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Notes & Tips

Conjugation of a peptide to mannan and its confirmation by tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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ABSTRACT

The conjugation of polysaccharides to peptides is essential for antigen delivery and vaccine development. Herein, we show that tricine SDS-PAGE in combination with Coomassie Blue staining was adequate to determine the conjugation efficacy of a peptide (epitope 35–55 of myelin oligodendrocyte glycoprotein) to mannan. In addition, tricine SDS-PAGE and periodic acid–Schiff stains were able to monitor the redox state of mannan. Using the described protocol, more than 99.9% of a peptide containing five lysines at its N-terminus was confirmed conjugated to mannan.

The synthesis of peptide–saccharide conjugates is widely used in modern synthesis with applications in diverse fields, from chemistry to medicine [1,2]. Subunit vaccines can be produced by conjugation of peptides to polysaccharides, resulting in the targeting of the conjugate to specific cells [3]. The conjugation occurs by formation of a Schiff base between the free amines of the peptide and the aldehydes of the oxidized carbohydrates. The process is widely used for the coupling of carbohydrates to proteins and is facilitated by the protein free amino groups [4]. On the other hand, coupling of peptides to carbohydrates may be hindered by the lack of free available amino groups. Of particular interest for the scientific community and health services are the peptides from myelin proteins conjugated to oxidized or reduced mannan which are potentially pathogenic in multiple sclerosis [5–7]. Although the synthesis and the promising *in vitro* and *in vivo* efficacy of these molecules have been described [5–7], the extent of conjugation and the redox condition of the participating sugars are not routinely assessed by a simple, low cost and efficient analytical method. Capillary electrophoresis (CE)[1] for example, has been used previously for the detection and confirmation of conjugation between a cyclic peptide and an oxidized mannan. The peptide contained an additional $[LysGly]_5$ linker that provided amine groups for the formation of Schiff bases with the aldehydes of the oxidized mannan [8]. The analysis was performed at borate (pH 9.3) and phosphate (pH 5.1) buffers at 30 kV with normal polarity and showed that the conjugated product was heterogeneous [8]. Tricine SDS-PAGE combined with Coomassie Blue or silver staining has been used for the electrophoretic analysis of proteins with molecular weights below 30 kDa [9]. In this study tricine SDS-PAGE and Coomassie Blue stain [10] were used to confirm the conjugation $MOG_{35–55}$ to mannan via $(LysGly)_5$ linker, and tricine SDS-PAGE followed by periodic acid–Schiff stain (PAS) [11] and 2,4-dinitrophenylhydrazine (DNP) colorimetric assay [12] to evaluate the presence of aldehydes in the redox state of the mannan moiety.

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[1] *Abbreviations used:* CE, capillary electrophoresis; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MOG, myelin oligodendrocyte glycoprotein; PAS, periodic acid–Schiff stain; DNP, 2,4-dinitrophenylhydrazine; CLTR-Cl, chlorotrityl chloride; HPLC, high-performance liquid chromatography; $NaIO_4$, sodium periodate; $NaBH_4$, sodium borohydride.

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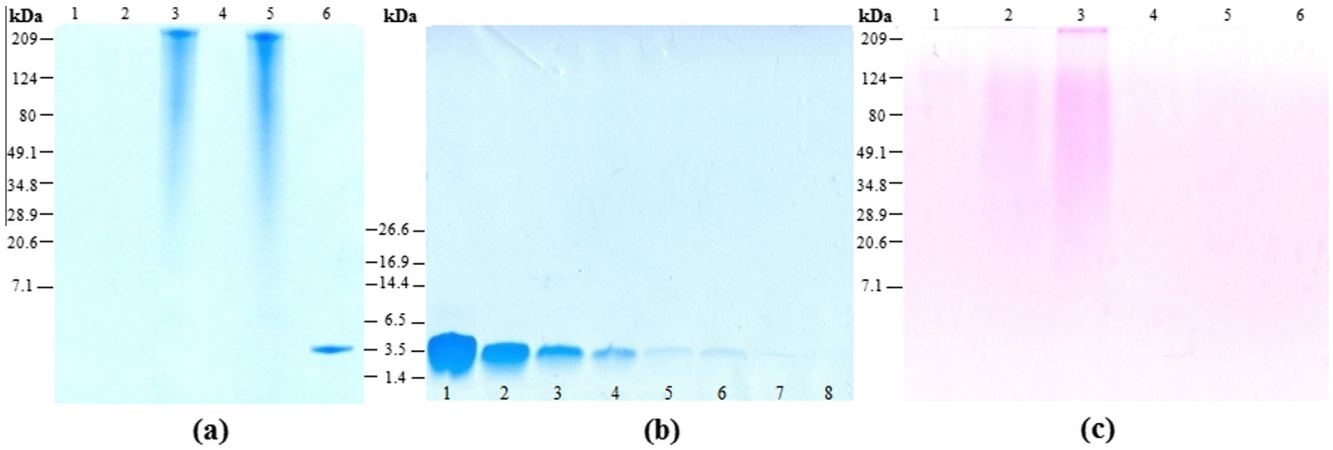

Fig.1. (a) Confirmation of conjugation by tricine SDS-PAGE and Coomassie Brilliant Blue staining: Lane 1, mannan; Lane 2, oxidized mannan; Lane 3, conjugate of oxidized mannan–$(LysGly)_5$–MOG_{35-55} peptide; Lane 4, reduced mannan; Lane 5, conjugate of reduced mannan–$(LysGly)_5$–MOG_{35-55} peptide; Lane 6, $(LysGly)_5$–MOG_{35-55}. Amounts of 78.75 μg for the various forms of mannan and 7.5 μg for the peptide were loaded per lane. (b) Detection limit of free peptide using Coomassie Brilliant Blue; lanes 1–8 correspond to $(LysGly)_5$–MOG_{35-55} in different concentrations. Lane 1, 3.75 μg; Lane 2, 1.875 μg; Lane 3, 0.75 μg; Lane 4, 0.375 μg; Lane 5, 0.1875 μg; Lane 6, 75 ng; Lane 7, 37.5 ng; Lane 8, 18.75 ng. (c) Periodic acid–Schiff-stained gels of mannan samples after tricine SDS-PAGE: Lane 1, mannan; Lane 2, oxidized mannan; Lane 3, conjugate of oxidized mannan–$(LysGly)_5$–MOG_{35-55} peptide; Lane 4, reduced mannan; Lane 5, conjugate of reduced mannan–$(LysGly)_5$–MOG_{35-55} peptide; Lane 6, $(LysGly)_5$–MOG_{35-55}. Amounts of 78.75 μg for the various form of mannan and 7.5 μg for the peptide were loaded per lane.

The peptide analogue [$(LysGly)_5$–MOG_{35-55}] was synthesized by Fmoc/tBu methodology using 2-chlorotrityl chloride resin (CLTR-Cl) and N^a-Fmoc (9-fluorenylmethyloxycarboxyl) side chain protected amino acids [13,14]. The purity of the peptides was more than 97% by analytical high-performance liquid chromatography (HPLC). Mannan (14 mg poly-mannose from *Saccharomyces cerevisiae*) was oxidized to aldehydes using sodium periodate (0.002 mM $NaIO_4$, 0.1 M phosphate, pH 6, 1 h, 4 °C in dark) and purified by size exclusion chromatography (PD-10 desalting columns). The collected oxidized mannan (2 ml, ~10.5 mg, or 5.25 mg/ml) was mixed with 1 mg of peptide analogues (0.5 mg/ml) and incubated at room temperature for at least 24 h. Conjugation of the peptide to oxidized mannan occurred via formation of a Schiff base between the amino group of Lys and the aldehydes of oxidized mannan (0.1 M carbonate–bicarbonate pH 9, 24 h, dark, room temperature). Unreacted aldehydes and remaining Schiff bases in the oxidized mannan–peptide conjugate were reduced to alcohols and amines, respectively, by sodium borohydride (0.025 mM $NaBH_4$, 3 h at room temperature) (Supplementary Fig. 1) [4–6].

Tricine SDS-PAGE was used as the method examining the completion of the coupling reaction instead of the previously described CE analysis [8]. Mannan, oxidized mannan, oxidized mannan–peptide conjugate, reduced mannan, reduced mannan–peptide conjugate, and free peptide $(LysGly)_5$–MOG_{35-55} were diluted (50:50) in tricine sample loading buffer (Bio-Rad Laboratories, Inc.) without 2-mercaptoethanol, and heated to 100 °C for 5 min. Samples were loaded on a tricine gel containing a 3.5% stacking gel and a 16.5% separating gel. Electrophoresis was performed at 4 °C, initially at 120 V for 10 min until the samples migrated into the stacking gel, followed by 150 V for 95 min (analysis in running gel). Immediately after, gels were stained either in 0.1% Coomassie Brilliant Blue G-250 in 50% methanol and 10% acetic acid for 0.5 h, or in Schiff's reagent solution for 0.5 h. Destaining of gels was accomplished by soaking into fixing solution for 0.5 h followed by overnight immersion in ultrapure water (Coomassie stain) or only by immersion in ultrapure water (PAS).

The redox status of mannan obtained was measured by an alternative method based on the reaction of aldehydes with DNP to form the stable 2,4-dinitrophenylhydrazone derivative absorbing at 405 nm. Mannan and conjugates (0.7 ml of various forms, 5.25 mg/ml) were mixed with a DNP solution (0.007 mM, 0.5 ml of 0.68 mg in 5% sulfuric acid, 33% ethanol) and incubated at room temperature for 1 h. The mixture was purified by size exclusion chromatography and the yellow fraction containing the aldehyde–DNP conjugate was collected and its absorbance measured at 405 nm (A_{405}). Results for this method represent the average of three experiments.

Tricine SDS-PAGE followed by Coomassie stain (Fig. 1) showed that the method could be used for the confirmation of the conjugation reaction. Free peptide [$(LysGly)_5$–MOG_{35-55}] and its conjugates to oxidized or reduced mannan stained well with Coomassie Brilliant Blue (Fig. 1a, all lanes). The free peptide (lane 6) was detected as a band with an apparent molecular weight of 3.0–3.5 kDa. The mannan–peptide conjugates (lanes 3 and 5) appeared as a diffuse smear with an apparent molecular weight of 20–200 kDa. The observed diffusion is explainable in view of the heterogeneity of the mannan moiety containing different numbers of mannose units in both the main and the side chains (variable mannosylation). The missing band at 3.5 kDa in the mannan–peptide conjugate (Fig. 1, lanes 3 and 5) confirmed that conjugation was complete. Thus, the relatively simple and inexpensive SDS-PAGE method could yield results similar to those of the more complicated CE technique [8]. Notably, no significant conjugation was observed for analogues with 1, 2, or 3 LysGly repeats as a linker (data not shown).

The amount of free peptide that could be imprinted visibly after electrophoresis was also calculated. Peptide concentration range was of 2.5×10^{-1} (3.75 μg) to 12.5×10^{-4} mg/ml (18.75 ng). The detection threshold for the free $(LysGly)_5$–MOG_{35-55} by the Coomassie stain was 2.5×10^{-3} mg/ml (or 37.5 ng on the gel, Fig. 1b, lane 7). As described in the procedure for the synthesis of the mannan–peptide conjugate, the peptide concentration at the beginning of conjugation was 0.5 mg/ml (7.5 μg per lane, Fig. 1a). Analysis of the conjugation reaction by tricine SDS-PAGE and Coomassie staining showed that no peptide was detectable after conjugation (Fig. 1a, lanes 3 and 5). Given that the detection limit of the Coomassie stain for the peptide was 37.5 ng, at least 99.9% of the peptide was confirmed conjugated to mannan.

We examined the sensitivity of the method by silver staining (not shown) which is generally considered more sensitive than the Coomassie stain, able to detect proteins up to 0.25 ng per lane [15]. The reproducible detection limit of the free peptide using silver stain was 75 ng per lane (data not shown). This result

contrasts the lower detection limit of the Coomassie Brilliant Blue for this work (37.5 ng per lane). Silver staining is based on the reductive precipitation of silver on macromolecules under alkaline conditions. It could be possible that this deposition was reduced on the smaller peptides due to their size and simpler conformation [16].

We examined whether tricine SDS-PAGE combined with PAS could be used to monitor redox changes in mannan. Samples containing mannan only (Fig. 1a: lane 1, mannan; lane 2, oxidized mannan; lane 3, reduced mannan) did not stain with Coomassie as polysaccharides do not react with the particular stain but are viewed specifically by PAS [17]. The usual procedure for the PAS staining of sugars starts with their oxidation followed by their staining with Schiff's reagent [18]. Herein (Fig. 1c), the stain of mannan polysaccharide was performed only by the Schiff reagent as mannan was previously oxidized with periodic acid to prepare it for conjugation to peptide. Schiff staining examined the presence of free aldehydes in mannan–$(LysGly)_5$–$MOG_{35–55}$ conjugates. Oxidized mannan and oxidized mannan–peptide conjugates stained positively and appeared as diffuse smears with apparent molecular weights of 20–200 kDa (Fig. 1c, lanes 2 and 3). The diffused electrophoresed samples represent the heterogeneity of the mannan moiety. The positive stain in lane 3 (Fig. 1c) indicated that aldehydes did not disappear completely after the conjugation of peptide to oxidized mannan. On the other hand, the negative stain for reduced mannan and reduced mannan–peptide conjugates (Fig. 1c, lanes 4 and 5) showed the complete reduction of aldehydes to alcohols after treatment with $NaBH_4$. Therefore tricine SDS-PAGE and PAS stain could follow up changes in the redox state of mannan.

To confirm the redox status of mannan obtained by tricine SDS-PAGE and PAS, we measured changes of total carbonyls by DNP. Aldehydes were increased after oxidation of mannan (A_{405} from 0.004 ± 0.001 to 0.750 ± 0.031), while conjugation of oxidized mannan to the $(LysGly)_5$–$MOG_{35–55}$ peptide resulted in their slight decrease (A_{405} of 0.622 ± 0.023). The presence of aldehydes in mannan after its conjugation to the peptide reflected the excess of mannan used. Reduction of mannan and mannan–$(LysGly)_5$–$MOG_{35–55}$ peptide conjugates by sodium borohydride eliminated the excess aldehydes (A_{405} of 0.003 ± 0.002 and 0.009 ± 0.001, respectively) by presumably converting them to alcohols. The peptide itself absorbed insignificantly (A_{405} of 0.0012 ± 0.0001). The DNP assay confirmed the results obtained by tricine SDS-PAGE and PAS.

In summary, tricine SDS-PAGE with Coomassie Brilliant Blue stain was used to confirm the conjugation of a peptide ($MOG_{35–55}$) containing five extra lysines [$(LysGly)_5$–$MOG_{35–55}$] to the aldehydes of a sugar (oxidized mannan). Inclusion of the extra five lysines to the peptide sequence gave quantitative conjugation. Tricine SDS-PAGE and PAS could confirm the redox state of mannan as reconfirmed by the DNP assay. The method permitted the estimation of free peptide in the conjugate up to 0.015% in the final conjugate solution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2015.06.010.

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