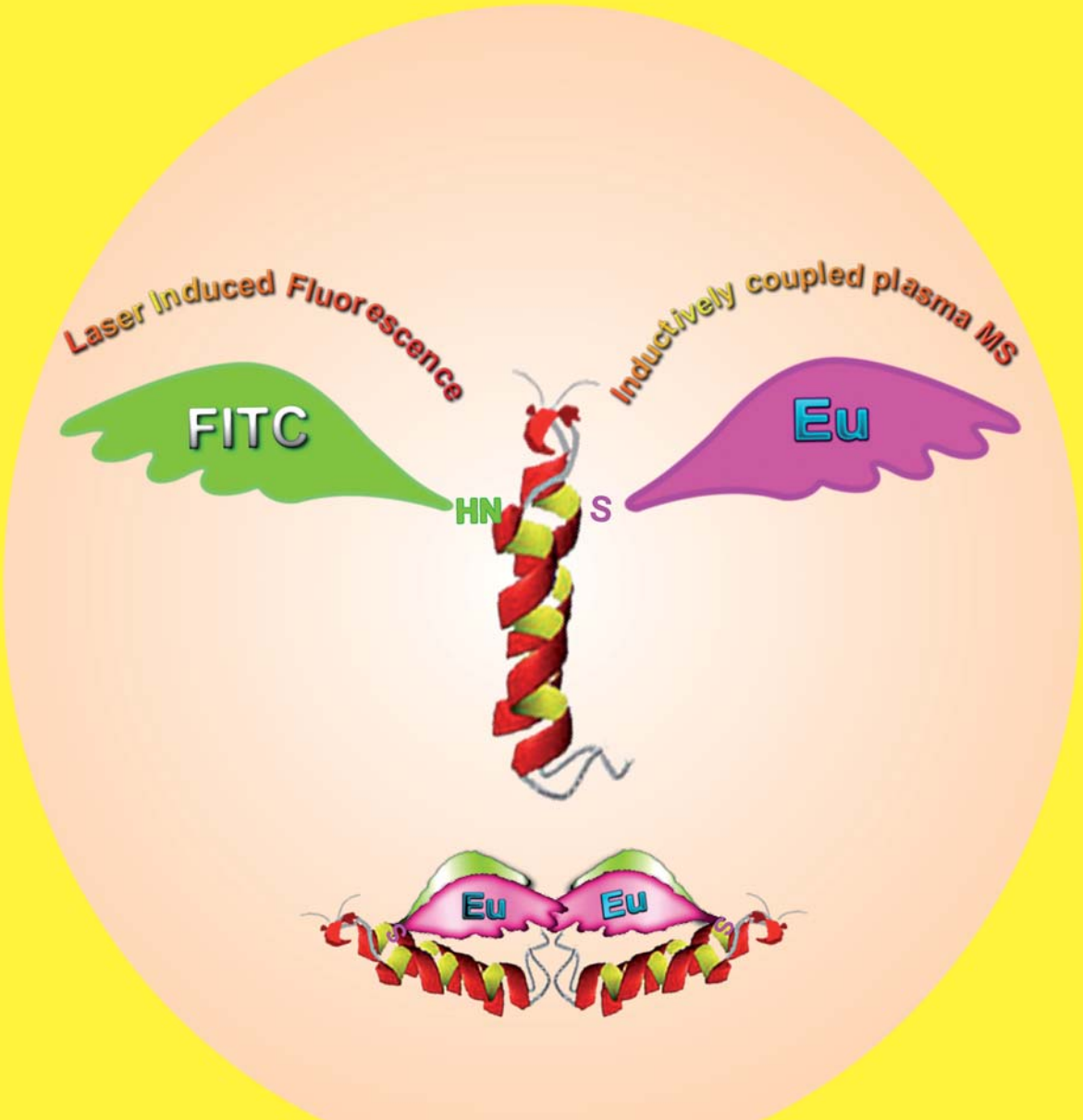
JAAS

Journal of Analytical Atomic Spectrometry

www.rsc.org/jaas

Volume 26 | Number 6 | June 2011 | Pages 1101–1304



Themed issue: 2010 Asia Pacific Winter Conference on Plasma Spectrochemistry

ISSN 0267-9477

RSC Publishing

COMMUNICATION
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International Year of CHEMISTRY 2011

0267-9477(2011)26:6;1-8

JAAS

Dynamic Article Links

Cite this: *J. Anal. At. Spectrom.*, 2011, **26**, 1175

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COMMUNICATION

A dual-labelling strategy for integrated ICPMS and LIF for the determination of peptides†

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Received 6th January 2011, Accepted 18th March 2011
DOI: 10.1039/c1ja00010a

In this proof-of-concept study, a novel dual-labelling strategy for conjugating –SH and –NH_2 of a peptide was developed, in which an element tag (1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid-10-maleimidoethylacetamide loaded with europium) and a fluorescent tag (fluorescein isothiocycanate) were employed. Its feasibility was demonstrated using HPLC-UV/ESI-MS for evaluating labelling-efficiency and HPLC-ICPMS with ^{153}Eu isotope dilution as well as CE-LIF for the determination of peptides.

Labelling strategy has been recognized as one effective way to improve the selectivity and sensitivity of peptide/protein imaging and quantification. To date, several labelling strategies have been reported. One of them is generally based on conjugating fluorescent tags onto target peptides/proteins to achieve their analysis using subsequent optical detection techniques, and successful relevant examples were documented in two recent reviews.[1,2] Another, which is worthy of mentioning here, is based on labelling elemental tags onto target peptides/proteins allowing the determination of the peptides/proteins using element-selective atomic spectroscopic techniques such as ICPMS, in which gold nanoparticles, lanthanides, iodine and ferrocene as well as organic mercurials were used in the indirect or direct labelling modes.[3–21]

Apart from the above mono-labelling methods, a few dual-labelling strategies towards different functional groups in one biomolecule have been proposed. For exmaple, Nygren *et al.* presented a dual-labelling approach of a binding protein using *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine and succinimidyl-6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoate for subsequently labelling the introduced –SH and a N-terminal –NH_2, allowing specific fluorescence detection of the native protein.[22] Karst *et al.* reported a dual-element labelling method towards –SH and –NH_2 with ferrocene-based reagents to investigate the tripeptide glutathione and large peptide insulin coupling with HPLC-ICPMS, suggesting multiple labelling leads to a strong increase of bioanalysis quantifiable information.[23] However, such dual-labelling methods were aimed at detection or determination of peptides/proteins using only one technique (fluorescence or ICPMS). But it is often desired to determine the same labelled peptides/proteins *via* multiple techniques to satisfy different purposes and applications. A dual-labelling strategy with fluorescent tags and elemental tags allows the combination of fluorescence-based spectrometry and mass-based ICPMS to obtain comprehensive information of the target peptides/proteins not limited to achieving their unambiguous quantification. Such a strategy is very scarce.[24]

In this proof-of-concept study, we tried to develop a novel strategy for labelling one peptide with an elemental tag and a fluorescent tag, in which 1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid-10-maleimidoethylacetamide loaded with europium (MMA-DOTA-Eu) was used for conjugating the peptide *via* the specific reaction between –SH and MMA, and with a typical fluorescent tag (fluorescein isothiocycanate, FITC) for subsequently conjugating the peptide *via* the reaction between –N=C=S and –NH_2 (Fig. 1), then determining the peptide using both ^{153}Eu isotope dilution ICPMS and CE-LIF. To the best of our knowledge, this is the first report of the dual-labelling strategy with both fluorescent and nonradioactive-element tags for the quantification of peptides using both ICPMS and LIF.

Specificity and efficiency of the proposed dual-labelling strategy are crucial for the further determination and/or absolute quantification experiments. A peptide, vasopressin (Vas) (containing a disulfide bond –S–S– and one N-terminal –NH_2), was chosen as a model peptide to investigate the optimum labelling conditions through HPLC-UV/ESI-MS. After complete reduction of the –S–S– in Vas with TCEP at 37 °C for 30 min, the influence of the molar ratio of MMA-DOTA to –SH on the labelling efficiency and specificity was investigated (Fig. S1†). The results obtained suggested that Vas in 100 mmol L^{-1} MOPS (pH 6.8) for 40 min at 47 °C was completely labelled when the ratio of MMA-DOTA to –SH was greater than 5. This was also confirmed by ESI-MS (Fig. S2a and b†). The deconvolution molecular weight (DM) of the labelled Vas was 2140.2, in agreement with the theoretical molecular weight (TM) of Vas-MMA-DOTA (527.54 × 2 + 1083.9 = 2139.0), and there no signal of unlabelled Vas was observed, indicating that the two exposed –SHs in the reduced Vas were completely labelled by two MMA-DOTA. Eu^{3+}

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† Electronic supplementary information (ESI) available: Instruments, agents, experiment procedures for optimzing labelling conditions, ESI-MS data and CE separation of peptides. See DOI: 10.1039/c1ja00010a

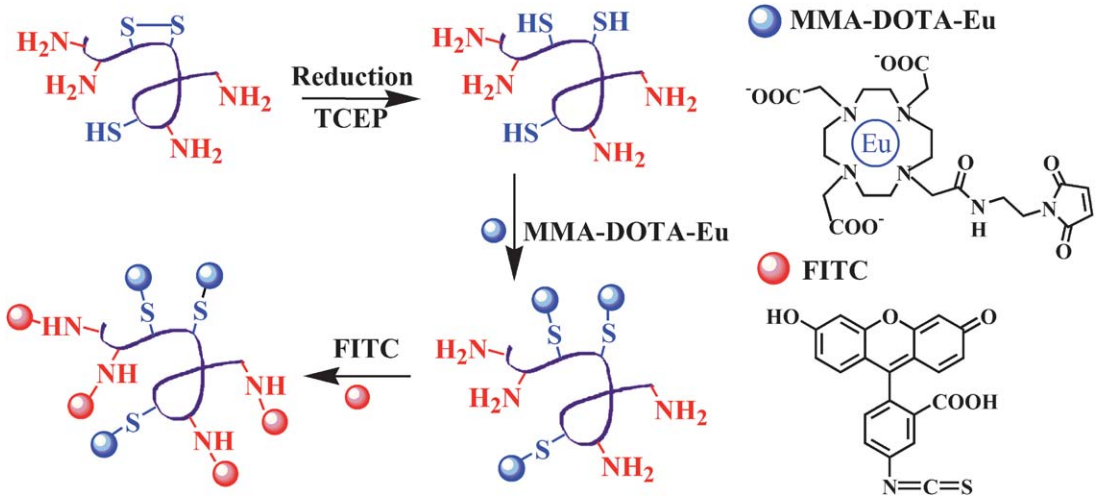

Fig. 1 Scheme for labelling –SH and –NH_2 in a peptide with MMA-DOTA-Eu and FITC.

is easily loaded *via* the coordination between Eu^{3+} and the oxygen and nitrogen atoms in the moiety of DOTA at pH 5.8 buffer using 500 mmol L^{-1} MES, and the excess Eu^{3+} depleted using EDTA.[20]

After loading Eu into DOTA in the above Vas-MMA-DOTA, the labelling step towards –NH_2 with FITC was carried out. An elevating temperature was helpful to accelerate the labelling reaction but too high a temperature led to the decrease of the fluorescence response and the generation of byproducts.[25] Besides, 50 °C was found to be the optimal temperature for conjugating FITC to –NH_2 and 200 mmol L^{-1} Na_2CO_3-$NaHCO_3$ solution (pH 9.0) was selected as the best buffer for this derivatization procedure; important parameters including the molar ratio of FITC to –NH_2 and reaction time that affected the labelling efficiency were further investigated using HPLC-UV/ESI-MS. 25 times excess in molar ratio of FITC over –NH_2 was found to be the best to complete the labelling reaction of Vas-MMA-DOTA-Eu with FITC (Fig. S3†). This was also confirmed by ESI-MS as shown in Fig. S2d.† In addition, results from the time-dependent labelling experiments suggested that 7 h was the best choice (Fig. S4†).

GGYGGC (containing 1 –SH and 1 N-terminal –NH_2) and somatostatin (Som, containing one –S–S– and 1 N-terminal –NH_2 and 2 lysine –NH_2) were also selected to demonstrate this dual-labelling strategy. The labelling efficiencies were corroborated by ESI-MS (Fig. S2e and f†), which indicated that one MMA-DOTA-Eu and one FITC were labelled onto GGYGGC, and two MMA-DOTA-Eu and two FITC were labelled onto Som. It should be noted that one lysine –NH_2 adjacent to cysteine missed being labelled by FITC, implying that when the lysine –NH_2 is close to the MMA-DOTA-Eu labelled cysteine in the amino acid sequence of Som, steric hindrance arising from both MMA-DOTA-Eu and FITC becomes obvious, obstructing the further labelling of the adjacent lysine –NH_2 in Som. This phenomenon suggested that small-size labelling tags should be designed and synthesized to avoid the possible steric hindrance encountered during the labelling process of peptides and proteins in the future, especially when the dual-labelling strategy is applied for quantitative analysis of peptides and proteins that have complex and unknown amino acid sequence and configuration.

Subsequently, absolute quantification of the three model peptides in a biological sample was performed with the proposed dual-labelling strategy using postcolumn ^{153}Eu isotope dilution HPLC-ICPMS (Fig. 2; detailed experimental conditions can be found in the ESI†) and CE-LIF (Fig. S7 and detailed experimental conditions are in the ESI†). The results listed in Table 1 indicate that the determined values using HPLC-ICP-IDMS and CE-LIF were in good agreement with each other, and in accordance with the values added in the sample.

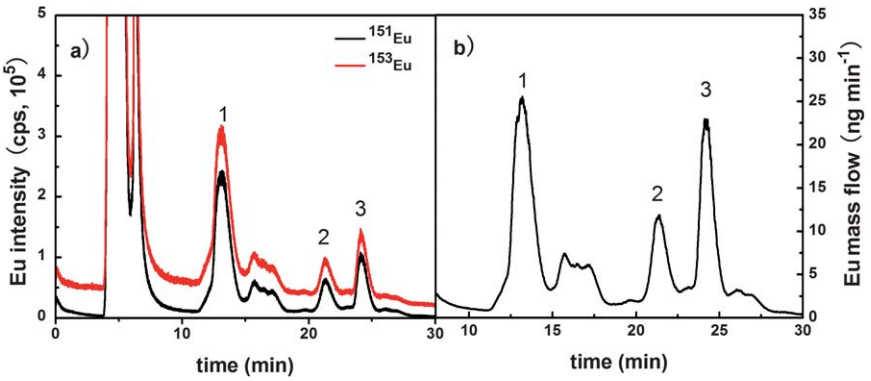

Fig. 2 a) Typical ^{151}Eu and ^{153}Eu isotope chromatograms of the three dual-labelled peptides. b) Eu mass flow chromatogram translated from a). Peak 1, GGYGGC; 2, Vas; 3, Som. Detailed experimental conditions can be found in the ESI.†

Table 1 Comparison of CE-LIF and HPLC-ICPMS for the analysis of the dual-labelled peptides ($n = 3$)

	LOD (nmol L^{-1})			Peptide concentration (μmol L^{-1})					
				Added			Found		
	Vas	Som	GGYGGC	Vas	Som	GGYGGC	Vas	Som	GGYGGC
CE-LIF	16.7	11.1	54.0	15.0	30.0	100.0	15.49 ± 0.72	28.59 ± 0.54[a]	100.98 ± 1.41
HPLC/ICPMS	0.130	0.130	0.259				15.73 ± 0.90	30.84 ± 0.35	101.74 ± 1.12

[a] The concentration of Som was calculated on the basis of two labelled $-NH_2$ with FITC.

Besides, the LODs of the three model peptides obtained using HPLC-ICP-IDMS were two orders of magnitude lower than those using CE-LIF (Table 1), reaching 0.130, 0.130 and 0.259 nmol L^{-1} for Vas, Som and GGYGGC, respectively, based on the Eu detection limit (3σ, 0.259 nmol L^{-1}) obtained in the mass flow chromatograms, suggesting that HPLC-ICP-IDMS is a superior platform for quantification of peptides. Moreover, it should be pointed out that this dual-labelling strategy offers a possibility to achieve not only the quantification of target peptides and proteins but also *in situ* imaging of them in a same biological sample in the future. The design and synthesis of a so-called all-in-one label[26] containing both an element and fluorescence moeity, which can be detected with multiple techniques, will speed up the steps towards high-resolution optical imaging and high-sensitive absolute quantification of peptides/proteins. Such research is ongoing in our lab.

Acknowledgements

This study is financially supported by the National Natural Science Foundation of China (21035006 and 20775062) and the 973 National Basic Research Program (2009CB421605).

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