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IRBM
IRBM 35 (2014) 66–71

ANR TECSAN 2010

Detection of urinary modified nucleosides by a bulk acoustic wave MIP sensor – Results and future work

L.A. Agrofoglio [a,*,1], A. Krstulja [a], C. De Schutter [a], P. Favetta [a], R. Delépée [a], V. Roy [a], C. Dejous [b,1], H. Hallil [b], J.-L. Lachaud [b], N. Lebal [b], N. Omar Aouled [b], V. Raimbault [b], D. Rebière [b], S. Dulong [c], F. Levi [c,1], C. Junot [d], F. Théodoro [d], A. Pruvost [d,1], R. Vidal [e,1]

[a] *ICOA UMR CNRS 7311, Université d'Orléans, Orléans, France*
[b] *University Bordeaux, IMS laboratory, CNRS UMR 5218, IPB, Talence, France*
[c] *Rythmes Biologiques et Cancers, UMRS 776 INSERM et Université Paris Sud, Villejuif, France*
[d] *Laboratoire d'Études du Métabolisme des Médicaments - iBiTec-S CEA Saclay, Gif-Sur-Yvette, France*
[e] *Merck Millipore, Pithiviers, France*

Received 10 February 2014; received in revised form 13 February 2014; accepted 13 February 2014
Available online 20 March 2014

Abstract

This paper presents an overview of the main actions and projects of the ANR TECSAN "CancerSensor" during the 2011–2014 period. The project aims to develop and validate a quantitative, non-invasive therapeutic tool to detect selected urinary modified nucleosides as biomarkers of colorectal cancer chemotherapy and to monitor in fine the efficiency of the chemotherapy. A methodology has been developed to generate a bulk acoustic wave molecularly imprinted polymer sensor (SAW-MIP) for the detection of various nucleosides and nucleotides of interest. A rapid and sensitive mass spectrometry method has been also developed.

1. Introduction

Modified nucleosides, derived predominantly from transfer ribonucleic acid (tRNA), have been shown to be excreted in abnormal amounts in the urine of cancer patients. Around 20 of them have been already used as tumour markers for instance: for breast cancer [1,2], for colon and liver cancer [3], for lung cancer [4]. Pseudouridine, 1-methyladenosine, 1-methylguanosine, 2-methylguanosine, 1-methylinosine, 2-pyridone-5-carboxamide-N1-ribofuranoside are some of the modified nucleosides (Fig. 1). These modified nucleosides cannot be reutilized or further degraded, but they are excreted in the urine as intact molecules because they do not have a salvage pathway [5]. The urinary excretion of such modified nucleosides is relatively low but it is an indication of the higher turnover rate of tRNA in tumour tissues [6].

Although different analytical techniques have been reported for the determination of urinary nucleoside levels, their detection is still challenging due to the low level of modified nucleosides but also to the complexity of urine (in a less intend, blood) biological matrix. Actual well-established analytical techniques (SPE-HPLC-UV, CE-UV, LC-MS/MS, GC-MS/MS) are restrictive. Thus, to develop and validate a quantitative, non-invasive diagnosis tool to monitor the efficacy of chemotherapy in patients with colorectal cancer, we have developed an acoustic-biosensor based on molecularly imprinted polymers (MIP). It includes advances which will take the sensitivity down to a low-concentration single-molecule level.

2. Results for the period 2011–2014

From 10 selected modified nucleosides mainly found in patients with histological diagnosed malignant tumours (colorectal cancers), we wish:

* Corresponding author.
E-mail address: luigi.agrofoglio@univ-orleans.fr (L.A. Agrofoglio).
[1] Project manager.

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http://dx.doi.org/10.1016/j.irbm.2014.02.009

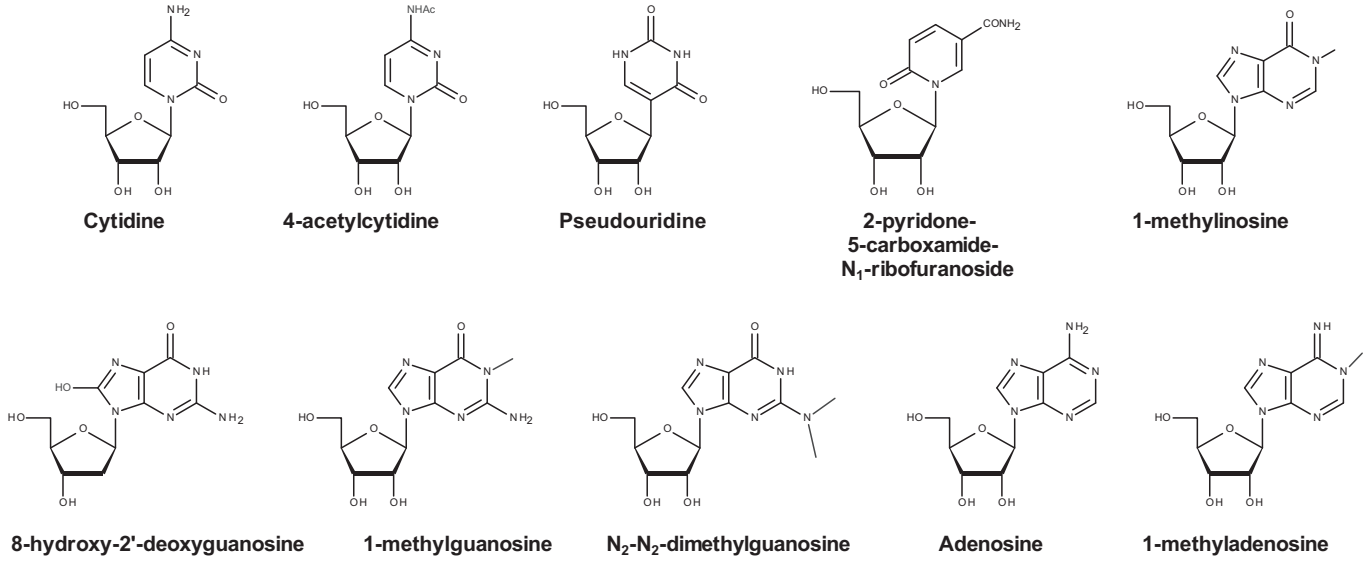

Fig. 1. Most common nucleoside urinary cancer markers.

- to develop an acoustic-sensor and reagent strips for a new simple, sensitive, non-invasive, cost-effective and real-time urinalysis of trace modified nucleosides tumour cancer;
- to use molecularly imprinted polymer technology, which has demonstrated speed and selectivity, to fix it on the acoustic-sensor or on a reagent strips;
- to use those tools to the analysis of more than 3000 urinary samples from patients under chemotherapy for colorectal cancer;
- to use those tools to correlate the chemotherapy treatment with the circadian time of infusion and the circadian variations of clearance.

During the last period, we have developed (1) various MIP for nucleosides detection, (2) a bulk acoustic wave MIP sensor for the adenosine-5'-monophosphate (AMP) (taken as a model); (3) a new rapid and sensitive mass spectrometry quantitation of nucleosides with direct urine injection.

2.1. Design of various MIP of nucleosides

The molecularly imprinted polymer (MIP) generates "tailor-made" receptors created to recognize and rebind a target molecule (substrate) with high selectivity and affinity. The principle is to imprint a target molecule called "template" in a three-dimensional polymer network. After extraction of the "template", the obtained cavity is complementary to the template (by electrostatic bonds) in terms of size, conformation, and given functional groups position. This "molecular memory" printed on a polymer is then likely to highly selectively recognize and bind the original template. The selectivity of the MIP depends on various factors, such as the size and shape of the cavity and rebinding interactions. The technology has been adopted with specific selectivity to prepare sorbents, coatings, and stationary phases in the field of analytical chemistry [7]. For instance, molecularly imprinted polymers (MIPs) were prepared for adenosine-5'-monophosphate (AMP). Different functional monomers interacting with the phosphate moiety were tested, and the MIP giving the best specific binding of AMP was composed with one equivalent of 2-(dimethylamino)ethyl methacrylate (DMAEM - a ternary amine-based functional monomer that can be ionized under pH 8.4) and ten equivalents of acrylamide compared to AMP. The imprinted polymer was selective for AMP towards others nucleotides or deoxy analogues [8] (Fig. 2).

Similar approaches were done to imprint pseudouridine, and various purine nucleosides (guanosine, 8-hydroxy-2'-deoxyguanosine, N1-methyl guanosine, N7-methyl guanosine). For purine nucleosides, two compositions were tested, using a simple functional monomer like methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) as crosslinker. Accordingly, two ratios of template/monomer/crosslinker where tested in two porogen types because of the problems in solubility. As porogens, acetonitrile and 30/70 (v/v) methanol/acetonitrile where used. Azobisdimethylvaleronitrile (ABDV) was used as low temperature thermal initiator. To compare the effect of different polymer formations on the rebinding efficiency, along with traditional bulk polymers synthesis a precipitation polymerization was also assayed. Polymers where tested and characterized by HPLC chromatography (HPLC analysis using MIPs as stationary phases, frontal analysis of MIPs), and by solid phase extraction (SPE) method to assess the extraction efficiency and applicability of the synthesized polymers. The solvent used was organic phase composed of 1% acetic acid in acetonitrile.

2.2. Design of a bulk acoustic wave MIP sensor for detection of AMP

The present work deals with the development of a Love-wave MIP-based sensor platform for the detection of Adenosine Mono Phosphate (AMP) as a model of target in liquid medium. Deposition process and characterization of the MIP layer, as

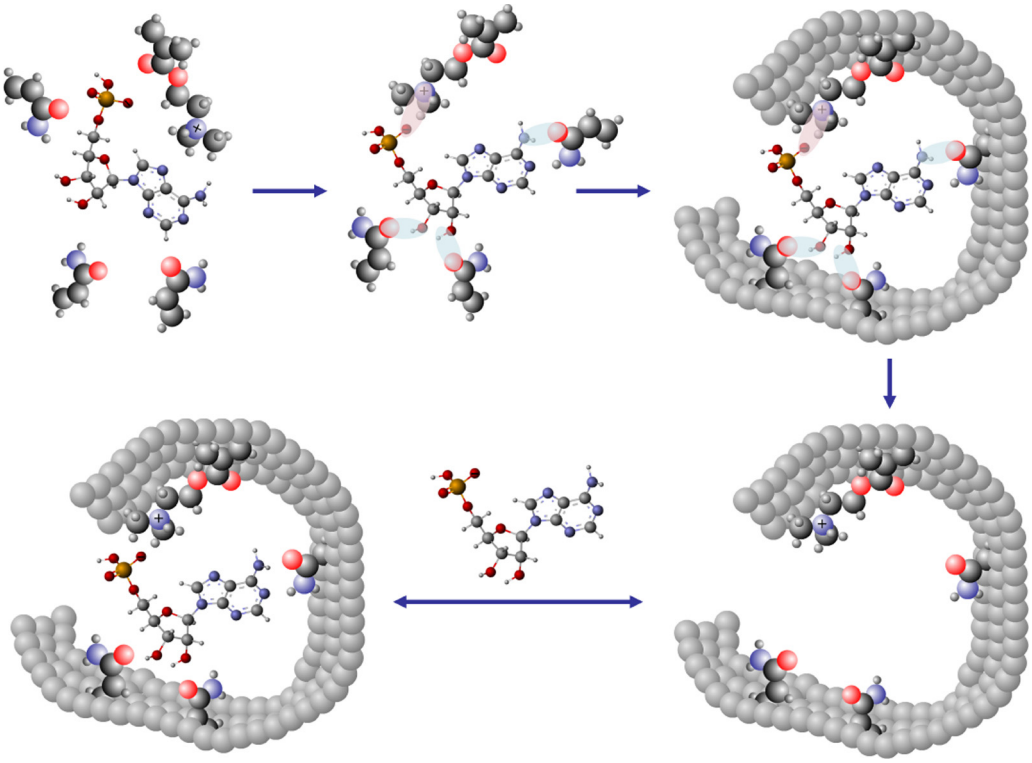

Fig. 2. Schematic representation of imprinted strategy for AMP [8].

well as real-time response of the MIP-coated sensor to various concentrations of AMP have been investigated:

- Love-wave device: the sensor is composed of a microelectronic component (Surface Acoustic Wave or SAW device) coated with a polymeric thin film as recognition layer. The 118 MHz SAW device [9] is based on the propagation of a surface guided shear horizontal wave (Love-wave) suitable for liquid applications, generated piezoelectrically with interdigitated electrodes (IDTs). This delay-line is mounted in an oscillating configuration for real-time monitoring with good resolution;
- Sensitive MIP film: a process for thin film MIP coating based on commercial nucleotides AMP as a nucleoside analog was developed. Thin and porous AMP-based MIP layers, localized on the propagation path between input and output IDTs, were successfully spin-coated on the sensor surface;
- Microfluidic set up: a chip with a microfluidic chamber (Fig. 3) is maintained by pressure above the sensor. This chip is made out of polydimethylsiloxane (PDMS) using a standard soft lithography process [10]. Two syringe pumps linked to an actuated valve inject the selected liquid: buffer solution (acetic acid/hydroxylamine 1 mM pH 7) or AMP in buffer solution, into the microfluidic chip with 5 μL/min flow rate.

Optical profilometry and Scanning Electron Microscopy (SEM) on the MIP-coated films showed their surface morphology. Then, the characterization of the MIP-coated SAW sensors under solvents vapors (Ethanol, Toluene), revealed similar behavior compared to mesoporous titania coatings and put to evidence the MIP rebinding ability [11]. Finally, on Fig. 4 is represented the real-time response of the acoustic wave oscillator with MIP for different concentrations of AMP. The MIP-based sensor showed a frequency shift of 150 Hz for 5 ppm AMP concentration.

This oscillator configuration and microfluidic chip allow detection of nucleotides in real time compared with static measurements reported in [12]. Further studies aim systematic measurements for repeatability characterization, then AMP-MIP layer replacement by a nucleoside-based MIP layer.

2.3. Liquid chromatography-mass spectrometry methods for urinary biomarkers detection

A reference method for the absolute quantification of selected modified nucleosides as potential biomarkers in human urine has been developed by liquid chromatography coupled to mass spectrometry.

We first developed a classical approach for absolute quantification by LC-MS/MS using a triple quadrupole instrument operated in the selected reaction monitoring (SRM) mode. However, sensitivity and specificity issues (interferences due to the presence of endogenous isobaric compounds, i.e., having the same nominal mass than the nucleosides to monitor) led us to stop using the triple quadrupole analyzers and to continue our method development on a Q-Exactive (an Orbitrap based instrument, ThermoFisher Scientifics, Courtaboeuf, France), which is an ultra-high resolution mass spectrometer.

In recent years, significant progress has been made in high-resolution mass spectrometers with especially higher sensitivity

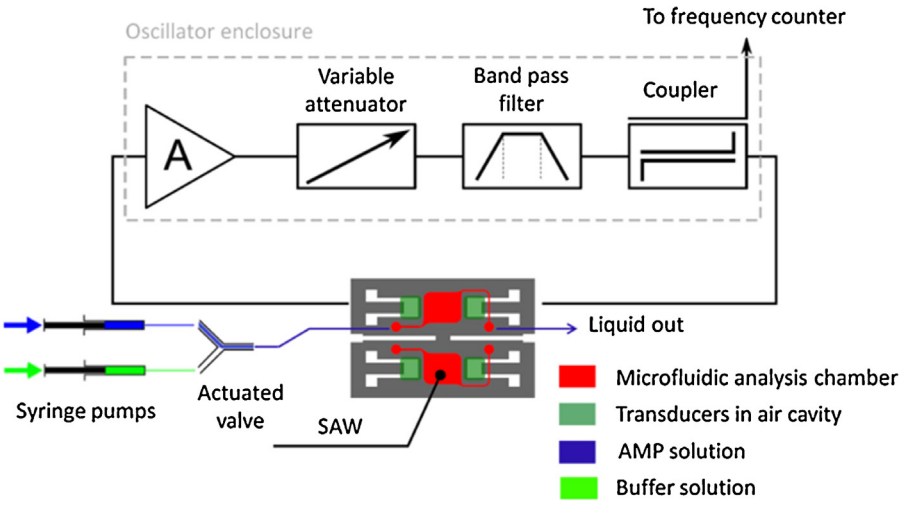

Fig. 3. Description of the experimental microfluidic monitoring apparatus.

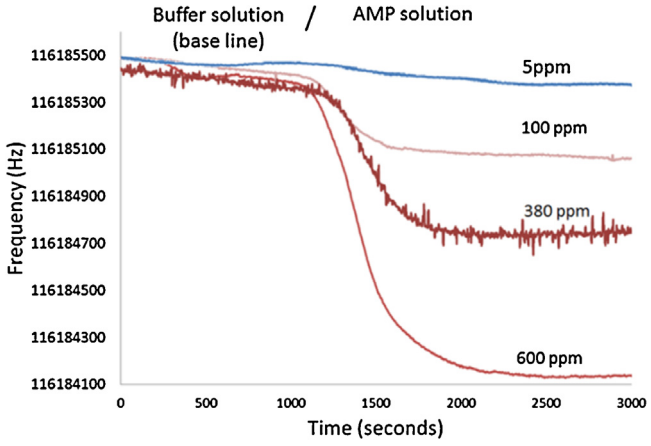

Fig. 4. Real-time frequency of the acoustic wave oscillator with MIP for different concentrations of AMP.

and duty cycle, making the Orbitrap mass analyzer a powerful tool for qualitative/discovery metabolomics. Quantification on HRMS instruments is most often done in the SIM and/or full scan modes. It means that the two stages of mass filtering (as done on a QqQ instrument) are avoided and selectivity is solely based on the capability of the high resolution and high mass accuracy to separate the ions of interest from background ions. As a consequence of the full scan acquisition and the absence of ion fragmentation, HRMS offers some attractive features for endogenous metabolites quantification, with for instance the monitoring of virtually all ions generated during electrospray ionization, even those with low fragmentation efficiency, a constant scan time regardless of the number of monitored compounds, and without any compound specific optimization [13]. There is also a clear advantage of HRMS for isobaric compound having similar MS/MS spectra [13b], due to the capacity of the instrument to extract ion chromatograms with a very narrow m/z window (a few ppm). On the contrary, SRM can differentiate isomers by looking at typical fragment ions while full scan HRMS cannot [14].

The recently developed Q-Exactive [15], composed of the Orbitrap mass analyser combined with a quadrupole mass filter as front-end, should further increase sensitivity and linearity performance of HRMS based quantification. Clearly, the quadrupole mass filtering is beneficial for SIM acquisition in complex samples by selecting a restricted m/z range and therefore enriching low abundant analytes before their introduction into the Orbitrap mass analyzer [16]. For this reason, we choose to develop and validate the quantification of urinary nucleosides using such an instrument.

Among the nine targeted nucleosides to be monitored, 3 of them were selected to be used as labeled internal standards for the entire quantification process (Adenosine [ribose-^{13}C5], 8OH, 2'deoxyguanosine (^{15}N5) and 1-methyl-adenosine-D3). Owing to the fact that nucleosides are highly concentrated in the urine, this allowed us to avoid a sophisticated and time consuming sample preparation. The solid phase extraction previously performed on a OASIS MCX cartridge at the beginning of the method development, was replaced by a simple urine dilution in water by a factor of 5. Then, a 10 μL aliquot of the diluted urine was injected into the analytical system. The ultra-high pressure chromatographic (UHPLC) method was developed using a reverse phase column. The separation was performed on a column Acquity UPLC BEH Shield® 1.7 μm 2.1 × 100 mm (Waters, France). Mobile phases were distributed in a gradient mode (Dionex Ultimate 3000 system). Detection by high-resolution mass spectrometry in full scan mode is accomplished using a Q-Exactive (Orbitrap technology, ThermoFisher Scientifics) after ionization in positive mode. The total analysis runtime was 7 minutes (Fig. 5).

This method was successfully validated according to the European recommendations (www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf).

3. Future plans (work in progress)

In the next future, we will continue our activities on three directions:

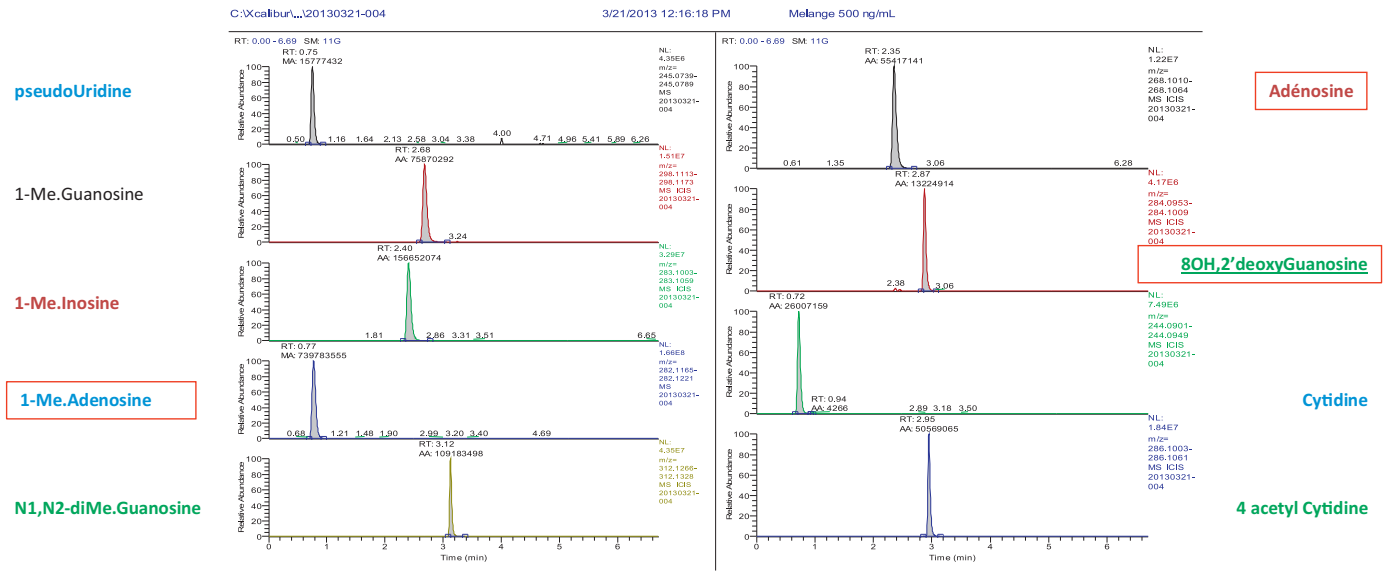

Fig. 5. MS chromatograms of the 9 monitored nucleosides. Nucleosides framed in red were also used as internal standards (stable isotopes labeling).

- the development of an acoustic wave MIP sensor for pseudouridine nucleoside, which is predominantly found in urine from cancer patients;
- as the liquid chromatography-mass spectrometry methods for urinary biomarkers detection is now available, it will be applied for the analysis of urine samples collected from large cohorts of cancer patients;
- apart from detecting and identifying tumour markers in the urine samples, the effect of circadian chemotherapy on the amount of cancer markers, using the developed tools will be explored. In fact, the circadian timing system is composed of molecular clocks, which drive 24-h changes in xenobiotic metabolism and detoxification, cell cycle events, DNA repair, apoptosis, and angiogenesis. As a result, circadian timing can modify 2- to 10-fold the tolerability of anticancer medications in experimental models and in cancer patients.

This is to date an almost unexploited dimension for diagnosis information. By combining and supporting these novel optical methods with state-of-the-art affinity molecule MIP technology, tumour biomarkers, acoustic-biosensor, will be exploited to extract a maximum amount of information out of very small amounts of sample material. Last, involvement of pharmaceutical companies will be encouraged, so as to broaden our view in detection of biomarkers and in use of acoustic wave MIP sensors for medical applications, favor the valorization of our research. Given the high incidence of cancer (including colorectal cancer), this project has a very high relevance on medical end-users.

Acknowledgement

The authors sincerely thank the National Research Agency (ANR-10-TECS-0004; http://www.agence-nationale-recherche.fr/) for financial support. The acoustic wave MIP sensor part of this project was in part granted by the Polytechnical Institute of Bordeaux (IPB). Monique Benoît and Veronique Conédéra (Technological Network RTB, LAAS, CNRS, Toulouse-France), for realizing acoustic wave delay lines, as well as Bernard Plano (IMS-Bordeaux) for SEM images.

References

[1] (a) Rasmuson T, Björk GR. Urinary excretion of pseudouridine and prognosis of patients with malignant lymphoma. Acta Oncol 1995;34:61;
(b) Rasmuson T, Björk GR, Damber L, Jacobsson L, Jeppsson A, Stigbrand T, et al. Tumor markers in mammary carcinoma. Acta Oncol 1987;26:261;
(c) Henneges C, Bullinger D, Fux R, Friese N, Seeger H, Neubauer H, et al. Prediction of breast cancer by profiling of urinary RNA metabolites using Support Vector Machine-based feature selection. BMC Cancer 2009:9;
(d) Cho SH, Choi MH, Lee WY, Chung BC. Evaluation of urinary nucleosides in breast cancer patients before and after tumor removal. Clin Biochem 2009;42:540;
(e) Frickenschmidt A, Froehlich H, Bullinger D, Zell A, Laufer S, Gleiter CH, et al. Metabonomics in cancer diagnosis: mass spectrometry-based profiling of urinary nucleosides from breast cancer patients. Biomarkers 2008;13:435–49.

[2] (a) Nakano K, Nakao T, Schram KH, Hammargren WM, McClure TD, Petersen E. Urinary excretion of modified nucleosides as biological marker of RNA turnover in patients with cancer and AIDS. Clin Chim Acta 1993;218:169;
(b) Zheng YF, Kong HW, Xiong JH, Lv S, Xu GW. Clinical significance and prognostic value of urinary nucleosides in breast cancer patients. Clin Biochem 2005;38:24.

[3] (a) Yang J, Xu G, Kong H, Zheng Y, Pang T, Yang Q. Artificial neural network classification on high-performance liquid chromatography of urinary and serum nucleosides for the clinical diagnosis of cancer. J Chromatogr B 2002;780:27;
(b) Jeng LB, Lo WY, Hsu WY, Lin WD, Lin CT, Lai CC, et al. Analysis of urinary nucleosides as helper tumor markers in hepatocellular carcinoma diagnosis. Rapid Commun Mass Spectrom 2009;23:1543.

[4] McEntire JE, Kuo KC, Smith ME, Stalling DL, Richens JW, Zumwalt RW, et al. Classification of lung cancer patients and controls by chromatography of modified nucleosides in serum. Cancer Res 1989;49:1057.

[5] Weissman SM, Eisen AZ, Lenrio M. Pseudouridine metabolism III: studies with isotopically labeled pseudouridine. J Lab Clin Med 1962;60:40.

[6] (a) Borek E, Baliga BS, Gehrke CW, Kuo KC, Belman S, Troll W, et al. High turnover rate of transfer RNA in tumor tissue. Cancer Res 1977;37:3362;

(b) Sander G, Topp T, Wieland J, Heller-Schoöch G, Schoöch G. Possible use of urinary modified RNA metabolites in the measurement of RNA turnover in the human body. Hum Nutr Clin Nutr 1986;40C:103;
(c) Schöch G, Sander G, Topp H, Heller-Schöch G. Modified nucleosides and nucleobases in urine and serum as selective markers for the whole-body turnover of tRNA, rRNA and mRNA: future prospects and impact. In: Gehrke CW, Kuo KC, editors. Chromatography and modification of nucleosides. Part C. Modified nucleosides in cancer and normal metabolism methods and applications. Amsterdam: Elsevier; 1990. p. C389–442;
(d) Seidel A, Brunner S, Seidel P, Fritz GI, Herbarth O. Modifed nucleosides: an accurate tumour marker for clinical diagnosis of cancer, early detection and therapy control. Br J Cancer 2006;94:1726.

[7] (a) Takeuchi T, Haginaka J. Separation and sensing based on molecular recognition using molecularly imprinted polymers. J Chromatogr B 1999;728:1;
(b) Wulff G. Fitting molecules into polymeric receptors. Chemtech 1998;11:19;
(c) Wulff G. Molecularly imprinting in cross-linked materials with the aid of molecular templates-a way towards artificial antibodies. Angew Chem Int Ed Engl 1995;34:1812;
(d) Piletsky SA, Turner APF. Electrochemical sensors based on molecularly imprinted polymers. Electroanalysis 2002;14:317;
(e) Remcho VT, Tan ZJ. MIPs as chromatographic stationary phases. Anal Chem 1999;71:A248;
(f) Haupt K. Imprinted polymers: the next generation. Anal Chem 2003;75:A377;
(g) Haupt K, Mosbach K. Molecularly imprinted polymers and their use in biomimetic sensors. Chem Rev 2000;100:2495;
(h) Mayes AG, Mosbach K. Molecularly imprinted polymers-useful materials for analytical chemistry. Trends Anal Chem 1997;16:312.

[8] Breton F, Delépée R, Agrofoglio LA. Molecular imprinting of AMP by an ionic-noncovalent dual approach. J Sep Sci 2009;32:3285.

[9] Lebal N, Hallil H, Dejous C, Plano B, Krstulja A, Delepee R, et al. Association of a Love wave sensor to thin film molecularly imprinted polymers for nucleosides analogs detection. IEEE NEMS Conference 2013;550 (doi:10.1109/NEMS. 2013.6559790).

[10] Raimbault V, Rebière D, Dejous C, Guirardel M, Lachaud JL. Molecular weight influence study of aqueous poly(ethylene glycol) solutions with a microfluidic Love wave sensor. Sensors Actuators B 2010;144: 318.

[11] Omar Aouled N, Lebal N, Hallil H, Delépée R, Agrofoglio L, Rebière D, et al. Study of gaseous compounds adsorption with a Love wave sensor based on Molecularly Imprinted Polymeric thin film. Sensors Transducers 2013;149:37.

[12] Gronewold TMA, Baumgartner A, Quandt E. Discrimination of single mutations in cancer-related gene fragments with a surface acoustic wave sensor. Anal Chem 2006;78:4865.

[13] (a) Kiefer P, Portais JC, Vorholt JA. Quantitative metabolome analysis using liquid chromatography-high-resolution mass spectrometry. Anal Biochem 2008;382:94;
(b) Lu W, Clasquin MF, Melamud E, Amador-Noguez D, Caudy AA, Rabinowitz JD. Metabolomic analysis via reversed-phase ion-pairing liquid chromatography coupled to a stand alone orbitrap mass spectrometer. Anal Chem 2010;82:3212.

[14] Ramanathan R, Jemal M, Ramagiri S, Xia YQ, Humpreys WG, Olah T, et al. It is time for a paradigm shift in drug discovery bioanalysis from srm to hrms. J Mass Spectrom 2011;46:595.

[15] Michalski A, Damoc E, Hauschild JP, Lange O, Wieghaus A, Makarov A, et al. Mass spectrometry-based proteomics using Q Exactive, a high-performance benchtop quadrupole Orbitrap mass spectrometer. Mol Cell Proteomics 2011;10. M111.011015.

[16] Gallien S, Duriez E, Crone C, Kellmann M, Moehring T, Domon B. Targeted proteomic quantification on quadrupole-orbitrap mass spectrometer. Mol Cell Proteomics 2012;11:1709.