Efficient Monitoring of Enzymatic Conjugation Reaction by Surface-Enhanced Laser Desorption/Ionization Time of Flight Mass Spectrometry for Process Optimization

Teresa Freire,[†] Jacques D'Alayer,[‡] and Sylvie Bay*[,†]

Unité de Chimie Organique URA CNRS 2128 and Plate-forme d'Analyse et de Microséquençage des Protéines, Institut Pasteur, 25-28 rue du Dr. Roux, 75724 Paris, Cedex 15, France. Received September 5, 2005;
Revised Manuscript Received January 16, 2006

Efficient analysis of bioconjugation reactions is one the most challenging task for optimizing and eventually achieving the reproducible production of large amount of conjugates. In particular, the complexity of some reaction mixtures precludes the use of most of the existing methods, because of the presence of large amounts of contaminants. As an alternative method, we used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) for monitoring an in vitro enzymatic transglycosylation of *N*-acetylgalactosamine (GalNAc) residues to a recombinant mucin protein MUC6. For this reaction, catalyzed by the uridine 5′-diphospho-*N*-acetylgalactosamine:polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts), we used either a recombinant ppGalNAc-T1 or a mixture of ppGalNAc-Ts contained in MCF7 tumor cell extracts. In the present study, we show that SELDI-TOF MS offers unique advantages over the traditional methodologies. It is a rapid, accurate, sensitive, reproducible, and very convenient analytical method for monitoring the course of a bioconjugation, even in heterogeneous samples such as cell extracts. SELDI-TOF MS proved very useful for optimizing the reaction parameters of the transglycosylation and for achieving the large scale preparation of Tn antigen-glycosylated mucins for antitumor immunotherapy applications.

INTRODUCTION

Bioconjugation technology has been widely used in nearly every discipline of life sciences research (*1*, *2*). One of the application areas is the preparation of hapten−carrier conjugates for immunization purposes, antibody production, and vaccine research. Indeed, small hapten molecules such as carbohydrates cannot elicit an efficient immune response on their own. To make them immunogenic, they must be coupled to a suitable carrier molecule, typically a protein. The characteristics of the resulting conjugate play a critical role in the intensity and the quality of the immune response. In particular, several groups have reported the influence of the hapten density on the level, the specificity, and the affinity of the produced antibodies (*3*−*6*). Careful follow-up of the conjugation is therefore very important to achieve the reproducible production of conjugates.

Different approaches are commonly employed to analyze these conjugates and to ascertain their optimal preparation. The choice of the method depends on the physicochemical properties of both hapten and carrier, as well as on the cross-linking strategy. The most frequently used procedures involve mass spectrometry, sometimes associated with high performance liquid chromatography (HPLC) (*4*, *6*−*8*), gel filtration (*2*), absorption (*9*) and fluorescence (*6*, *7*) spectroscopy, gel electrophoresis (*5*, *6*, *8*), and colorimetric assay for reactions involving sulfhydryl groups (*10*), amino groups (*11*), or carbohydrate residues (*12*). Although efficient, these existing methods suffer from several drawbacks. First, they often require further treatment of the samples, they are time-consuming, and they are not easy to perform when multiple samples are to be analyzed. Moreover, they are usually not very accurate and only give a rough estimate of the conjugate's molecular mass and integrity. Finally, the complexity of some reaction mixtures (cell extracts, sera, tissue homogenates, etc.) can affect the effectiveness of the analysis adversely, due to the presence of large amounts of other compounds (lipids, detergents, salts, other proteins, etc.).

Therefore, there is a need for rapid and sensitive analytical methods for monitoring bioconjugation reactions, particularly with complex and heterogeneous samples. Additionally, such efficient methods are essential in development, for optimizing the process and scaling up the production of the conjugates, while ensuring a batch-to-batch consistency.

ProteinChip array technology or surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) allows the analysis of complex protein mixtures by combining two powerful techniques: chromatography and mass spectrometry. After selective retention on a chip surface, the compounds of interest are subsequently analyzed by a laser desorption/ionization mass spectrometer. This technique has been successfully used in many fields such as biomarker discovery, study of biomolecular interactions, protein profiling, analysis of posttranslational modifications, etc. (*13*, *14*).

As compared to these applications, very few examples have been described so far showing the use of SELDI-TOF for the monitoring of a reaction (whether chemical or enzymatic) or for the analysis of the resulting product. Recently, the direct analysis of peptides or proteins after either enzymatic digestion (*15*−*17*) or limited acid hydrolysis (*18*) has been successfully performed on-chip, with the aim of identifying protein sequences. On-chip enzymatic reactions and subsequent characterization have also been performed in order to study posttranslational modifications. Using this method, the degree of glycosylation of a recombinant antibody has been monitored with a deglycosylation procedure using PNGase F (*19*) and the

* Corresponding author. Tel: (33) 1 45 68 83 98; Fax: (33) 1 45 68 84 04; e-mail: sbay@pasteur.fr.

[†] Unité de Chimie Organique URA CNRS 2128.

[‡] Plate-forme d'Analyse et de Microséquençage des Protéines.

10.1021/bc0502661 CCC: $33.50
Published on Web 02/24/2006

phosphorylation state of a peptide or a protein has been evaluated after the action of a kinase (*20*) or a phosphatase (*21*). This last example included a time course study. In another characterization study, Hubalek et al. (*41*) have described the analysis of biotinylated tryptic peptides after digestion of biotinylated recombinant human monoamine oxidases and subsequent purification on an affinity column. Similarly, a peptide enzymatically released from a synthetic peptide related to the proteinase-activated receptor 2 was identified by SELDI-TOF MS (*22*). Interestingly, SELDI-TOF MS has also allowed the course of the autoactivation process of a bacterial protein to be followed, directly from the culture supernatant (*23*). Finally, the utility of SELDI-TOF MS has been demonstrated for monitoring the attachment of bacterial oligosaccharides to a protein, by a conjugation method using squaric acid diester chemistry (*24*, *25*). However, the reaction was a chemical ligation in a simple mixture composed of the synthetic linker-derivatized oligosaccharide, the protein carrier, and the buffer. To our knowledge, SELDI-TOF MS has never been used for monitoring an in vitro enzymatic conjugation reaction in a complex mixture.

Among the hapten molecules, carbohydrates are of particular interest since they are part of bacterial determinants, and they are also tumor-associated antigens (TAA). As a result, a large number of carbohydrate−protein conjugates have been developed as vaccines against infectious diseases (*26*) and cancer (*27*). In previous works, we and others have prepared various conjugates displaying the Tn antigen (α-D-GalNAc−Ser/Thr) (*28*−*30*) which is a carbohydrate TAA overexpressed in breast, lung, prostate, and colon cancers (*31*, *32*). The resulting glycopeptides (*28*) or glycoproteins (*29*, *30*) have been shown to be highly promising vaccine candidates for targeting cancers. However, the preparation of such conjugates relies on multistep tedious syntheses and/or time-consuming purifications. To circumvent these difficulties, we developed an enzymatic approach for producing a protein glycoconjugate with a high Tn density.

Tn-mucins are attractive targets for antitumor immunotherapy since carbohydrates have been shown to be an essential part of tumor-associated structures within the mucins (*33*). As the protein backbone, we chose the MUC6 gastric mucin (*34*) which has been described in different tumors, including lung (*35*, *36*) and breast (*37*, *38*) carcinomas. This mucin is a natural substrate of the uridine 5′-diphospho-*N*-acetylgalactosamine:polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts, EC 2.4.1.41) which are the enzymes responsible for the Tn antigen synthesis in vivo.

To achieve the large scale preparation of MUC6-Tn conjugates for antitumor immunotherapy, we performed the in vitro enzymatic GalNAc antigen transfer onto the mucin acceptor. We describe herein the monitoring of the conjugation by SELDI-TOF MS and we show that this method is very rapid and efficient for optimizing the reaction parameters, including in complex mixtures.

EXPERIMENTAL PROCEDURES

MUC6 Cloning and Expression in *E. coli*. A cDNA clone containing one tandem repeat of MUC6 was isolated from total cDNA of MCF7 breast cancer cells by RT-PCR and cloned into pGem-T (Promega, France). The PCR products were designed to encode a half tandem repeat (87 amino acids) that were amplified with *Pfu* DNA polymerase and the primers MUC6-F, 5′-cgggatccTCCACCTCCTTGGTGACT-3′ and MUC6-2R 5′-ggaagcttATTAGGATGGTGTGTGGA-3′ (lowercase characters indicate restriction sites for *BamH*I and *Hind*III in the forward and reverse primers, respectively). Following digestion with *BamH*I and *Hind*III, each product was cloned into the pET28a(+) vector (Novagen, Fontenay-sous-Bois, France), so as to encode for a protein carrying a six-histidine tail at the N-terminus. *E. coli* DH5α transformants were selected on LB plates containing 50 μg/mL kanamycin, and the positive clones were confirmed by PCR and sequencing. Plasmids were purified from selected clones and used to transform *E. coli* BLi5 chemically competent cells. The recombinant was expressed in *E. coli* BLi5 by induction with 1 mM IPTG and purified over Ni^{2+}-nitriloacetic acid columns under denaturing conditions according to the manufacturer's (Qiagen, Germany) instructions. The MUC6 protein was characterized by amino acid analysis (AAA), SELDI-TOF MS, and electrospray mass spectrometry (ESMS: 12148.05; calculated: 12147.45). These analyses, together with a N-terminal sequencing, show that the protein lacks the N-terminal methionine residue.

Breast Cancer Cell Line Extracts. Breast cancer cell line MCF7 was grown to 90% confluence in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% fetal bovine serum, 1 mM pyruvate, and 2 mM glutamine and 5% CO_2 at 37 °C. After trypsinization, cells were washed three times with phosphate-buffered saline (PBS), resuspended in 250 mM sucrose, and homogeneized. Cells were then centrifuged at 3000*g* for 10 min at 4 °C. The resulting supernatant was again centrifuged at 100000*g* for 1 h at 4 °C. The pellet was resuspended in 0.1 M imidazole pH 7.2 and 0.1% Triton X-100. Cell extracts were aliquoted and stored at −80 °C. Protein concentration was determined by the BCA method (Sigma Chemical Co., St Louis, MO).

Recombinant Bovine Polypeptide GalNAc Transferase 1. A soluble form of the bovine ppGalNAc-T1 (ppGalNAc-T1), kindly given by F. Piller and V. Piller, was expressed in the yeast *Pichia pastoris* KM71H strain (Invitrogen, Cergy Pontoise, France) using the pPICZαA expression vector (Invitrogen). The recombinant ppGalNAc-T1 (from amino acids 52 to 559) carrying a N-terminal 6His-tag was expressed (induction in 0.5% methanol for 120 h) and purified on Ni-NTA-agarose (Qiagen, Hilden, Germany). The specific activity of the recombinant ppGalNAc-T1 was tested as previously described (*39*) and estimated to 3 U/mg protein (1 unit transfers 1 μmole of GalNAc per min at 37 °C to the acceptor peptide $[STP]_5$).

In vitro GalNAc Transfer to MUC6 Protein. Microsome fractions of MCF7 breast cancer cells were incubated at 37 °C for 48 h with uridine 5′-diphospho-*N*-acetylgalactosamine (UDP-GalNAc) and purified recombinant MUC6 (40−80 μM) in 50 mM imidazole pH 7.2 containing 50 mM $MnCl_2$ and 0.1% Triton-X100. When recombinant ppGalNAc-T1 was used, the MUC6 purified protein (40−80 μM) was incubated with UDP-GalNAc in 50 mM MES, pH 6.5 for 24 h at 37 °C. Aliquots were taken at different times and frozen at −20 °C. The different reaction conditions are detailed in Table 1. The resulting MUC6-Tn were purified using Ni-NTA-agarose (Qiagen, Hilden, Germany) (only when cell extracts were used) and then subjected to reversed phase HPLC using a Perkin-Elmer pump system with an UV detector at 230 nm. The column was a Symmetry 300 C18 (5 μm, 300 Å, 3.9 × 250 mm) (Waters, France). Elution was carried out with a linear gradient of 10−60% acetonitrile in 0.1% trifluoracetic acid in water at a flow rate of 1 mL/min (over 30 min). The peak was collected and then lyophilized. The MUC6-Tn glycoproteins were characterized by amino acid analysis and SELDI-TOF MS.

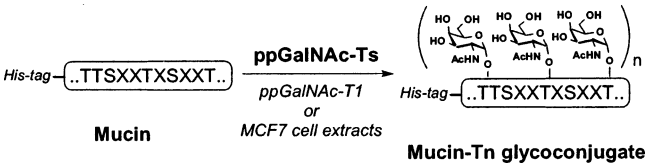
Reaction Monitoring by SELDI-TOF. The SELDI-TOF mass spectrometer was purchased from Ciphergen Biosystems (Fremont, CA). IMAC30 ProteinChip array surfaces were activated with 100 mM $NiCl_2$ at room temperature for 15 min and then washed with water and PBS. Spots were incubated with total glycosylation mix aliquots for 40 min at room temperature using the Bioprocessor system and then washed

Table 1. Conditions of the Tranglycosylation Reactions and the Average GalNAc Amount of the Resulting Conjugates

figure	panel	UDP-GalNAc amount (equiv)[a]	enzyme amount (μg of protein/10 μg mucine)	observed average molecular mass (Da)[c]	change in mass (ΔM)	average GalNAc amount/mucin ($\Delta M/M_g$)[d]
1	A	0	1 (ppGalNAc-T1)	12144.3	-	-
1	B	2	0.01 (ppGalNAc-T1)	13170.0	1025.7	5 (5.05)
1	C	2	0.04 (ppGalNAc-T1)	13778.8	1634.5	8 (8.04)
1	D	2	0.2 (ppGalNAc-T1)	15018.2	2873.9	14 (14.14)
1	E	2	1 (ppGalNAc-T1)	16036.1	3891.8	19 (19.15)
1	F	1	1 (ppGalNAc-T1)	15806.6	3662.3	18 (18.02)
1	G	0.5	1 (ppGalNAc-T1)	15598.8	3454.5	17 (17.0)
3	A	0	130 (MCF7 cell extracts)[b]	12144.8	-	-
3	B	2	130 (MCF7 cell extracts)[b]	17037.3	4892.5	24 (24.08)

[a] The molar equivalent amount is expressed as compared to potential *O*-glycosylation sites (47 serine and threonine residues). [b] 65 μg was added at the beginning of the reaction and then at 24 h. [c] The average molecular mass of the neoglycoconjugate was calculated from the medium peak which is marked with an arrow on Figures 1 and 3. [d] Calculated average mass of GalNAc $M_g = 203.193$ Da.

Scheme 1. Enzymatic Transglycosylation of GalNAc from UDP−GalNAc to the MUC6 Mucin Using Either Recombinant ppGalNAc-T1 or MCF7 Tumor Cell Extracts



with 0.1% Triton-X100 in PBS (2 × 5 min), PBS (3 × 2 min), and 5 mM HEPES (2 × 5 min). The matrix (2 × 0.6 μL of sinapinic acid saturated in 50% acetonitrile/0.5% TFA) was applied on each spot and allowed to air-dry. Chips were then read in the PBS II Reader, and each array spot was laser-sampled. Spectra were generated at a laser setting of 220 with a detector sensitivity of 8 and an acceleration energy of 20 kV. The laser intensity at these values corresponds to ~8 μJ. Optimization range of the time lag focusing was set between 10 and 20 kDa. The instrument was externally calibrated with bovine ubiquitin (8564.8 Da), bovine cytochrome C (12230.9 Da), and bovine β-lactoglobulin (18363.3 Da) (all from Ciphergen Biosystems) with the matrix and the settings as described above for the MUC6 samples. Spectra were treated using the Ciphergen ProteinChip software 3.2.1.

RESULTS AND DISCUSSION

The recombinant MUC6 protein was subjected to the transglycosylation reaction from the carbohydrate activated donor UDP-GalNAc in the presence of the enzyme, either a recombinant ppGalNAc-T1 (*39*) or a mixture of ppGalNAc-Ts contained in MCF7 tumor cell extracts (Scheme 1). Small aliquots of the reaction were withdrawn at different times and directly analyzed by SELDI-TOF MS after immobilization on the ProteinChip surface through immobilized metal affinity capture (IMAC30). The reaction progress was monitored by the shift to higher masses, caused by the transfer of GalNAc residues, as compared with the mass of the starting material (Figures 1 and 3). The spectra displayed a set of peaks showing the incremental molecular masses of the conjugates and allowing to determine the average hapten−protein stoichiometry, as well as the distribution of the conjugates. The transglycosylation reactions were studied under various experimental conditions (Table 1).

The ppGalNAc-T1 was shown to catalyze very efficiently the in vitro transfer of GalNAc residues onto the MUC6 protein (Figure 1). The extent of transfer was first analyzed with 2 equiv of UDP-GalNAc donor (the molar equivalent amount refers to the potential *O*-glycosylation sites, i.e., the total serine and threonine residues) and variable amounts of the recombinant ppGalNAc-T1, after 24 h incubation. As shown in Figure 1

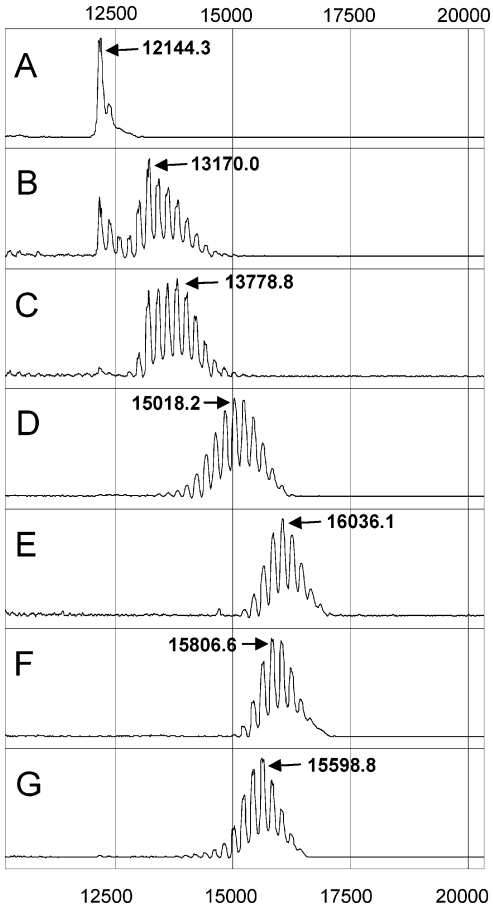


Figure 1. SELDI-TOF MS analysis of the progress of the GalNAc transglycosylation on MUC6 (molecular mass: 12144.3 Da, Panel A) using ppGalNAc-T1, depending on the donor (panels B−E) or the enzyme amount (panels E−G). The panel A (incubation reaction without UDP-GalNAc) served as a control for the starting material, i.e., the nonglycosylated MUC6 protein. The average mass/charge (*m*/*z*) values (in daltons) are shown in each panel on the medium peak marked with an arrow. The conditions of the tranglycosylation reactions, and the average GalNAc amount of the resulting conjugates are given in the Table 1.

(Panels B−E), the addition of enzyme results in a pronounced and progressive spectra shift showing significant increase in glycosylation with an average Tn amount from 5 (Panel B) to 19 (Panel E). Further addition of enzyme alone or enzyme together with UDP-GalNAc did not significantly improve the transfer (data not shown). Likewise, longer incubation period than 24 h did not produce any observable increase in molecular mass, showing that a maximum Tn level has been reached, at least under these experimental conditions (data not shown).

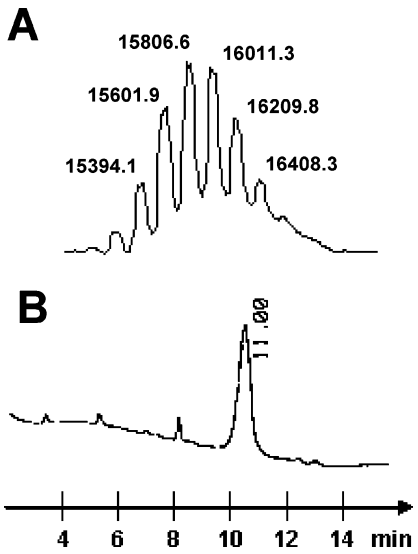


Figure 2. Comparison of the resolution by SELDI-TOF MS and HPLC analysis. Products from the experiment of Figure 1F (1 equiv of UDP-GalNAc and 1 μg of ppGalNAc-T1/10 μg of MUC6) were analyzed by SELDI-TOF MS (A) and reversed-phase HPLC (B). Enlarged SELDI-TOF mass spectrum shows the mass increment details (*m*/*z* values in daltons). Chromatographic conditions: Waters Symmetry C18 (5 μm, 300 Å, 3.9 × 250 mm), flow rate of 1 mL/min, gradient with water (0.1% trifluoracetic acid)/acetonitrile (10−60%) over 30 min.

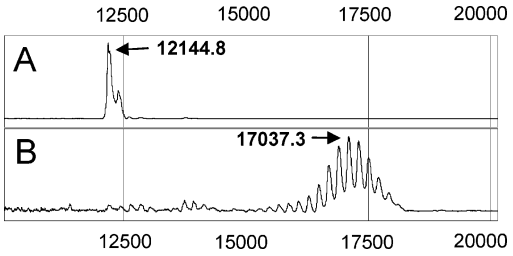


Figure 3. SELDI-TOF MS analysis of the conjugation reaction using MCF7 tumor cell extracts. The panel A (incubation reaction without UDP-GalNAc) served as a control for the starting material, i.e., the nonglycosylated MUC6 protein. The average mass/charge (*m*/*z*) values (in daltons) are shown in both panels on the medium peak marked with an arrow. The conditions of the tranglycosylation reaction, and the average GalNAc amount of the resulting conjugate are given in the Table 1.

The effect of the amount of donor is presented in Figure 1E−G. When lower quantities of UDP-GalNAc were used, the SELDI-TOF MS profile was found to be comparable, although a slight decrease in transfer was observed with 0.5 equiv of donor (Figure 1G).

To produce large amounts of conjugates while saving the expensive donor UDP-GalNAc, we chose the experimental conditions of Figure 1F for scaling up the reaction (1 equiv of UDP-GalNAc and 1 μg of enzyme/10 μg of MUC6). When the transglycosylation was performed on semipreparative quantities (mg range of mucin), the number of transferred GalNAc residues was found to be virtually the same as the one obtained at the analytical level (data not shown). As a result, the preparation of milligrams of neoglycoconjugates has been achieved, and the evaluation of their immunological properties will be reported separately (T. Freire et al., *Glycobiology*, in press).

Enlargement of the representative spectrum with the selected conditions (Figure 1F) shows the mass increment details (Figure 2A). The difference between each peak corresponds to the expected average mass of a single GalNAc residue (203.193 Da), demonstrating that SELDI-TOF MS allows a good resolution, at least in this molecular mass range. In contrast, the reverse phase HPLC profile did not show any separation of the different species (Figure 2B).

SELDI-TOF MS was then applied to the analysis of a transglycosylation in complex mixtures, by using the ppGalNAc-Ts contained in extracts from MCF7 tumor cells (Figure 3). The mass distribution obtained with the crude mixture was similar to the one observed with the recombinant enzyme. However, the transfer rate was better since an average density of 24 Tn/protein molecule was achieved after 48 h of reaction and two additions of cell extracts (Figure 3B). With the ppGalNAc-T1 and the cell extracts, the maximum Tn level was found to be, respectively, 40% and 51% of the potential glycosylation sites. The fact that the maximum Tn-protein stoichiometry was higher in this last case suggests that the additional GalNAc may have been transferred by other ppGalNAc-Ts that are present in the cell extracts. Interestingly, the presence of numerous contaminants in the cell extracts hardly affect the signal-to-noise ratio, showing that SELDI-TOF can be useful for such complex analysis.

We show here that the monitoring of a chemical conjugation reaction can be done for an enzymatic reaction in a crude and complex mixture (cell extracts) without any interference from contaminants, although such monitoring with simple samples has been reported previously by P. Kováč and co-workers (*24*, *25*). These results imply that SELDI-TOF MS is a powerful tool for monitoring a bioconjugation reaction. Indeed, this technique provides a very efficient alternative to the traditional analysis methods.

First, it is a sensitive and accurate method since the conjugates could be efficiently analyzed from as few as 10 ng (1 pmol range), at least in the molecular mass range of 10000−20000 with mass increments of approximately 203 Da. The traditional protein carriers which are used for immunization purposes have usually higher molecular mass, from approximately 60000 Da (bovine serum albumin or BSA, diphtheria toxoid) to several million Da (keyhole limpet hemocyanin or KLH). The accuracy and resolution of SELDI-TOF MS will be most probably limited in these higher molecular mass ranges, in particular for the KLH. This will also depend on the molecular mass of the hapten. To overcome these problems, the use of Zip Tip pipette tips can be considered. This method involves a microscale cleanup of the sample which then can be applied to high performance mass spectrometers (MALDI or electrospray), allowing efficient analyses in the high molecular weight range. However it is noteworthy that, for low molecular weights, ProteinChip array technology remains simpler since it allows the purification step and the mass analysis on the same device. This eliminates the need for a transfer step and results in an optimal recovery of the sample.

Second, SELDI-TOF MS is very rapid since it can be performed directly on the crude reaction without the need to purify or to derivatize the sample prior to analysis. In addition, the fact that several samples can be easily analyzed in parallel can be very valuable for studying the impact of various conditions on the efficiency of the conjugation. SELDI-TOF MS is therefore potentially very useful for high-throughput optimization strategies. Finally, the present study indicates that SELDI-TOF MS is suitable for analyzing complex crude samples from in vitro enzymatic conjugations. It may thus be directly applicable to the monitoring of intracellular reactions performed in vivo. Such analyses have already been reported on crude fermentation or cell culture sources in order to optimize recombinant protein production (*19*, *40*) or to analyze the secretion and autoactivation of a bacterial protein (*23*). Similar studies on mucin proteins are currently underway in the laboratory.

ACKNOWLEDGMENT

The authors are thankful to V. Piller and F. Piller for providing the recombinant ppGalNAc-T1 and to C. Leclerc for the critical review of the manuscript. We gratefully acknowledge financial support from the Association pour la Recherche sur le Cancer and the ECOS France-Uruguay Program. T.F. was financed by la Ligue Nationale contre le Cancer, la Fondation pour la Recherche Médicale, and a Vasant and Kusum Joshi grant.

LITERATURE CITED

(1) Niemeyer, C. M. (2004) Bioconjugation protocols: Strategies and methods. *Methods in Molecular Biology*, Volume 283, Humana Press, Totowa, NJ.

(2) Hermanson, G. T. (1996) *Bioconjugate techniques*, Academic Press, New York.

(3) Houen, G., Olsen, D. T., Hansen, P. R., Petersen, K. B., and Barkholt, V. (2003) Preparation of bioconjugates by solid-phase conjugation to ion exchange matrix-adsorbed carrier proteins. *Bioconjugate Chem. 14*, 75−79.

(4) Oda, M., Sato-Nakamura, N., and Azuma, T. (2004) Molecular characterization of monovalent and multivalent hapten-protein conjugates for analysis of the antigen−antibody interaction. *Anal. Biochem. 333*, 365−371.

(5) Pawlowski, A., Kallenius, G., and Svenson, S. B. (2000) Preparation of pneumococcal capsular polysaccharide-protein conjugate vaccines utilizing new fragmentation and conjugation technologies. *Vaccine 18*, 1873−1885.

(6) Singh, K. V., Kaur, J., Varshney, G. C., Raje, M., and Suri, C. R. (2004) Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. *Bioconjugate Chem. 15*, 168−173.

(7) Weller, M. G., Diemer, M., Wersching, C., Niessner, R., and Sochor, H. (2003) Development of antibodies for the detection of *N*-acetyl-glufosinate. *J. Agric. Food Chem. 51*, 6668−6675.

(8) Adamczyk, M., Gebler, J. C., and Mattingly, P. G. (1996) Characterization of protein-hapten conjugates. 2. Electrospray mass spectrometry of bovine serum albumin-hapten conjugates. *Bioconjugate Chem. 7*, 475−481.

(9) Pauillac, S., Naar, J., Mouratou, B., and Guesdon, J. L. (2002) Application of a modified version of Habeeb's trinitrophenylation method for the characterization of hapten-protein conjugates in a reversed micellar medium. *J. Immunol. Methods 263*, 75−83.

(10) Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) Ellman's reagent: 5,5′-dithiobis(2-nitrobenzoic acid)- -a reexamination. *Anal. Biochem. 94*, 75−81.

(11) Sashidhar, R. B., Capoor, A. K., and Ramana, D. (1994) Quantitation of epsilon-amino group using amino acids as reference standards by trinitrobenzene sulfonic acid. A simple spectrophotometric method for the estimation of hapten to carrier protein ratio. *J. Immunol. Methods 167*, 121−127.

(12) Manzi, A. E., and Varki, A. (1993) Glycobiology. *A Practical Approach*, Oxford University Press, New York.

(13) Tang, N., Tornatore, P., and Weinberger, S. R. (2004) Current developments in SELDI affinity technology. *Mass Spectrom. Rev. 23*, 34−44.

(14) Issaq, H. J., Veenstra, T. D., Conrads, T. P., and Felschow, D. (2002) The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. *Biochem. Biophys. Res. Commun. 292*, 587−592.

(15) Caputo, E., Moharram, R., and Martin, B. M. (2003) Methods for on-chip protein analysis. *Anal. Biochem. 321*, 116−124.

(16) Cool, D. R., and Hardiman, A. (2004) C-terminal sequencing of peptide hormones using carboxypeptidase Y and SELDI-TOF mass spectrometry. *Biotechniques 36*, 32−34.

(17) Merchant, M., and Weinberger, S. R. (2000) Recent advancements in surface-enhanced laser desorption/ionization-time-of-flight-mass spectrometry. *Electrophoresis 21*, 1164−1177.

(18) Lin, S., Tornatore, P., King, D., Orlando, R., and Weinberger, S. R. (2001) Limited acid hydrolysis as a means of fragmenting proteins isolated upon ProteinChip array surfaces. *Proteomics 1*, 1172−1184.

(19) Cleverley, S., Bengio, S., Boschetti, E., and Spencer, J. (2003) Direct "on-chip" protein-expression monitoring. *Genet. Eng. News 23*, 2.

(20) Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science 282*, 1318−1321.

(21) Vorderwülbecke, S., Cleverley, S., Weinberger, S. C., and Wiesner, A. (2005) Protein quantification by the SELDI-TOF-MS-based ProteinChip System system. *Nature Methods 2*, 393−395.

(22) Dulon, S., Leduc, D., Cottrell, G. S., D'Alayer, J., Hansen, K. K., Bunnett, N. W., Hollenberg, M. D., Pidard, D., and Chignard, M. (2005) *Pseudomonas aeruginosa* elastase disables PAR2 in repiratory epithelial cells. *Am. J. Respir. Cell Mol. Biol. 32*, 411−419.

(23) Boyle, M. D., Romer, T. G., Meeker, A. K., and Sledjeski, D. D. (2001) Use of surface-enhanced laser desorption ionization protein chip system to analyze streptococcal exotoxin B activity secreted by *Streptococcus pyogenes*. *J. Microbiol. Methods 46*, 87−97.

(24) Chernyak, A., Karavanov, A., Ogawa, Y., and Kovac, P. (2001) Conjugating oligosaccharides to proteins by squaric acid diester chemistry: rapid monitoring of the progress of conjugation, and recovery of the unused ligand. *Carbohydr. Res. 330*, 479−486.

(25) Saksena, R., Chernyak, A., Karavanov, A., and Kovac, P. (2003) Conjugating low molecular mass carbohydrates to proteins. 1. Monitoring the progress of conjugation. *Methods Enzymol. 362*, 125−139.

(26) Ada, G., and Isaacs, D. (2003) Carbohydrate-protein conjugate vaccines. *Clin. Microbiol. Infect. 9*, 79−85.

(27) Danishefsky, S. J., and Allen, J. R. (2000) From the laboratory to the clinic: a retrospective on fully synthetic carbohydrate-based anticancer vaccines. *Angew. Chem., Int. Ed. 39*, 836−863.

(28) Lo-Man, R., Vichier-Guerre, S., Perraut, R., Dériaud, E., Huteau, V., BenMohamed, L., Diop, O. M., Livingston, P. O., Bay, S., and Leclerc, C. (2004) A fully synthetic therapeutic vaccine candidate targeting carcinoma-associated Tn carbohydrate antigen induces tumor specific antibidies in non-human primates. *Cancer Res. 64*, 4987−4994.

(29) Kuduk, S. D., Schwarz, J. B., Chen, X.-T., Glunz, P. W., Sames, D., Ragupathi, G., Livingston, P. O., and Danishefsky, S. J. (1998) Synthetic and immunological studies on clustered modes of mucin-related Tn and TF O-linked antigens: the preparation of a glycopeptide-based vaccine for clinical trials against prostate cancer. *J. Am. Chem. Soc. 120*, 12474−12485.

(30) Slovin, S. F., Ragupathi, G., Musselli, C., Olkiewicz, K., Verbel, D., Kuduk, S. D., Schwarz, J. B., Sames, D., Danishefsky, S., Livingston, P. O., and Scher, H. I. (2003) Fully synthetic carbohydrate-based vaccines in biochemically relapsed prostate cancer: clinical trial results with alpha-N-acetylgalactosamine-O-serine/threonine conjugate vaccine. *J. Clin. Oncol. 21*, 4292−4298.

(31) Springer, G. F. (1984) T and Tn, general carcinoma autoantigens. *Science 224*, 1198−1206.

(32) Freire, T., and Osinaga, E. (2003) Immunological and biomedical relevance of the Tn antigen. *Immunology* (Spain) *22*, 27−38.

(33) Grinstead, J. S., Koganty, R. R., Krantz, M. J., Longenecker, B. M., and Campbell, A. P. (2002) Effect of glycosylation on MUC1 humoral immune recognition: NMR studies of MUC1 glycopeptide-antibody interactions. *Biochemistry 41*, 9946−9961.

(34) Toribara, N. W., Roberton, A. M., Ho, S. B., Kuo, W. L., Gum, E., Hicks, J. W., Gum, J. R., Jr., Byrd, J. C., Siddiki, B., and Kim, Y. S. (1993) Human gastric mucin. Identification of a unique species by expression cloning. *J. Biol. Chem. 268*, 5879−5885.

(35) Hamamoto, A., Abe, Y., Nishi, M., Fujimori, S., Ohnishi, Y., Yamazaki, H., Oida, Y., Miyazaki, N., Inada, K., Ueyama, Y., Iwasaki, M., Inoue, H., and Nakamura, M. (2005) Aberrant expression of the gastric mucin MUC6 in human pulmonary adenocarcinoma xenografts. *Int. J. Oncol. 26*, 891−896.

(36) Nishiumi, N., Abe, Y., Inoue, Y., Hatanaka, H., Inada, K., Kijima, H., Yamazaki, H., Tatematsu, M., Ueyama, Y., Iwasaki, M., Inoue, H., and Nakamura, M. (2003) Use of 11p15 mucins as prognostic factors in small adenocarcinoma of the lung. *Clin. Cancer Res. 9*, 5616−5619.

(37) De Bolos, C., Garrido, M., and Real, F. X. (1995) MUC6 apomucin shows a distinct normal tissue distribution that correlates with Lewis antigen expression in the human stomach. *Gastroenterology 109*, 723−734.

(38) Pereira, M. B., Dias, A. J., Reis, C. A., and Schmitt, F. C. (2001) Immunohistochemical study of the expression of MUC5AC and MUC6 in breast carcinomas and adjacent breast tissues. *J. Clin. Pathol. 54*, 210−213.
(39) Duclos, S., Da Silva, P., Vovelle, F., Piller, F., and Piller, V. (2004) Characterization of the UDP-N-acetylgalactosamine binding domain of bovine polypeptide alphaN-acetylgalactosaminyltransferase T1. *Protein Eng. Des. Sel. 17*, 635−646.
(40) Savage, C. (2003) Ciphergen segments proteomics applications. *Genet. Eng. News 23*, 1.
(41) Hubalek, F., Pohl, J., and Edmondson, D. E. (2003) Structural comparison of human monoamine oxidases A and B: mass spectrometry monitoring of cysteine reactivities. *J. Biol. Chem. 278*, 2612−2618.

BC0502661