

# Polymerase Chain Reaction and Immunoassay—Matrix Assisted Laser Desorption Mass Spectrometry Using Tag-Mass Technology: New Tools to Break Down Quantification Limits and Multiplexes

J. Stauber,<sup>†</sup> M. El Ayed,<sup>†</sup> M. Wisztorski,<sup>†</sup> R. Day,<sup>‡</sup> I. Fournier,<sup>\*,†</sup> and M. Salzet<sup>\*,†</sup>

University de Lille1, CNRS, MALDI Imaging Team, Laboratoire de Neuroimmunologie des Annélides, Villeneuve d'Ascq F-59655, France, and Université de Sherbrooke, Département de Pharmacologie, Faculté de médecine, Sherbrooke, Québec, J1H 5N4, Canada

We present a new development of the Tag-Mass concept based on a photocleavable linker with tagged molecules for polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) quantification coupled to mass spectrometry. PCR-MS and immunosorbent assay-MS with tagged oligonucleotides, bases, and antibodies will allow the acquisition of multiplexed information from genomic, transcriptomic, and proteomic experiments. This is a novel application of Tag-Mass from tissue imaging to fluid quantification and will open doors to several clinical applications ranging from biomarker-driven gene modulation to use at the patient's bedside following treatment.

Detection and quantification methods are nowadays unavoidable in the process for characterizing molecules. Since the development of the first quantification methods, new applications have been developed in both public and private domains, including antidoping control and clinical and fundamental research. Three major developments in the quantification domain, directly linked to technological advances of the 20th and 21st centuries, have allowed for increased sensitivity and reproducibility, two key factors in quantification.

The first methods of quantification for biological assays were colorimetric chemical methods, which are linked to the specific reactivity of substrates present in urine<sup>1</sup> or blood.<sup>2</sup> These methods are not often used or are employed for a peculiar domain of study. Other more general methods, such as the enzyme-linked immunosorbent assay (ELISA),<sup>3</sup> Lowry method<sup>4,5</sup> and Biruet method,<sup>6</sup> have been used for many years. For these approaches, increasing

the sensitivity and reproducibility appear to be the major foci for improving quantification performances. These methods measure components down to a concentration of approximately 1 mg/mL. In ELISA, with the sandwich method,<sup>7,8</sup> the use of a more specific antibody increases the sensitivity to 0.01 mg/mL.

At the end of the 1970s, the development of novel analytical chemical methods such as mass spectrometry (MS) opened the door for expression method studies in the 1980s and innovative discoveries using proteomics in the 1990s. The most current methods routinely employed are ELISA and polymerase chain reaction (PCR). These methods are both robust and sensitive. In 1995, novel techniques using antibodies for assessing the component of interest<sup>9</sup> before analysis by MS were developed; these techniques were refined in 1997 to allow assessment of single nucleotide polymorphisms.<sup>10,11</sup> The technique consisted primarily of using a set of nucleoside triphosphates for selective PCR replication of DNA. One or more of the nucleoside triphosphates were made much heavier by attaching a chemical group, but in such a way that replication was not disturbed. With the use of this technique, a single nucleotide polymorphism in DNA fragments of 40–50 base pairs could easily be made visible by mass spectrometry without further manipulation.<sup>10,11</sup>

Two novel developments have recently been published, with one based on the use of cryodetection for large masses. This technique was used to analyze an antigen–antibody complex.<sup>12</sup> Conversely, Lou et al.<sup>13</sup> used antibodies conjugated to a polymer tag that contained multiple metal chelates and was loaded with lanthanide ions to analyze antibody complexes. Interchain disulfide bonds in the antibody were partially reduced, and the polymer

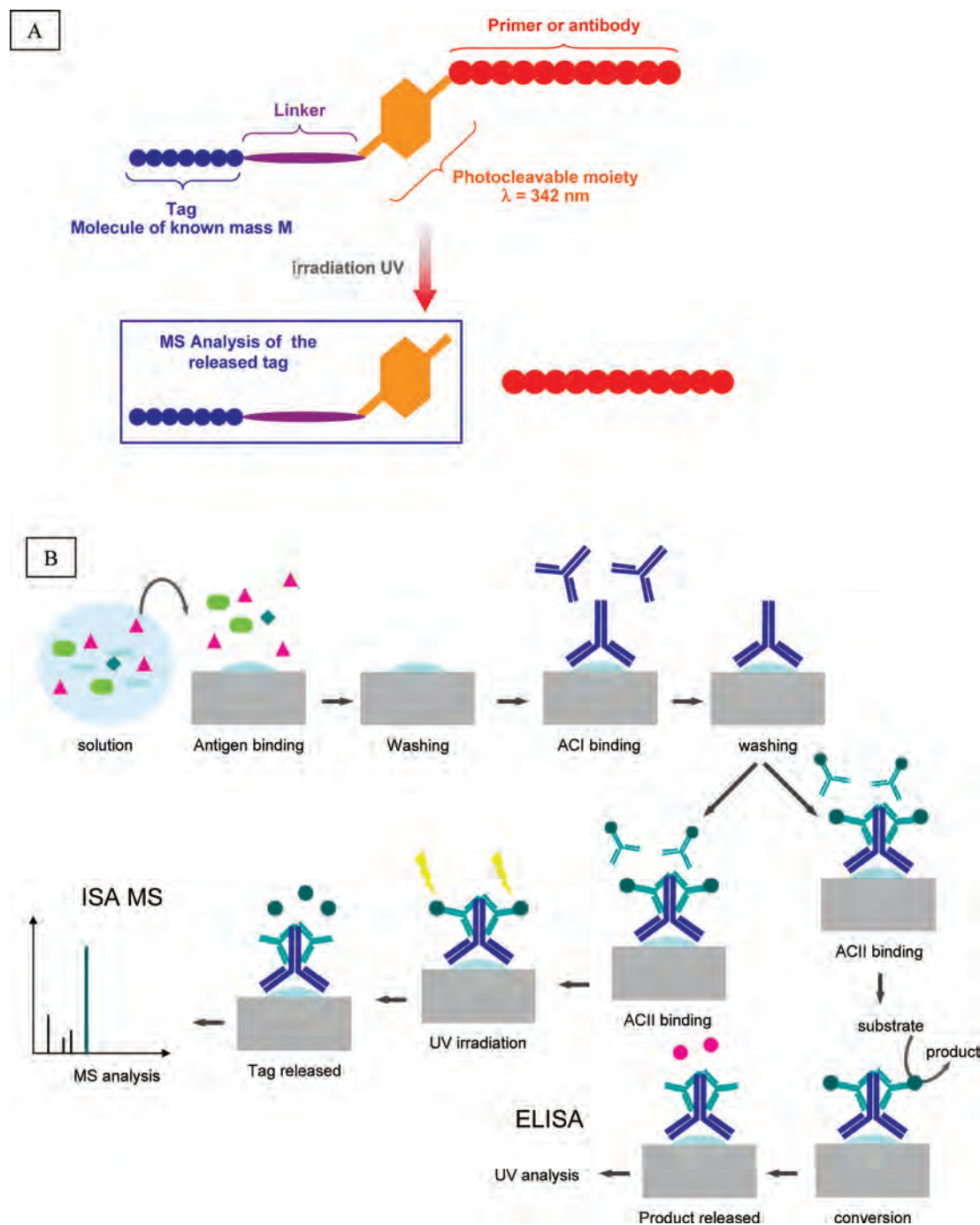
\* To whom correspondence should be addressed. E-mail: michel.salzet@univ-lille1.fr (M.S.); isabelle.fournier@univ-lille1.fr (I.F.).

<sup>†</sup> University de Lille1, CNRS.

<sup>‡</sup> Université de Sherbrooke.

- (1) Gaskell, J. F. *J. Physiol.* **1907**, *36*, 142–148.
- (2) Berger, H.; Antener, I.; Brechbuehler, T.; Stalder, G. *Ann. Paediatr.* **1964**, *202*, 465–482.
- (3) Engvall, E.; Perlmann, P. *Immunochemistry* **1971**, *8*, 871–874.
- (4) Traul, K.; Larson, D.; Stephens, R.; Wolff, J.; Munch, K.; Mayyasi, S. *J. Clin. Microbiol.* **1975**, *2*, 253–260.
- (5) Stephens, R.; Traul, K.; Woolf, D.; Lowry, G.; Lelek, J. *J. Clin. Microbiol.* **1975**, *1*, 225–233.

- (6) Fields, J. P.; Chodosh, S. *Clin. Chem.* **1970**, *16*, 773–775.
- (7) Kato, K.; Hamaguchi, Y.; Okawa, S.; Ishikawa, E.; Kobayashi, K.; Katunuma, N. *J. Biochem.* **1977**, *81*, 1557–1566.
- (8) Kato, K.; Hamaguchi, Y.; Okawa, S.; Ishikawa, E.; Kobayashi, K. *J. Biochem.* **1977**, *82*, 261–266.
- (9) Nelson, R. W.; Krone, J. R.; Bieber, A. L.; Williams, P. *Anal. Chem.* **1995**, *67*, 1153–1158.
- (10) Luft, F. C. *J. Mol. Med.* **1997**, *75*, 692–693.
- (11) Little, D. P.; Braun, A.; Darnhofer-Demar, B.; Frilling, A.; Li, Y.; McIver, R. T., Jr.; Koster, H. *J. Mol. Med.* **1997**, *75*, 745–750.
- (12) Nazabal, A.; Wenzel, R. J.; Zenobi, R. *Anal. Chem.* **2006**, *78*, 3562–3570.
- (13) Lou, X.; Zhang, G.; Herrera, I.; Kinach, R.; Ornatsky, O.; Baranov, V.; Nitz, M.; Winnik, M. A. *Angew. Chem., Int. Ed.* **2007**, *46*, 6111–6114.



**Figure 1.** (A) Schematic diagram of the photocleavable linker/tag system for indirect detection after photodissociation under the MALDI UV laser wavelength. (B) Schematic representation of ELISA versus ISA-MS procedure.

was attached through its terminal maleimide group to sulfhydryl residues on the Fc portion of the antibody.<sup>13</sup> This concept is similar to one our laboratory developed for specific matrix assisted laser desorption ionization (MALDI) imaging, which allows transcriptome and proteome identification using Tag-Mass.<sup>14</sup> In the Tag-Mass method, the presence of the probe is revealed by the signal in the MS spectra of the Tag reporter. This is obtained by using tagged probes for which reporters are linked to the probe via a photocleavable group. Photocleavage is specific of UV

irradiation at a wavelength close to the wavelength of the laser used in MALDI allowing the release of the reporter during the MALDI analysis (Figure 1A). The reporter has been chosen to be easily detectable in MALDI and indirectly give information on the presence of the probe. Moreover, a quantification assay combined with a modified antibody for detection by IPC-MS was recently developed.<sup>15</sup> The sensitivity of this novel method was similar to that of the classical ELISA method. This method, however, allows multiplexing, whereas ELISA does not.

(14) Lemaire, R.; Stauber, J.; Wisztorski, M.; Van Camp, C.; Desmons, A.; Deschamps, M.; Proess, G.; Rudlof, I.; Woods, A. S.; Day, R.; Salzert, M.; Fournier, I. *J. Proteome Res.* **2007**, *6*, 2057–2067.

(15) Baranov, K.; Volkova, O.; Chikhaev, N.; Mechetina, L.; Laktionov, P.; Najakshin, A.; Tarantin, A. *J. Immunol. Methods* **2008**, *332*, 73–81.

On the basis of previously proposed Tag-Mass technology, we have developed two novel quantification methods for both transcriptomes and proteomes, the PCR-MS method and the immunosorbent assay-MS method (ISA-MS) (Figure 1B). These methods have revealed to be very sensitive. Using ISA-MS, we were able to detect approximately 250 amol of antigen per well. Thus, immunosorbent assay combined with MS detection is approximately 1000-fold more sensitive than the classical ELISA method.

## MATERIAL AND METHODS

**Materials.**  $\alpha$ -Cyano-4-hydroxycinnamic acid (HCCA), aniline (ANI), 3-hydroxypicolinic acid (3-HPA), trifluoroacetic acid (TFA), angiotensin II, des-arg-bradykinin, substance P, ACTH 18-39, ACTH 7-38, and bovine insulin were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France) and used without any further purification. Acetonitrile p.a. and methanol p.a. were from J.T. Baker.

For Tag synthesis, solvents (DMF, dichloromethane) purchased from Biosolve were of peptide synthesis grade and used without modification. Amino acids, 4-[4-[1-(Fmoc-amino)ethyl]-2-methoxy-5-nitrophenoxy] butanoic acid (photocleavable linker) were purchased from Novabiochem. *N*-Methylmorpholine (NMM), *N,N'*-diisopropylcarbodiimide (DIPCD), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC), and *N,N*-diisopropylethylamine (DIPEA) were purchased from Aldrich. Trifluoroacetic acid, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/*N*-hydroxybenzotriazole (TBTU) were purchased from Biosolve, and goat antirabbit IgG and PD 10 (G 25 resin column) were purchased from Sigma. 3-Maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) was purchased from Pierce.

**Solid Ionic Matrixes Synthesis (SIM).** A total of 50 mg of HCCA was dissolved in 20 mL of methanol. An equimolar amount of base aniline was then added. The solution was mixed for 1 h and the solvent evaporated in a vacuum evaporator for 45 min ( $T = 50\text{ }^{\circ}\text{C}$ ,  $P = 40\text{ mbar}$ ). The resulting solid ionic matrix [HCCA<sup>-</sup>, ANI<sup>+</sup>] was placed in a desiccator for 30 min to eliminate residual solvent and stored at  $-20\text{ }^{\circ}\text{C}$ . Immediately before use, the ionic matrixes were prepared by dissolving 10 mg of compound in 1 mL of acetonitrile/water (2:1, v/v, 0.1% TFA).

**Peptide-Tagged Photocleavable Molecule Synthesis.** *Photocleavable Tagged Oligonucleotide.* The peptide was synthesized using Symphony (Protein Technologies Inc.) and purified using a Delta-Pak C18 15  $\mu\text{m}$  100A column (Waters Corp., Milford, MA). The oligonucleotide was synthesized from 3' to 5' using Expedite (Applied BioSystem). The amine function with photocleavable linker was added at the 5' terminus before cleavage and deprotection. These steps were performed using a 28% NH<sub>4</sub>OH solution over a 24 h period in the dark. The amino oligonucleotide was then purified using a Delta-Pak C18 15  $\mu\text{m}$  300A column. The amino function of the oligonucleotide was coupled with a heterobifunctional reagent containing a maleimide function. The maleimido oligonucleotide was solubilized in water and added to 1.2 equivalents of peptide in solution. The mixture was then stirred for 16 h. The oligo-peptide conjugate was then purified using a Delta-Pak C18 15  $\mu\text{m}$  300A column and characterized using MALDI-MS (see Mass Spectrometry).

*Photocleavable Tagged Antibody.* Peptides were custom-made by Eurogentec S.A. using solid phase peptide synthesis (SPPS)

on a 0.25 mmol scale using 9-fluorenylmethyloxycarbonyl (Fmoc) amino-terminus protection (standard synthesis protocols for the equivalent of Fmoc-AA) with double coupling reactions (twice at 40 min each) and TBTU/NMM as an activator on a Symphony synthesizer (Rainin Instrument Co., Woburn, MA). The photocleavable linker (4 equiv) was introduced manually using DIPCD/DIPEA (2 h) as an activator. Purifications were performed using RP-HPLC on a Waters Delta-Pak C18 [15  $\mu\text{m}$ -100A-25  $\times$  100 mm] column using a Waters liquid chromatography system consisting of a model 600 solvent delivery pump, a Rheodine injector, and an automated gradient controller (solvent A, H<sub>2</sub>O/0.125% TFA; solvent B, CH<sub>3</sub>CN/0.1% TFA; gradients, 5–15% to 30–60% B in 20 min). Detection was carried out using a model M2487 variable wavelength UV detector connected to the Waters Millennium software control unit. Quality control was performed by analytical RP-HPLC on a Waters Delta-Pak C18 column [5  $\mu\text{m}$ -100A-150  $\times$  3.9 mm] (solvent A, H<sub>2</sub>O/0.125% TFA; solvent B, CH<sub>3</sub>CN/0.1% TFA; gradient, 100% A to 60% B in 20 min) using a Waters Alliance 2690 separation module equipped with a Waters 996 photodiode array detector and by MALDI-TOF MS (see Mass Spectrometry).

Functional analysis using the photolinker derivatized peptide A was performed as follows: a solution of 0.5 mg of MBS in 300  $\mu\text{L}$  of DMF was added to a solution of 4 mg of goat antirabbit IgG in 2 mL of PBS and mixed for 30 min. The solution was then desalted on a PD 10 column using 50 mM phosphate buffer at pH 6. A solution of 1 mg of the photocleavable derivatized peptide in 300  $\mu\text{L}$  of DMF and 1 mL of PBS was then added and stirred for 3 h at room temperature. The reaction mixture was then dialyzed overnight against PBS (membrane cutoff 12–14 000).

*Synthesis of dUTP–Peptide Conjugates with a Photocleavable Linker.* To prepare the triphosphate, an Fmoc-protected CPG resin was required. The succinylate was prepared from GT115A (100 mg). The sample was relatively pure but contained a small amount (by TLC) of a higher running nontritylated compound. Since it was not possible to purify the succinate, the reaction was slightly modified to use 1.5 equiv. The reaction did not go to completion (TLC indicated that it reached greater than 50% completion), as determined by comparison of the intensity of the components on TLC (254 nm) and the intensity of the DMT cation on treatment with HCl fumes. Since the nonsuccinylated product did not react, the resin was functionalized using this mixture. However, the loading concentration was very low (5.4  $\mu\text{mol g}^{-1}$  [180 mg]).

The resin was detritylated using 2% TCA/DCM washed with DCM until the DMT cation was completely removed. It was then dried using argon, and the resin was soaked in pyr/DMF 1:3 (0.4 mL) for 5 min before a solution of 0.1 M Eckstein's reagent in dioxane was added (0.1 mL). The reaction was allowed to stand for 15 min. The resin was then washed (dioxane, MeCN) and dried (argon). The resin was again soaked in a solution of 0.5 M bis(tributylammonium) pyrophosphate in anhydrous DMF and tri-*n*-butylamine for 20 min, washed (DMF, MeCN), and dried (argon). The product was oxidized (iodine/water/pyridine/THF for 30 min), washed (MeCN), and dried (argon). The Fmoc protecting group was removed (20% piperidine/DMF, 0.5 mL, 20 min) and the resin washed thoroughly (DMF, MeCN) and dried (argon). The product was then washed with DCI and a solution of DCI/photolabile amino linker CEP (1:1, 0.5 mL) was added.

The reaction was allowed to stand for 20 min. The solution was removed, and the resin was washed (MeCN) and dried (argon). A mixture of cap A/cap B (1:1, 0.5 mL) was added, and the resin was soaked for 5 min before removal of the capping reagents and washing/drying of the resin as before. The product was oxidized ( $I_2$ /THF/pyr/ $H_2O$ , 5 min), and the resin was washed and dried again. The product was then cleaved from the resin with  $cNH_4OH$  at room temperature for 30 min, then purified by anion exchange HPLC on a Dionex NucleoPac100 HPLC column using the solvent system buffer A (0.1 M  $NH_4Cl$  with 10% acetonitrile) and buffer B (1 M  $NH_4Cl$  with 10% acetonitrile) at a flow rate of 2.5 mL/min using 6Triphos.mth. This yielded three fractions (A, -7 min; B, -7.9 min; and C, -10.3 min). All three fractions were lyophilized overnight before desalting by reverse phase HPLC using buffer A (water) and buffer B (acetonitrile) at a flow rate of 4 mL/min. The three fractions were again lyophilized overnight before being suspended in 200  $\mu L$  of water. Mass spectrometry showed that CMM661A pk 1 was definitely not the triphosphate. Rather, the triphosphate was either CMM661pk 2 or 3, which had similar MS profiles (CMM662A was formed from CMM661A pk 2 and CMM663A was formed from CMM661A pk 3). Both samples were then used for the subsequent reaction. Bicarbonate buffer (10  $\mu L$ ) and the maleimide NHS ester (50  $\mu L$ ) were added to each sample, and the reactions were agitated overnight. The samples were diluted with Milli-Q water (500  $\mu L$ ) and filtered. The samples were then purified by RP-HPLC using buffer A (0.1 M TEAA) and buffer B (MeCN) at a flow rate of 4 mL/min with MeCN50.mth. The coupling of the peptide was carried out using these fractions.

**PCR-MS.** Total RNA were extracted from rat brains using Qiazol lysis reagent by homogenizing the samples with 1.4 mm ceramic beads ( $2 \times 45$  s, 6500 rpm) in a Precellys24 homogenizer (Bertin, Ozyme, France). RNA extractions were performed according to the manufacturer's instructions. Samples were then treated or not with RQ1 DNase1 (Promega).<sup>16</sup>

One sixth of the first-strand synthesis reaction was amplified for 40 cycles using 1 U Taq polymerase (Eurogentec, Liege, Belgium) and 100 pmol of each forward and reverse primer. The cycling parameters were 94 °C for 90 s, 65 °C for 90 s, and 72 °C for 120 s. Omitting RT or RNA from the reaction mixture resulted in negative control RT-PCR reactions. A 100 bp calmodulin messenger fragment was amplified with tagged or not primer (5' AA GCG GTA CGA GTA GCA 3' reverse primer and oligodT forward primer). Proenkephalin mRNA (mRNA) was also amplified. Tagged or untagged sense (5' CAG GAC TCC CCA AAG GAG AAC AGG A 3') and antisense (5' GAC GTA CCA GGC GGT AGC TGC ATT T 3') primers were deduced from 3' and 5' extremities of the proenkephalin transcript and used to amplify a 400 bp fragment.<sup>17</sup> All PCR products were subcloned into pGEM-T easy vector (Promega), and cDNA clones were sequenced on an ABI Prism 310 genetic analyzer (Applied Biosystems). For tagged nucleotides, the best ratio was 1:3 of tagged dUTP and desoxyribo thymine triphosphate (dTPP). The amplified PCR fragments, i.e., 100 pb for the calmodulin and 400 pb for the proenkephalin were

separated in 10% agarose-BET gel. The specific band was directly cut before extracted from the gel. A total of 1  $\mu L$  of the extracted PCR amplified fragment is analyzed in MALDI using 3HPA (3 hydroxypicolinic acid) for complete primers analysis or HCCA/ANI for analyzing the amplified probe to improve the reporter signal.

**Immunoassays.** *Classical Enzyme-Linked Immunosorbent Assays.* Indirect ELISA was conducted as previously described<sup>18,19</sup> with pig immunoglobulin (Sigma Aldrich) as the antigen (250 ng/well, then serial dilution). Briefly, antipig IgG raised in rabbit (Sigma Aldrich, P0916) was used as the primary antibody. Serial dilution of the primary antibody against serial dilution of the antigen were performed to determine the optimal concentration of primary antibody. These experiments show that broadest linear range is obtained for a dilution of 1:100. The secondary antibody was a peroxidase affinity-pure goat antirabbit IgG (H+L, Min XHu Ser Protease, Jackson) and was used at a dilution of 1:5000. In classical ELISA, antigen quantity is determined via the absorbance measurement ( $\lambda = 490$  nm) of a colorimetric reaction product generated by reaction of the peroxidase enzyme on its substrate (*ortho*-phenylenediamine, OPD).

*Indirect ISA-MS.* The hybridization technique was similar to classical ELISA. The pig immunoglobulin was coated on the microtitration plate wall (1 h, 37 °C). After incubation, the excess of antigen was removed by three washing baths in PBS (0.1 M, pH 7.4). The antipig IgG raised in rabbit primary antibody (dilution 1:100) was then added and incubated for 1 h at 37 °C. After three washing steps in PBS (0.1 M, pH 7.4) to remove the excess of primary antibody, the modified photocleavable antibody (modified antirabbit IgG raised in goat, Eurogentec SA, Belgium) was added and incubated for 1 h at 37 °C. Different dilutions of the tagged secondary antibody were tested to define the optimal conditions (1:16, 1:32, 1:64, 1:128, 1:256, 1:512). After antibody fixation, two washing steps using PBS (0.1 M, pH 7.4) followed by one washing step with pure water were performed. Pure water is used to avoid the presence of salts that would hamper MALDI matrix crystallization and thereof MALDI analysis. The complex was then placed under UV light for 15 min in order to release the reporter (peptide-linker) attached by UV-cleavage and linked to the antibody. This reporter was then quantified using MALDI (Figure 1B). In these experiments, bradykinin was used as the reporter. After photocleavage, the reporter corresponds to the bradykinin peptide bonded to a linker. The reporter has a mass of  $m/z$  1702. The peak intensity after normalization of the spectra allowed the quantitative measurement of the peptide tag to be correlated with the amount of antigen/well.

**Mass Spectrometry.** For classical analysis, 1  $\mu L$  of sample solution and 1  $\mu L$  of matrix solution were mixed on the MALDI sample one after each other before crystallization and let to dry at room temperature according to the dried-droplet preparation procedure. MALDI-TOF mass spectra were performed on a Voyager-DE STR mass spectrometer (Applied Biosystems, Framingham, MA) with delayed extraction (DE) and a 337 nm pulsed nitrogen laser. For routine analysis of oligopeptides, HCCA/ANI was used at concentrations of 10 mg/mL in ACN / $H_2O$  (2:1, v/v,

(16) Chopin, V.; Salzet, M.; Baert, J.; Vandenbulcke, F.; Sautiere, P. E.; Kerckaert, J. P.; Malecha, J. *J. Biol. Chem.* **2000**, *275*, 32701-32707.

(17) Tasiemski, A.; Hammad, H.; Vandenbulcke, F.; Breton, C.; Bilfinger, T. J.; Pestel, J.; Salzet, M. *Blood* **2002**, *100*, 553-559.

(18) Salzet, M.; Watez, C.; Slomianny, M. C. *Comp. Biochem. Physiol. Comp. Physiol.* **1993**, *104*, 75-81.

(19) Salzet, M.; Bulet, P.; Van Dorsselaer, A.; Malecha, J. *Eur. J. Biochem.* **1993**, *217*, 897-903.

0.1% TFA) and 3-HPA at 50 mg/mL in H<sub>2</sub>O/ammonium citrate (100 mM). For tagged PCR products, acquisition parameters were an acceleration voltage of 20 kV, first grid voltage of 94%, the guide-wire voltage at 0.05%, and an extraction delay time of 200 ns. Each recorded MS result was the average of 100 laser shots. For tagged antibodies, the acquisition was performed with a MALDI-TOF (Voyager STR, Applied Biosystem, Framingham, MA) with HCCA as the matrix. The accelerating voltage, 20 kV; grid voltage, 94%; extraction delayed time, 100 ns; guide wire, 0.05%; laser repetition rate, 3 Hz. For each spectrum, 800 shots over 15 spots were averaged. Peak intensity were subsequently measured on the spectra after baseline correction using DataExplorer 5.0 software (Applied BioSystem, Framingham, MA).

## RESULTS

**PCR-MS. Amplification with Tagged Oligonucleotides.** The strategy used was to synthesize PCR primers (tagged or not) for PCR amplification. The first step was to check the compatibility of tagged primers using this technique. The left primer (5' AA GCG GTA CGA GTA GCA 3') was used for PCR amplification against an oligodT primer. This primer was designed in order to amplify a ubiquitous protein (the calmodulin binding protein (CBP)) from the mouse brain.

Before amplification experiments the tagged left primer was analyzed by MALDI MS to check the efficiency of the photocleavage process. The tagged primer (or oligopeptide) used includes three moieties which are, respectively, the oligonucleotide sequence (5' AA GCG GTA CGA GTA GCA 3'), the photocleavable group, and the reporter group, here chosen to be a peptide of known sequence (H-RPPGFSPFRC-CONH<sub>2</sub>  $M_w(\text{avg}) = 1161.37$ ). The analysis was performed using 3-HPA matrix in order to provide both detection of the peptide and the whole oligopeptide. The recorded MS spectrum (Figure 2A) shows several signals. First, we can observe that the peptide tag is well detected. However, two signals are observed for the reporter, respectively, at  $m/z$  1163.9 and  $m/z$  1637.26 corresponding, respectively, to the  $[M + H]^+$  ion of the peptide (P) itself and to the  $[M + H]^+$  ion of the peptide bonded to a part of the linker (P-PC). This second form (PC-P) is the expected form after photocleavage of the structure since the structure presents a linker between the peptide and the photocleavable moiety. The second signal corresponding to the peptide (P) is attributed to a prompt fragmentation in the early moments of desorption because of the presence in the structure of a disulfide bridge because of the instability of such functions in the gas phase. For this reason the disulfide bridge was further on replaced by a maleimide group. Later experiments with the maleimide group reveal that prompt fragmentation process can be suppressed, and then only a single signal is observed for the reporter. Concerning Figure 2A, the higher mass signals are attributed to either the whole oligopeptide with respectively  $[M + H]^+$  ion at  $m/z$  6970.50 and  $[M + 2H]^{2+}$  at  $m/z$  3489.92; or the oligonucleotide (O) alone with, respectively,  $[M + H]^+$  ion at  $m/z$  5351.03 and  $[M + 2H]^{2+}$  at  $m/z$  2678.79. This demonstrates that photocleavage is not total. Nevertheless, we can observe that the reporter group signal is very well detected.

In a first PCR test series, several annealing temperatures (52, 54, 56, and 58 °C) were tested on total RNA extracted from mouse

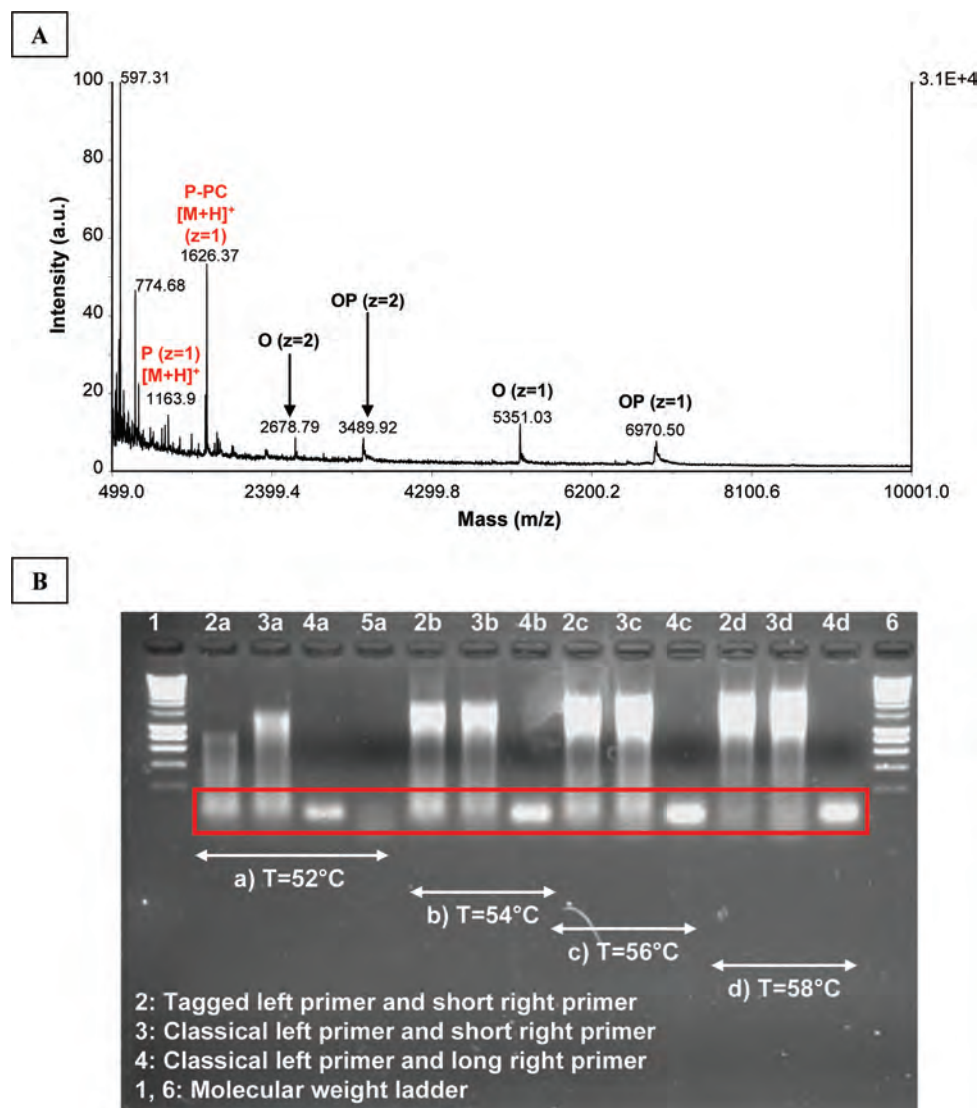
brain without using either with tagged primers (2, 6, 9, 12) performed or with classical primer (3, 7, 10, 13) (Figure 2B). In all cases, two bands are amplified one at 2.5 Kb and the other one at 100 bp whatever the primer used. This points out that the tagged primer can be used for PCR amplification. In a second step, RNA extractions were performed according to the manufacturer's instructions, and samples were then treated for DNA contamination by RQ1 DNase1 (Promega). PCR were performed with classical primers (4, 8, 11, 14). A single band at 100 pb is amplified. This demonstrates that the 2.5 Kb is amplified from the genomic DNA present in the extract whereas the 100 pb is amplified from the cDNA. These data reflects that tagged primers can be amplified either small or large PCR DNA fragment.

With the use of the same principle, proenkephalin was chosen as the model target mRNA (Figure 3), as previous studies have shown a low background for this molecule's mRNA distribution in the rat brain.<sup>20</sup> Standard untagged and tagged primers bearing the photocleavable linker/tag system were added to the proenkephalin cDNA sequence (Figure 3A). The left primer sequence was CAG GAC TCC CCA AAG GAG AAC AGG A, and the right primer sequence was GAC GTA CCA GGC GGT AGC TGC ATT T. For the tagged primers, the reporter peptide is linked to a spacer using a maleimide group to avoid prompt fragmentation itself bonded to the photocleavable group and the primer. The reporter peptide used is the same peptide than for previous experiments (H-RPPGFSPFRC-CONH<sub>2</sub>  $M_w(\text{avg}) = 1161.37$ ). Both types of primers were used for RT-PCR amplification and gave the same results in terms of product and yield of amplification. RT-PCR was used to make a 400 bp double-stranded cDNA oligonucleotide probe (Figure 3B). MALDI-MS analyses of the amplified band with either the left tagged primer and right free or the left free and right tagged primer revealed the same detected compound at an  $m/z$  of 1720.41, corresponding to the  $[M + H]^+$  ion of the P-PC form as expected from the structure after photodissociation. This confirmed the ability to detect the tagged peptide after PCR amplification (Figure 3C). Two other main signals are observed in the same  $m/z$  range. A first signal is observed at  $m/z$  1722.03 which corresponds to the  $[M + Na]^+$  ion of the P-PC form. A peak corresponding to the P-PC form with an oxygen loss (P-PC - O) is also observed at  $m/z$  1703.84. This last ion corresponds to a side product of the photodissociation process and has already been described previously.<sup>21</sup> Several other signals marked with asterisks are also detected. These signals are regularly spaced by 44 u and can be attributed to polymer (PEG) remaining in the solution even after purification.

**Desoxyribonucleotide Tag for PCR Amplification.** An alternative approach was developed based on the labeling of a single desoxyribonucleotide triphosphate (dNTP). For these experiments, desoxyribo uracile triphosphate (dUTP) was chosen and chemically modified with the same photocleavable linker/tag system described above (Figure 4A,B). In this new design, both the 5' and 3' ends of the nucleotide were kept free, allowing direct incorporation in the amplified cDNA. Use of a tagged nucleotide led to the incorporation of one tag for each T nucleotide,

(20) Lansac, G.; Dong, W.; Dubois, C. M.; Benlarbi, N.; Afonso, C.; Fournier, I.; Salzet, M.; Day, R. *J. Neuroimmunol.* **2006**, *171*, 57-71.

(21) Olejnik, J.; Ludemann, H. C.; Krzymanska-Olejnik, E.; Berkenkamp, S.; Hillenkamp, F.; Rothschild, K. *J. Nucleic Acids Res.* **1999**, *27*, 4626-4631.



**Figure 2.** (A) MALDI mass spectrum in the linear positive mode of an oligopeptide, including a 20-mer oligonucleotide sequence and the photocleavable tag/system with 3-HPA as a matrix. (B) PCR product run on agarose gel stained with ethidium bromide. (5' AA GCG GTA CGA GTA GCA 3') was used for PCR amplification against an oligodT primer. This primer was designed to amplify a ubiquitous protein (the calmodulin binding protein, or CBP) from the mouse brain (agarose 2%; migration voltage, 100 V; intensity, 150 mA). (1) Molecular size standard, (a) PCR conducted at  $T = 52^\circ\text{C}$ , (b) PCR conducted at  $T = 54^\circ\text{C}$ , (c) PCR conducted at  $T = 56^\circ\text{C}$ , (d) PCR conducted at  $T = 58^\circ\text{C}$ ; (2) PCR with left primer tagged and the right primer free; (3) PCR with the left primer free and right primer tagged; (4) PCR with left and right primers tagged; and (5) control  $\text{H}_2\text{O}$ .

increasing sensitivity. Finally, the use of tagged nucleotides is practical as these nucleotides can be incorporated using standard procedures, regardless of the probe being amplified. The dUTP-tagged system was used for amplifying the proenkephalin probe by RT-PCR (Figure 4C). Figure 4C illustrates the MALDI spectra obtained with the classical probe (b) or with the tagged one (a). The tagged probe gave a peak series at an  $m/z$  of 1128.23, 1163.06, 1201.75, and 1243.26 that corresponded to the tag peptide attached to the probe; these signals were not detected with the untagged probe. The characteristic signal of the tag corresponding to the peptide with part of the spacer peptide is observed at  $m/z$  1163.23 ( $[\text{M} + \text{H}]^+$  ion) with good S/N ratio and signal intensity, demonstrating that the maleimide group incorporation prevents fast fragmentation. At an  $m/z$  of 1201.75 and 1243.26, the peaks are separated by 38–40 u, corresponding to potassium cations linked to the reaction medium. The peak at  $m/z$  1128.23 comes

from a prompt fragmentation process of the lateral chain of the cysteine in front of the S–S bridge.

Taken together, these data suggest that it is possible to perform PCR-MS analyses with tagged oligonucleotides and tagged nucleotides. This opens the door for studies employing the multiplexing technique as we have demonstrated with a mixture of three different peptides tagged with three different oligonucleotides.<sup>14</sup> Previous work by Lipkin and colleagues developed MassTag-PCR technology based on 5'oligo(dT) sequences<sup>22</sup> and applied it to multiplex studies<sup>23–25</sup> for identification of a respiratory virus. This technology, however, is based on a simple mass spectrometric method, the PinPoint assay, for SNP typing of PCR products.<sup>22</sup> Our technique is completely different,

(22) Haff, L. A.; Smirnov, I. P. *Nucleic Acids Res.* **1997**, *25*, 3749–3750.

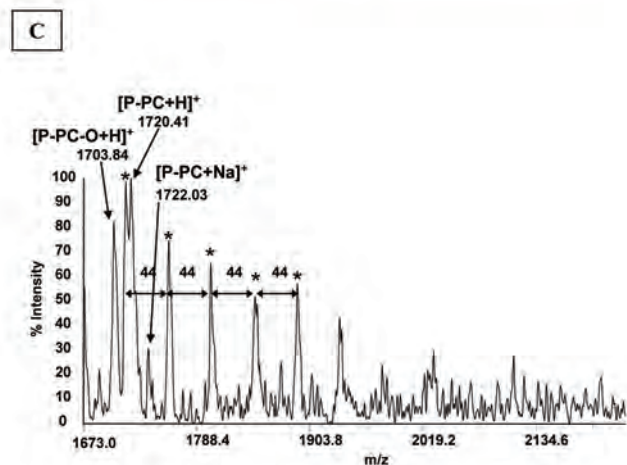
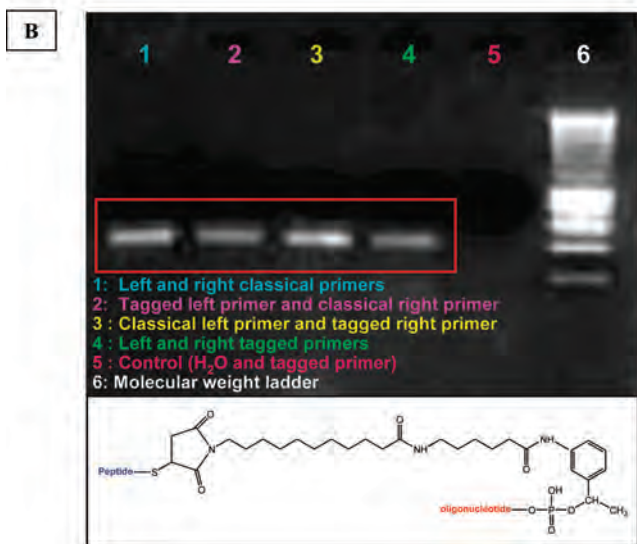
(23) Lamson, D.; Renwick, N.; Kapoor, V.; Liu, Z.; Palacios, G.; Ju, J.; Dean, A.; St. George, K.; Briese, T.; Lipkin, W. I. *J. Infect. Dis.* **2006**, *194*, 1398–1402.

**A**

```

TGATCGCTCTANGGGGTTCTGGCCGACGTCGCATGCTCCCGGTTGCCATGGCGGCCGCGGGAATTCGATTCAGGACTCCCCAA
AGGAGAACAGGATGCCATCAGGGAGACAGGACTCCCCGTGGAAGATAGGATACCTCCAGGAAGACAGAATGCCCCCAGG
CAGCCCTGGGACAGCGGAAACACTAGGGACCAAGCTATCCCTGGTCTACTCGGGATTTGTTGGGTTGTGGCGGGGTCAG
GAAAGATTGTCCCTGCTGGTCTGCTCCACGACCACCCACCCGGCAAGGTTCCCTCCTAGAGAACCTTGTGAGAGACAGAACG
GGTCCCCACAGGCGCATTTCTTCTTCCAACAGCCCATGGCGCAGTTCCCTGAGACTTGCATCTGGCTGCTAGCGCTGGGTCCT
GCCTCTGGCTACAGTGCAGGCAGACTGCAGCCAGGACTGCGCTTAAATGCAGCTACCGCTGGTACGTCAATCACTAAGTG
GAATTCGGCGCCGCCTTGCANGTCGACCATATGGGGANAACCTCCCCAACCCGTTGGATGCANTAGCTTGAGTATTCTATAN
NNGTCNCTAAAAAACTTGGCGTAAANCATTGGGCAATANCTTGTTTCCTNNGGGGGGGAAAAATGTTTATCCNCTCCAAAT
CCNCCCAAACNATTCNAACCCGGAANNATNAAANGGGTNAANACCTTGGGGGGGGNCCN

```



**Figure 3.** (A) Rat proenkephalin sequence obtained after cloning and sequencing of the PCR amplified cDNA (orange, vector arm; red, primers; black, the amplified probe of 413 bp). (B) Agarose gel stained with ethidium bromide showing proenkephalin cDNA fragments amplified with different types of tagged or untagged primers. Inset: structure of the oligonucleotide tagged by the photocleavable linker/tag system. (C) MALDI mass spectrum of the tagged primers amplified by PCR after extraction from the agarose gel and purification in the reflectron positive mode (matrix, HCCA/ANI).

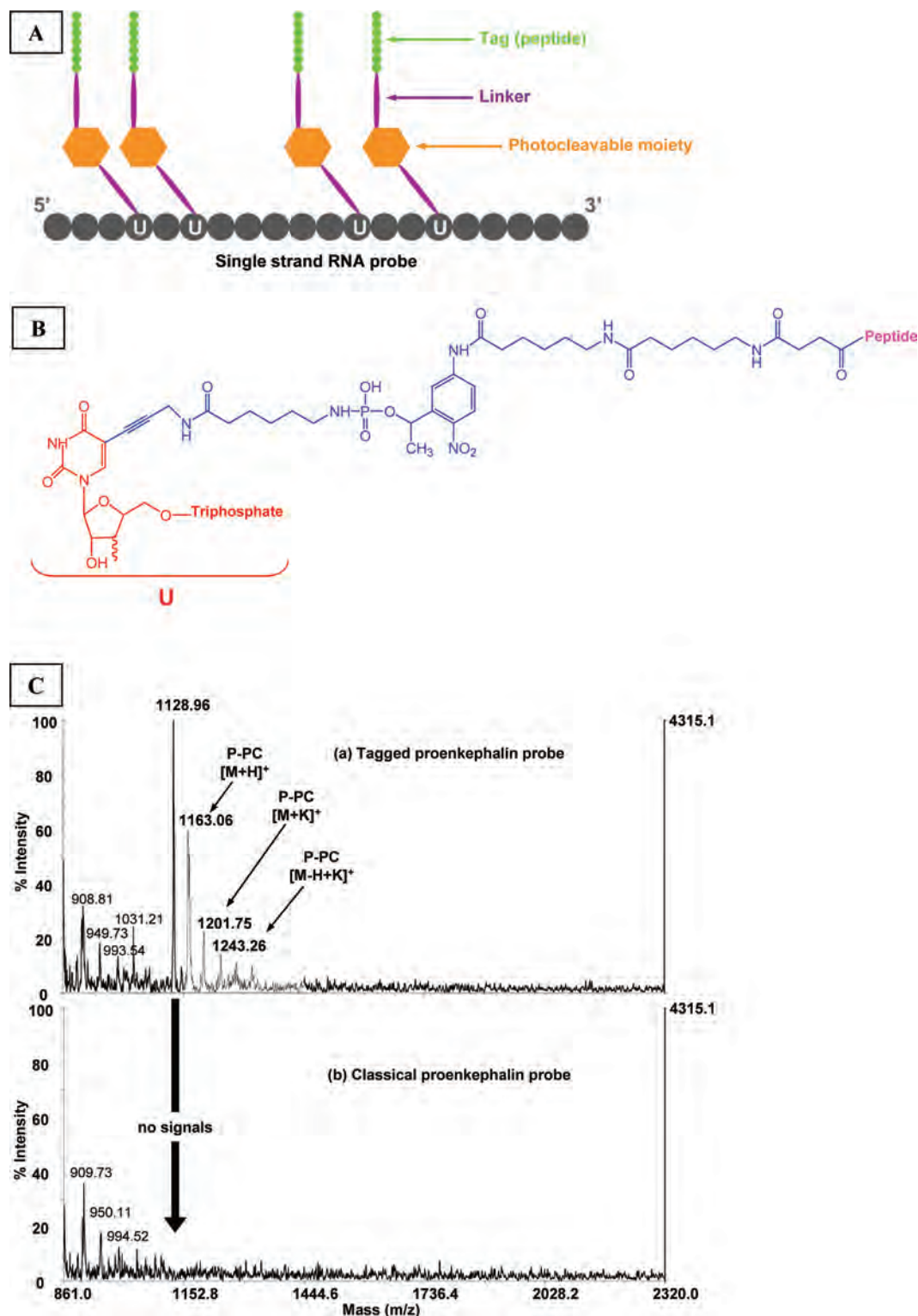
although the idea is similar. Thus, Tag-Mass can be another technique used for identification of pathogens exhibiting resistance.

**ISA-MS.** The ISA-MS method is identical to the classical ELISA method with the exception of the detection mode. In the classical ELISA method (Figure 1B), detection is based on oxidation of peroxidase in the presence of *o*-phenyldiamine dihydrochloride to yield a colored product that is detected by absorbance at 490 nm. The absorbance is linearly dependent on the concentration of the antigen based on a fixed concentration of primary and secondary antibodies (in our case, 1:100 for the primary and 1:5000 for the secondary). In ELISA, the linearity is limited by (i) in the low concentration range, the limit of detection, and (ii) in the high concentration range by the maximum capacity of adsorption of antigens on the plate wells. This gives curves presenting a typical shape with a linear region in the middle and two stages at the extremities (Figure 5). In ISA-MS, no color was detected, but the secondary antibody was connected by a photocleavable link to a peptide tag (bradykinin). After the reaction, the microtitration plate was then placed under UV light in order to release the reporter. In the present experiments, the peptide with the spacer generated by photocleavage gives a signal at  $m/z$  1703.87 for the  $[M + H]^+$

ion of the P-PC form and  $m/z$  1688.8 for the  $[M + H]^+$  ion of the  $[(P-PC) - O]$  as previously observed as a side photodissociation product. After UV exposure, solution was analyzed in MALDI (Figure 5A). The signal of the tag peptide is dominating this portion of the spectrum and is observed with a good sensitivity and signal/noise ratio (Figure 5A). A first experiment was performed to verify the linearity between the tagged antibody and the tag peptide released (Figure 5B). As observed from Figure 5B, the peak intensity is linearly varying with antigen concentration (picograms per milliliter) on a wide range (a few to 2500 pg/mL) except for the highest concentrations ( $\sim$ 5000 pg/mL) and lowest ones  $<5$  pg/mL). In the higher concentration, the curves are presenting a stage depicting that the maximum binding capacity of the antigen has been reached. In the lower mass range, values are dramatically decreasing traducing limit of detection effects. Optimal experimental conditions for antigen quantification (i.e., maximum of linear evolution) were determined by varying the secondary antibody concentration by serial dilution. In Figure 5C are reported the evolution of the peak intensity measured by MALDI MS versus the concentration of antigen for different secondary antibody dilutions (1:16, 1:32, 1:64, 1:128). It is clear that linearity on the larger antigen concentration range is observed for a dilution of the secondary antibody of 1:64. For other dilutions, linearity is also observed but on a narrower concentration range. For a 1:64 dilution of the secondary antibody linearity is observed between 9 and 5000 pg/mL showing a broad range of linearity.

(24) Palacios, G.; Briese, T.; Kapoor, V.; Jabado, O.; Liu, Z.; Venter, M.; Zhai, J.; Renwick, N.; Grolla, A.; Geisbert, T. W.; Drosten, C.; Towner, J.; Ju, J.; Paweska, J.; Nichol, S. T.; Swanepoel, R.; Feldmann, H.; Jahrling, P. B.; Lipkin, W. I. *Emerg. Infect. Dis.* **2006**, *12*, 692–695.

(25) Renwick, N.; Schweiger, B.; Kapoor, V.; Liu, Z.; Villari, J.; Bullmann, R.; Miething, R.; Briese, T.; Lipkin, W. I. *J. Infect. Dis.* **2007**, *196*, 1754–1760.

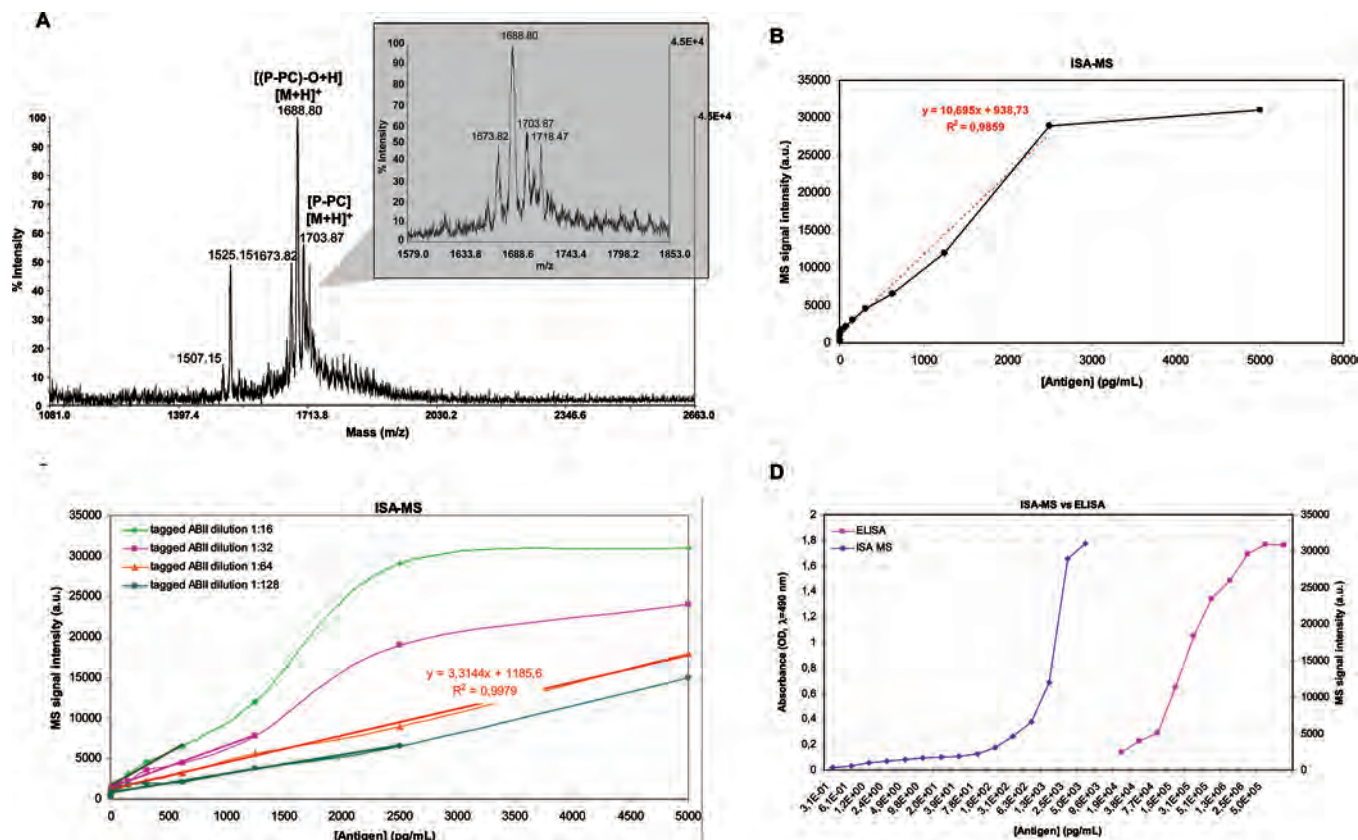


**Figure 4.** (A) Schematic representation of the incorporation of the tagged U nucleotide in a single-strand RNA probe. (B) Structure of the U-tagged nucleotide. (C) MALDI mass spectrum in the reflectron positive mode of the U-tagged probe (proenkephalin) in a solution with HCCA/ ANI as matrix.

Below 5 pg/mL, the signal drastically decreases below the linearity curve because the detection limit is reached. For a dilution of 1:16, the maximum binding is reached at 1250 pg/mL and 2500 for a dilution of 1:32 whereas for 1:64 the curve is still linear at a value of 5000 pg/mL. In our experiments, we thus decided to use 1:64 as the dilution for the secondary antibody in indirect ISA-MS. Under these conditions, we attempted to compare the sensitivity

of the ISA-MS method to the classical ELISA method (Figure 5D). The results showed that the limit of detection with ISA-MS (few picograms per milliliter) is 1000-fold lower than for ELISA (few nanograms per milliliter). With ISA-MS it is possible to detect up to 200 amol of antigens whereas only 200 fmol were detected using classical ELISA. The ISA-MS method is therefore approximately 1000-fold more sensitive than the classical ELISA method, which





**Figure 5.** (A) MALDI mass spectrum in the linear positive mode of the tagged antibody after ISA-MS experiment and UV postcleavage. (B) ISA-MS titration curves of MS signal intensity vs antigen concentration. (C) ISA-MS titration curves of MS signal intensity vs antigen concentration with secondary antibody dilutions ranging from 1:16 to 1:128. (D) Comparison of the titration of antigen concentration between the ISA-MS and ELISA methods.

is highly promising for titration of biological markers present at low concentration like pathology markers in body fluids. ISA-MS is clearly more sensitive than ELISA with UV detection. Although, various strategies of assays and different detections were proposed in the last 10 years, currently, much detection are performed by on fluorescence measurements which also prove to be very sensitive. Fluorescence assays can be performed according very different strategies, including beads grafted with antibodies, microarrays<sup>26–30</sup> with fluorescence detection, or by classical ImmunoSorbent Assays with fluorescence detection (FLISA). Fluorescence is much more sensitive than UV detection and present a limit of detection ranging from hundreds of picograms per milliliter up to 10 pg/mL according to the strategy used. In this respect, ISA-MS is at least as sensitive as fluorescence detection, but sensitivity of ISA-MS can still improve taking into account the increasing sensitivity of new mass spectrometers or by changing the reporter for an even more easily detectable reporter. ISA-MS as fluorescence presents the great advantage of multiplexing capabilities. In fact, multiplexing conditions can

be easily reached only by varying the mass of the reporter. It is also possible to access more easily comparative analysis by using isotope coded reporter (e.g., deuterated reporters) that will present exactly the same response for MS analysis but giving different specific MS signals. This is more difficult with fluorescence since fluorophores can present different response in term of detection and are also not always incorporated with the same rate, which can render quantification more difficult. Also, we must notice that fluorescence can be hampered by the lifetime of the fluorescence signal, quenching problems, and residual fluorescence. Such problems are avoided by using MS measurement using photo-cleavable tags. Thus, ISA-MS is a good alternative to other detection systems for titration of antigens that could be used in multiplexing conditions with a good limit of detection.

## CONCLUSIONS

In the present study, we developed novel multiplex quantitative methods using Tag-mass concept, i.e., PCR-MS and ISA-MS. The PCR-MS based on the Tag-mass technology is an alternative to MS-PCR<sup>31</sup> or PCR-MS.<sup>32</sup> The PCR-MS assay offers the additional, unique advantages of type-specific identification and quantitation. This technology is well established for SNP (single nucleotide

(26) Kondo, Y.; Ahn, J.; Komatsubara, R.; Terada, A.; Yasuda, T.; Tsuge, I.; Urisu, A. *Allergol. Int.* **2009**, *58*, 295–299.  
 (27) Li, N.; Tang, H.; Gai, H.; Dong, X.; Wang, Q.; Yeung, E. S. *Anal. Bioanal. Chem.* **2009**, *394*, 1879–1885.  
 (28) Oelschlaeger, P.; Srikant-Iyer, S.; Lange, S.; Schmitt, J.; Schmid, R. D. *Anal. Biochem.* **2002**, *309*, 27–34.  
 (29) Sasakura, Y.; Kanda, K.; Fukuzono, S. *Anal. Chim. Acta* **2006**, *564*, 53–58.  
 (30) Swartzman, E. E.; Miraglia, S. J.; Mellentin-Michelotti, J.; Evangelista, L.; Yuan, P. M. *Anal. Biochem.* **1999**, *271*, 143–151.

(31) Rust, S.; Funke, H.; Assmann, G. *Nucleic Acids Res.* **1993**, *21*, 3623–3629.  
 (32) Patel, D. A.; Shih, Y. J.; Newton, D. W.; Michael, C. W.; Oeth, P. A.; Kane, M. D.; Opipari, A. W.; Ruffin, M. T. t.; Kalikin, L. M.; Kurnit, D. M. *J. Virol. Methods* **2009**, *160*, 78–84.

polymorphism) studies.<sup>31,33</sup> With the Tag-mass concept, a novel dimension is open: the multiplexing ISA-MS also offers multiplexing and the quantitation less than a femtomole. These data are in line with the ones obtained in fluorescence ELISA but without quenching problems for ISA-MS. These novel tools will have application directly in clinics for bedside patients following or biomarker tracking in body fluids.

---

(33) Ran, P.; Li, Y. B.; Yan, J.; Zhang, B. L.; Zhang, J.; Shan, H. X.; Ye, Z. P.; Hou, Y. P.; Zhang, L.; Liao, M.; Wu, J. *Zhonghua Yixue Yichuanxue Zazhi* **2007**, *24*, 63–66.

## ACKNOWLEDGMENT

J.S. and M.E.A. contributed equally to this work. This study was supported by grants from the Centre National de la Recherche Scientifique (CNRS, Ministère de L'Education Nationale), the L'Enseignement Supérieur et de la Recherche, and the Agence Nationale de la Recherche (to I.F.).

Received for review June 28, 2009. Accepted October 1, 2009.

AC901416S