was storage at 10 mg/mL (Carraway and Kosland, 1972).

#### 2.2.2. Chemical activation of active-BSA or native-BSA through amino-reactive groups

2 mL of 10 mM Sulfo-SMCC solution was added to 1 mL of native-BSA or active-BSA at 10 mg/mL. After 1 h at RT, both separate aliquots maleimide-BSA and hyper-maleimide-BSA resulted from chemical activation of native-BSA and active-BSA, respectively, were exhaustively dialyzed separately against maleimide conjugation buffer pH 7.0 (50 mM Sodium Phosphate, 1 mM EDTA, pH 7.0).

#### 2.2.3. Peptide conjugation with active-BSA and hyper-maleimide-BSA

*2.2.3.1. Conjugation by maleimide chemistry.* 10 mg of each carrier protein maleimide-BSA or hyper-maleimide-BSA were incubated, during 2 h at RT, with 4 mg/mL of a sulfydryl-containing peptide in maleimide conjugation buffer. Then, both samples were dialyzed against 20 mM Phosphate buffered saline (PBS) (pH 7.2) using desalting column by gravity.

*2.2.3.2. Conjugation by carbodiimide chemistry.* 20 mg of the peptide were dissolved in 5 mL of EDC Conjugation Buffer and 10 mg of peptide were added to both native-BSA and active-BSA, EDC conjugation solutions from the first step of modification of BSA. In the next step, 5 mg of EDC were dissolved in distilled water at 10 mg/mL and were immediately added 2.5 mg of each aliquot of native and active-BSA. Both solutions were incubated during 2 h at RT and finally dialyzed against 20 mM PBS (pH 7.2) using desalting column by gravity.

#### 2.2.4. Characterizaton of chemically modified carrier proteins by Isolectric focusing

10 μg of each samples was re-suspended in rehydration buffer ( 8 M Urea, 2 M thiourea, 4% CHAPS, 1mDTT, 1.2% DeStreak, 0.5% IPG buffer 3–11 NL). Samples were applied to 24 cm IPG strips with a non linear pH gradient of 3 to 11 ( Amersham Biosciences) and rehydrated overnight using immobiline Drystrip Reswelling Tray for at least 18 h. Isoelectric focusing was performed at 500 V for 1 h, a 1000 V gradient over 1 h, a 4000 V gradient over 30 min, a 8000 V constant voltage. Current was limited at 50 μA per strip and temperature was 20 °C. Then IPG strips were removed and fixed in 35% methanol, 10% acetic acid and stained with Coomassie Brilliant Blue ( CBB) according to Kang et al., 2002 IPG (Kang, D., Gho, S.G., Suh, M. & Kang, C. Highly Sensitive and Fast Protein Detection with Coomassie Brilliant Blue in Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Bull. Korean Chem. Soc. 11, 1511–1512 (2002).

#### 2.2.5. Characterization of peptide-protein carriers conjugates by 2D-gels

Samples were precipitated with methanol/chloroform, and finally the pellet was re-suspended in re-hydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 5 mM TCEP, 15 mg DeStreak, 0.5% IPG buffer). The samples were applied to 7 cm IPG strips with a nonlinear pH gradient of 3 to 10 (Amersham Biosciences). Isoelectric focusing was performed at 50 V for 12 hours, 500 V for 1 h, 1000 V for 1 h, a voltage gradient ranging from 1000 to 8000 V for 30 min, and finally 5 h until the voltage reach 35000 V. Strips were treated with sodium dodecylsulfate