VIP *Very Important Paper*

Pyrene Tags for the Detection of Carbohydrates by Label-Assisted Laser Desorption/Ionisation Mass Spectrometry**

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Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) is widely used for the analysis of biomolecules. Label-assisted laser desorption/ionisation mass spectrometry (LALDI-MS) is a matrix-free variant of MALDI-MS, in which only analytes covalently attached to a laser desorption/ionisation (LDI) enhancer are detected. LALDI-MS has shown promise in overcoming the limitations of MALDI-MS in terms of sample preparation and MS analysis. In this work, we have developed a series of pyrene-based LDI reagents (LALDI tags) that can be used for labelling and LALDI-MS analysis of reducing carbohydrates from complex (biological) samples without the need for additional chemical derivatisation or purification. We have systematically explored the suitability of four pyrene-based LDI enhancers and three aldehyde-reactive handles, optimised sample preparation, and demonstrated the use of LALDI tags for the detection of lactose. We have also exemplified the potential of LALDI tags for labelling carbohydrates in biological samples by direct detection of lactose in cow's milk. These results demonstrate that LALDI-MS is a promising technique for the analysis of reducing carbohydrates in biological samples, and pave the way for the development of LALDI-MS for glycomics and diagnostics.

Introduction

Matrix-assisted laser desorption/ionisation (MALDI) is a soft ionisation technique for mass spectrometry (MS), in which analytes are co-crystallised with a suitable matrix that facilitates absorption of laser light as well as transfer of energy and charge to analytes.[1,2] MALDI-MS is a powerful analytical tool for studying high molecular weight biomolecules, such as oligonucleotides, lipids, and glycoconjugates.[3] Because of their low limits of detection and high-throughput capabilities, MALDI-MS instruments are increasingly being exploited as platforms for glycomics, proteomics and point-of-care diagnostics.[4–7] Despite the advantages and widespread use of MALDI-MS, the presence of a matrix also brings with it several disadvantages: analytes need to be able to mix and co-crystallise with the matrix, co-crystallised samples can contain significant inhomogeneities ("hot spots") leading to challenges with reproducibility and quantification, and matrix peak charge-sharing clusters can obscure low-molecular-weight (below ~800–1000 Da) analytes.[8] Moreover, because of the indiscriminate desorption/ionisation of species present in the matrix, contaminants such as salts, detergents, or more-readily ionised species can result in complicated spectra and analyte signal suppression, meaning purification of analytes may be needed before MALDI-MS analysis.[9] Analysis of biological species can be especially difficult because reagents commonly used in biological studies, such as buffers, salts and detergents, can affect both co-crystallisation and ionisation.[10] High salt concentrations can completely obscure or suppress analyte ionisation, necessitating the use of volatile buffers or sample clean up prior to MALDI-MS analysis. These limitations have prompted the development of various matrix-free LDI-MS approaches.[11] A particularly promising approach is label-assisted laser desorption/ionisation mass spectrometry (LALDI-MS), in which chemical tags (LDI enhancers) cause the selective desorption/ionisation from complex mixtures of the analytes to which they are attached, without the need for purification or an external matrix.[11]

Pyrenes have proven particularly suitable as LDI-enhancing tags. Amano et al. analysed oligosaccharides and glycopeptides derivatised with pyrenebutanoic hydrazide or 1-pyrenyldiazomethane by MALDI-MS.[12–15] In these experiment, analytes were first purified and then labelled in organic solvent on the MALDI target plate, before co-crystallisation with a matrix. In contrast, Kozmin et al. demonstrated the *direct* detection of pyrene-labelled small molecules, as their stable (radical) cations, by

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matrix-free LDI in a MALDI-MS instrument equipped with a standard 355-nm laser.[16] This enabled high-throughput reaction discovery by LALDI-MS, in which nanomoles of the pyrene-labelled substrates and products could be detected selectively against a complex background of reagents, catalysts and additives upon evaporation of the reaction solvents. Subsequently, the group of Basak demonstrated the use of pyrene-based reagents in LALDI-MS to detect zinc ions,[17] *cis*-1,2-diols and catechols,[18] and biogenic amines,[19] Kita, Kigoshi and co-workers used LALDI-MS to analyse tryptic peptides,[20–22] and Guo and co-workers described pyrene-based peptide probes for the measurement of protease activity.[23]

Carbohydrates are the most ubiquitous and structurally diverse group of biomolecules, and their detection and analysis by mass spectrometry is notoriously difficult.[24–27] Unlike proteins and nucleic acids, the biosynthesis of carbohydrates is not template-driven or under transcriptional control. Therefore, artificial amplification of a specific carbohydrate cannot be achieved and ultimately restricts the available material to the often minute heterogeneous quantities obtained from natural sources.[26] Compared to peptides, carbohydrates are not easily ionised. To circumvent this issue, chemical derivatisation, such as permethylation[28] or, less commonly, the addition of reducing-terminal functional groups[29] is often employed to promote ionisation, enable purification and/or separation, and improve the stability and MS analysis of glycans.[24] Such approaches have led MALDI-MS to be widely used for analysis and quantification of carbohydrates such as *N*- and *O*-glycans.[25,29–32]

We hypothesised that LALDI-MS could be a suitable method for the direct detection of carbohydrates from complex biological samples. Such an approach would require the in situ derivatisation of carbohydrates (i.e., in the sample itself rather than after isolation of carbohydrates) with suitable LDI enhancers, which we call LALDI tags (Figure 1). Herein, we report the development of a series of pyrene-based LALDI tags for the labelling of reducing carbohydrates in aqueous (biological) samples, and their subsequent detection by LALDI-MS. We have synthesised and characterised four different pyrene-based LDI enhancers, and optimised sample preparation, in order to achieve reproducible LALDI-MS measurements with good limits of detection. In addition, we have explored the use of different bioorthogonal handles for the labelling of a reducing carbohydrate, lactose. Using LALDI-MS, the resulting pyrene-tagged lactose derivatives could be detected in complex aqueous samples without the need for purification, desalting or derivatisation. As a proof-of-concept, we have exemplified the use of one LALDI tag for the direct detection (after in situ derivatisation) of lactose in cow's milk. This work highlights the potential of LALDI tag technology, and lays out a number of important principles for the design of LALDI tags and LALDI-MS experiments to enable future application in the detection of biomolecules in complex samples.

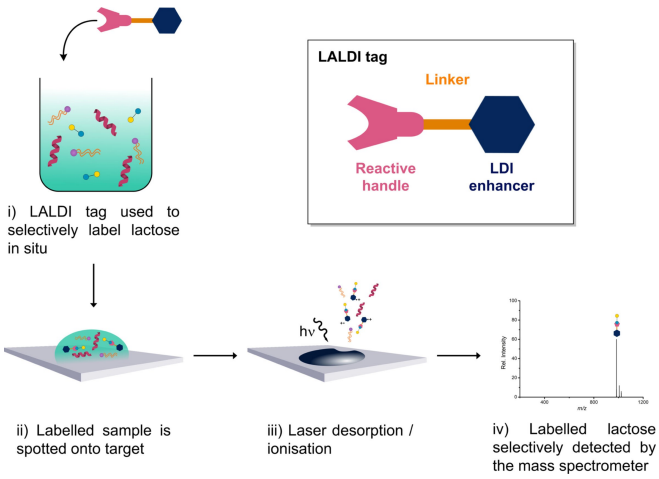


Figure 1. Concept: LALDI tags as a tool for MS-based carbohydrate detection and analysis. The figure shows the general workflow for the analysis of carbohydrates in a biological sample (in this study: lactose in cow's milk) using LALDI-MS. The inset shows the general LALDI tag design: an LDI-enhancing label (dark blue) to facilitate matrix-free laser desorption/ionisation, a linker (orange) to improve the aqueous solubility of the tag, and a reactive handle (pink) that provides a quick and reliable method to selectively label carbohydrates.

Results

Development of pyrene-based ionisation enhancers

In order to develop LALDI tags with good sensitivity and stability in LALDI-MS that would allow labelling reactions under aqueous conditions, we first considered the nature of the pyrene group and its linkage to a solubilising oligo(ethylene glycol) (OEG) linker. Most previous LALDI-MS studies used LDI enhancers based on pyrene butyramide (PyBA).[16,18,19,23] In addition, Yoneda et al. reported that compounds based on 6-amidopyrene (6-APy) have increased absorption at 355 nm (the wavelength of lasers commonly used in MALDI instruments), and that some 6-APy compounds could be observed by LALDI-MS with limits of detection down to 10 pmol.[20] However, the 6-APy tag readily fragmented to lose ketene, and the work suggested that careful design of linker chemistry is essential to avoid complex fragmentation patterns in LALDI-MS or subsequent tandem MS analysis.[20] Therefore, we designed and synthesised four different pyrene-OEG conjugates for analysis in LALDI-MS. Two were based on the previously reported PyBA (**1**) and 6-APy (**2**) moieties, and two on the novel 1-amidopyrene (1-APy; **3**) and pyrene urea (PyU; **4**) moieties (Figure 2a). The synthesis, purification and chemical analysis of these compounds are described in the *Supporting Information*. Compounds **1** and **2** exhibited UV/vis absorption profiles identical to those reported by Yoneda et al., with 6-APy derivative **2** exhibiting a bathochromic shift of the pyrene absorption band compared to PyBA derivative **1** (Figure 2b). 1-APy derivative **3** and PyU derivative **4** exhibited similar bathochromic shifts to **2**, consistent with the extended conjugation of compounds **2**–**4** compared to **1**. In addition, this shift was absent in an *N*-

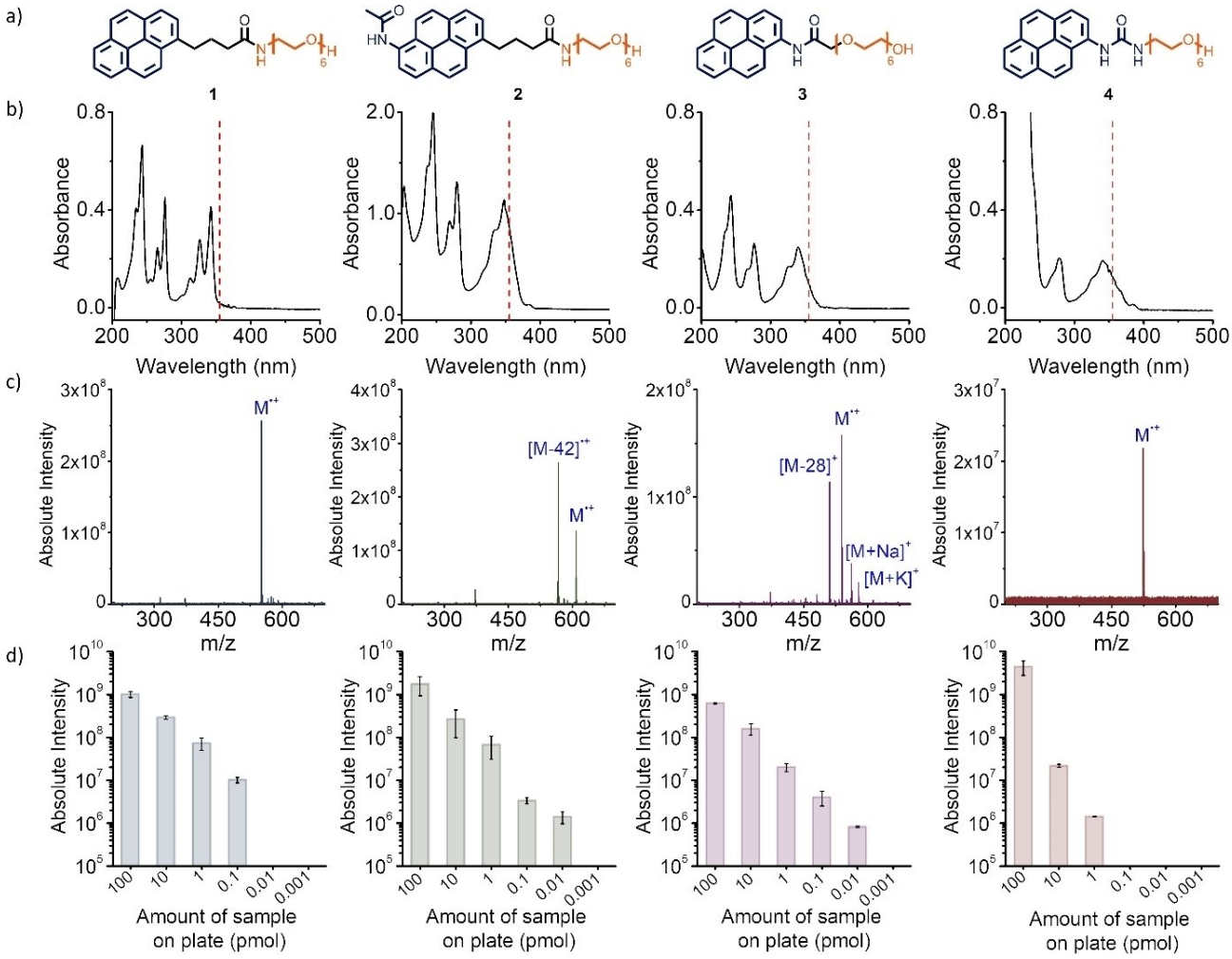


Figure 2. Performance of four different pyrene derivatives in LALDI-MS experiments. a) Structures of water-soluble pyrene-based LALDI reagents. b) UV/vis absorption spectra for compounds **1**–**4**. The red hashed line indicates the excitation wavelength applied in the LDI-MS instrument (355 nm). Note that the UV/vis spectra of pyrenes in the solid state have been reported to display broad absorption bands < 391 nm.[22,36] c) LALDI-mass spectra of compounds **1**–**4** (10 pmol samples). d) Determination of the limits of detection of compounds **1**–**4** by LALDI-MS. Bars represent the mean values for the sum of all positive mode ions related to each LALDI reagent, including the molecular ions ($M^{\bullet+}$), cationised molecules ($[M+Na]^+$, $[M+K]^+$), and major fragments (**2**: [*M*-ketene]$^{\bullet+}$, **3**: $[M-CO]^{\bullet+}$) detected by LALDI-MS of decreasing sample amounts. Error bars represent the standard deviation, where $n = 3$ (analysis of three individual spots from the same compound solution).

methylated variant of **3**, compound **S1** (Figure S1 in the *Supporting Information*), suggesting that *N*-methylation results in steric hindrance preventing full conjugation between amide and pyrene.

Compounds **1**–**4** were then analysed by LALDI-MS, comparing their stabilities (i.e., level of in-source fragmentation), lower limits of detection and ease of spectral interpretation. A 9.4 T (Bruker solariX XR) high-performance MALDI-FT-ICR mass spectrometer was used, allowing unambiguous assignment of elemental formulae of molecular ions, cationised molecules, and decomposition products with low- or sub-ppm mass accuracy (Figure 2c,d).[33] Compounds were dissolved in methanol, a known amount of each sample was spotted onto a target plate, and solvent was allowed to evaporate, resulting in films containing 1 fmol–100 pmol of each compound on the target plate. LALDI-MS analysis was performed on these films without addition of any other reagents, matrices or surface materials, with three independent spots measured for each sample (see the Experimental Section for details). Measurements were initially performed using samples spotted onto standard ground steel MALDI target plates, but low shot-to-shot reproducibility was obtained (Figure S2a). This was most likely the result of heterogeneities in the sample films. Indeed, scanning electron microscopy analysis of the sample films revealed the formation of 'coffee ring' patterns and accumulation of analytes in grooves on the target plate upon sample drying (Figure S2b,c).[34] Instead, use of a micro-focusing target plate designed to localise hydrophilic samples onto a smaller area[35] resulted in LALDI-MS measurements with improved limits of detection of 10 fmol–10 pmol, excellent shot-to-shot reproducibility, and good correlation between amount of analyte and signal intensity (Figure 2c,d). Therefore, this micro-

focusing target plate was used for all subsequent LALDI-MS experiments.

LALDI-mass spectra of compounds **1**–**4** all showed the radical cations ($M^{\bullet+}$) as the main peaks (Figure 2c). In many experiments, sodiated and potassiated molecules ($[M+Na]^+$, $[M+K]^+$) were detected as well, most likely as a result of salts in the solvents, reagents or potentially also on the target plate. Because we developed LALDI tags for analyses from biological samples containing high salt concentrations, no attempts were made to suppress the detection of these cationised molecules. 6-APy **2** and 1-APy **3** achieved favourable lower limits of detection of 10 fmol (Figure 2c,d). However, both compounds also exhibited some in-source fragmentation. For 6-APy **2**, a peak was observed at $[M-42]^{\bullet+}$ correlating with fragmentation at the terminal amide and loss of ketene, consistent with reports by Yoneda et al.[20] For 1-APy **3**, a peak was observed at $[M-28]^{\bullet+}$, which was not present in the synthetic product **3** according to LC–MS analysis. Accurate mass LALDI-MS (Figure 2) and product ion analysis (Figure S3), in combination with LALDI-MS analysis of a minimal 1-APy analogue **S2** (Figures S4–S6; which also displayed $[M-28]^{\bullet+}$ peaks), revealed that these signals were the result of the loss of CO. Collision-induced dissociation (CID) product ion analysis of the molecular ion of 1-APy **3** resulted in multiple expected product ions arising from fragmentations along the OEG and amide linkers (Figure S3a). However, the $[M-28]^{\bullet+}$ peak observed in LALDI mass spectra of 1-APy **3** was absent in the CID product ion spectrum of the molecular ion of **3**, suggesting that the loss of CO occurs in the MALDI source. *N*-Methylated 1-APy derivative **S1** displayed reduced LDI source stability compared to 1-APy **3**, with an increased relative abundance of $[M-28]^{\bullet+}$ and additional fragmentation observed in the LALDI mass spectrum (Figure S1). PyBA **1** and PyU **4** displayed little in-source fragmentation and so excellent stability, and could be detected down to 100 fmol and 1 pmol, respectively (Figure 2c,d). Comparison of compounds **1**–**4** in Table 1 suggests that 6-APy **2** and 1-APy **3** have the most favourable limits of detection, while PyBA **1** and PyU **4** have the highest stability. Because 1-APy derivatives are more stable (no loss of ketene) and easier to synthesise than 6-APy derivatives, and because PyBA derivatives can give complex fragmentation patterns in tandem MS analysis,[20] LDI enhancers **3** and **4** were selected for the development of LALDI tags for carbohydrate analysis.

Table 1. Summary of the performance of LDI enhancers **2**–**4** to PyBA **1** in LALDI-MS experiments. PyBA, pyrenebutyramide; 6-APy, 6-amidopyrene; 1-APy, 1-amidopyrene; PyU, pyrene urea.

LDI enhancer	MS stability	Limit of detection [pmol]
PyBA 1	no major fragmentation	0.1
6-APy 2	additional peak at $[M-42]^{\bullet+}$	0.01
1-APy 3	additional peak at $[M-28]^{\bullet+}$	0.01
PyU 4	no major fragmentation	1

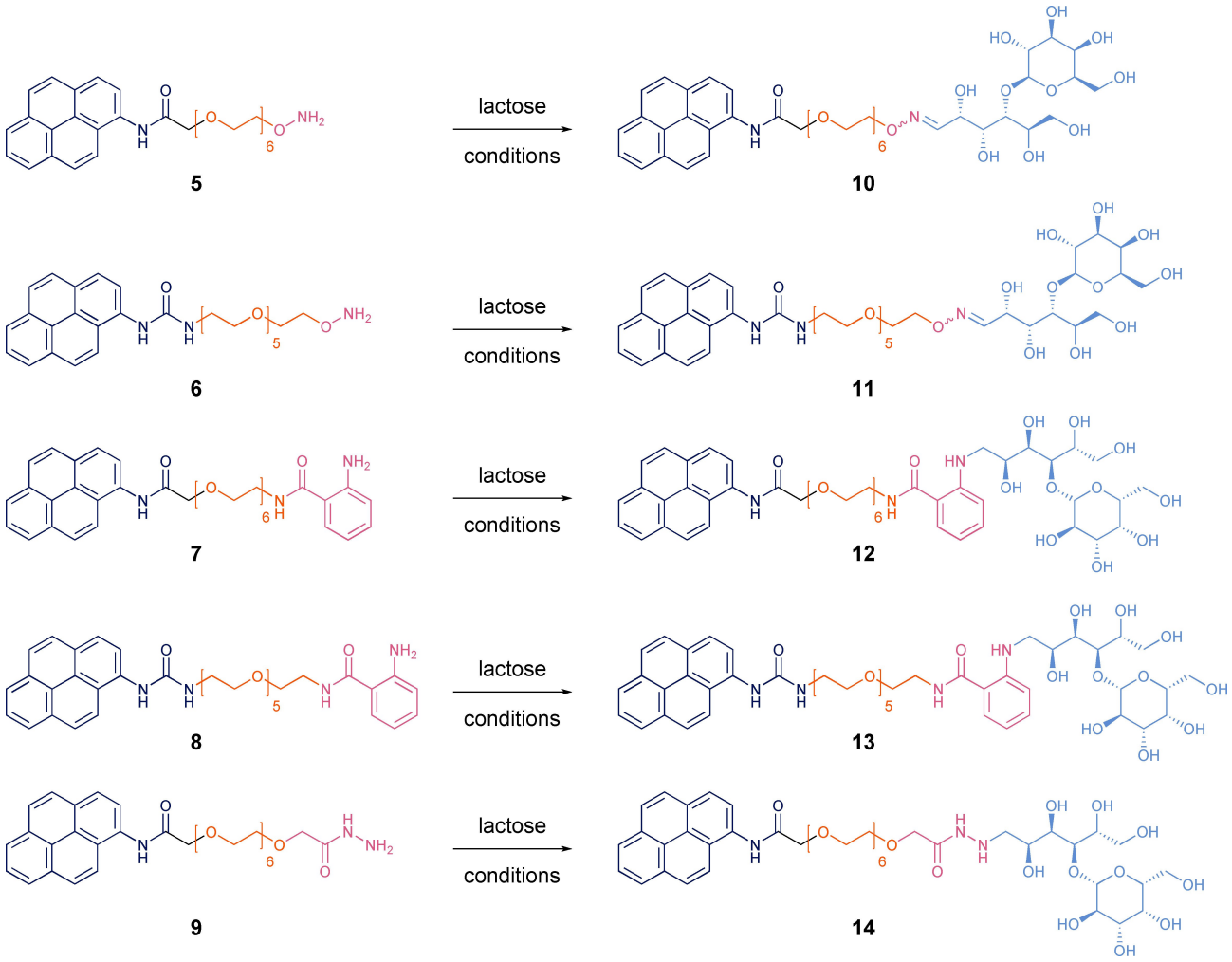
Development of LALDI tags for reducing carbohydrates

Labelling of reducing carbohydrates, such as *N*- or *O*-glycans released through enzymatic[37,38] or chemical cleavage techniques,[39,40] would require a suitably reactive handle to allow efficient, chemoselective formation of a stable conjugate product in the presence of a complex biological matrix. Inspired by common glycan labelling strategies, reviewed in Ref. 25, three reactive handles were chosen: hydroxylamine,[41–44] hydrazide,[45] and 2-aminobenzamide (2-AB).[46–48] Five unique LALDI tags were synthesised, combining the two selected LDI enhancers **3** and **4** and the three reactive handles through their solubilising linker (compounds **5**–**9**, Table 2). The synthesis, purification and chemical analysis of these LALDI tags are described in the *Supporting Information*. Compounds **6**, **8** and **9** were analysed by LALDI-MS to determine whether their detection and stability would be affected by the three different reactive handles (Figures S7–S9; Table S1). Analysis of these unreacted LALDI tags would also provide insight relevant for the analysis of in situ labelling reactions, allowing distinctions to be made between signals relating to the products, unreacted LALDI tags, and other signals in the spectra. Each of the reagents was detected by LALDI-MS, but the compounds (especially **9**) underwent more in-source fragmentation than the simple LDI enhancers **1**–**4**. This is most likely because of the reactivity of the handles, and the instability of *N*–*O* and *N*–*N* bonds that we observe in our LALDI-MS experiments (see, e.g., Figure S10) consistent with previous reports of *N*–*O* bond fragmentation in LALDI-MS.[20]

LALDI tags **5**–**9** were subsequently used for the labelling of lactose, which was considered an ideal model for the labelling reaction and LALDI-MS analysis because it is readily available, and because small glycans such as lactose can be difficult to detect by MALDI-MS without further chemical modifications (reviewed in Ref. 29), or the use of additives[49,50] or specialised nanoparticle supports.[51–53] Lactose was labelled with each of the five LALDI tags **5**–**9**, with high conversion assessed by LC–MS analysis (Table 2). The pyrene-labelled lactose derivatives **10**–**14** were purified by preparative HPLC to enable analysis of known quantities of well-defined analytes, in order to study performance of **10**–**14** in LALDI-MS analysis. According to analytical HPLC, compounds **10**–**13** were analytically pure (Figures S49–S52), while compound **14** contained contaminants resulting from the labelling reaction that could not be removed (Figure S53). LALDI-MS analysis was carried out on these samples (Figure 3; Table 2).

LALDI-MS analysis of **10**–**14** resulted in reproducible detection of pyrene derivatives (Figures 3 and S10–S15). Lactose oxime derivatives **10** and **11**, and the 2-AB-linked lactose derivatives **12** and **13** gave signals with good signal-to-noise ratios (Figures 3a,c-e and S10–S13). However, analysis of the hydrazide-linked lactose derivative **14** did not yield signals for the expected products; instead the spectrum was dominated by signals for low-molecular-mass species, presumably produced on in-source fragmentation occurring predominantly around the hydrazide linkage (Figures 3f and S14). Consistent with analysis of simple 1-APy LDI enhancer **3** (see above), loss of CO

Table 2. The use of LALDI tags **5**–**9** to detect lactose by LALDI-MS.



LALDI tag	Ratio LALDI tag:lactose	Reaction	Reaction conditions [a]	Product	% Label converted [b]	Molecular ion detected in LALDI-MS?	MS stability [c]	Limit of detection [pmol]
5	1:5	oxime ligation[54]	i	**10**	100	yes	+/−	1
6	1:5	oxime ligation[54]	i	**11**	100	yes	+	10
7	1:2	reductive amination[55]	ii	**12**	92	yes	++	10
8	1:2	reductive amination[55]	ii	**13**	89	yes	++	10
9	4:7	reductive hydrazination[56]	ii	**14**	64	no	−−	n.a.

[a] Reaction conditions: i) 3,5-diaminobenzoic acid, MeCN/sodium citrate buffer (0.1 M, pH 3, 1:1), RT, 24 h; ii) $NaBH_3CN$, DMSO/AcOH (7:3), 60 °C, 2 h. Additional details of the reaction conditions, purification and chemical analysis of the labelled lactose are given in the *Supporting Information*. [b] Conversion was calculated by comparing integrals of UV absorbance peaks for starting materials **5**–**9** and products **10**–**14** in the LC–MS analysis of the reaction mixture. [c] MS stability was assessed from i) the number of ions observed, in addition to the molecular species, on LALDI-MS analysis of each analyte, and ii) the relative abundance of the signals corresponding to the molecular ion ($M^{\bullet+}$) and cationised molecules ($[M+Na]^+$, $[M+K]^+$) compared to the additional ions (presumably fragment ions), then scored using an arbitrary scale from ++ to −−.

was clearly detected from 1-APy-labelled lactose derivative **10** (Figures 3a and S10). Loss of CO was also observed during analysis of 1-APy-labelled lactose **12**, although only low intensity signals for $[\mathbf{12}–CO]^{\bullet+}$ were observed (Figure S12). Both oxime-linked lactose derivatives **10** and **11** displayed in-source fragmentation at the oxime linkage (Figures 3a,c, S10 and S11). 2-AB linked lactose derivatives **12** and **13** were clearly detected as $[M+Na]^+$ (Figure 3d,e); only low intensity fragment ions – presumably arising on fragmentation around the benzamide and secondary amine groups – were observed (Figures 3d,e, S12 and S13). 1-APy **10** was found to have the lowest limit of detection, observed down to a sample size of 1 pmol, while

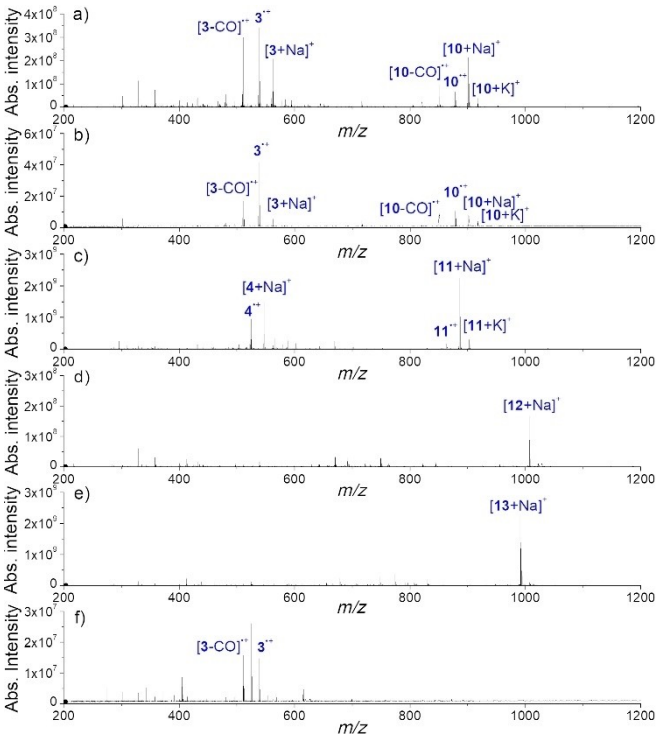


Figure 3. LALDI mass spectra for samples of LALDI-tagged lactose derivatives (100 pmol) with molecular ions, cationised molecules, and identified fragments. a) **10** (from MeOH); b) **10** (from HBSS); c) **11** (from MeOH); d) **12** (from MeOH); e) **13** (from MeOH); f) **14** (from MeOH). HBSS = Hanks' balanced salt solution. For a comparison of absolute signal intensities of **10**–**14** from MeOH, see Figure S15. For a comparison of LALDI mass spectra of **10**–**13** from HBSS, see Figure S16.

labelled lactose derivatives **11**–**13** were found to have a lower limit of detection of 10 pmol (Table 2). No lower limit of detection was determined for lactose hydrazide derivative **14** as neither the molecular ion nor cationised molecules were detected at any sample quantity. From these results, it was concluded that pyrene-labelled lactose compounds **12** and **13** were the most stable in LALDI-MS analysis (Table 2), while compound **10** resulted in the lowest limit of detection (while displaying *N*–*O* bond cleavage).

Use of LALDI-MS for the selective detection of lactose in complex sample matrices

To test the detection of LALDI-tagged lactose derivatives in a complex sample matrix, LALDI-MS analysis of **10**–**13** was repeated with the samples dissolved in the cell culture buffer Hanks' balanced salt solution (HBSS), which contains various buffer salts ($[Na^+]$ 142 mM, $[K^+]$ 5.80 mM, $[Mg^{2+}]$ 0.898 mM, $[Ca^{2+}]$ 1.26 mM), glucose (5.55 mM), and a phenol red indicator (26.6 μM). An image of the evaporated sample of LALDI-tagged lactose **10** in HBSS, captured by the mass spectrometer's target camera, is shown in Figure S16b. The crystalline appearance of the sample is consistent with the high abundance of buffer salts present in the samples analysed during these experiments. Each of the pyrene-labelled lactose derivatives **10**–**13** was clearly detected in the presence of HBSS, with the spectra obtained (Figures 3b and S16a) being nearly identical to those obtained from pure samples (Figure 3a,c–f). While the absolute signal intensities for samples in HBSS were generally lower than for samples in MeOH (Figures 3 and S16a), limits of detection were not affected (Tables 2 and S2). These results highlight the advantage of the LDI-enhancing label in selectively promoting ionisation and MS detection of only the labelled species, even in the presence of buffer, contaminants and high concentrations of salts.

After establishing that pure LALDI-tagged lactose derivatives could be selectively detected by LALDI-MS, we set out to exemplify the use of LALDI tags to label reducing carbohydrates directly in a biological sample. For this proof-of-concept, we chose cow's milk as the biological sample because of its high lactose concentration (4–5% *w*/*w*) and complex matrix;[57] if pyrene-based LALDI tags acted as a general MALDI matrix in this experiment, the abundance of lipids and proteins would be expected to cause interference. LALDI tag **6** was selected because the oxime ligation had been used successfully to label lactose in aqueous media (Table 2) and because the higher in-source stability of its PyU moiety compared to the 1-APy in LALDI tag **5** (Table 1 and Figure 3). Following labelling of a sample of cow's milk using LALDI tag **6** under oxime ligation conditions, the reaction mixture was diluted and centrifuged to remove curd. The supernatant was transferred by pipette to the MALDI plate and analysed directly by LALDI-MS (Figure 4), giving notably clean LALDI mass spectra displaying peaks corresponding to lactose derivative **11** (*m*/*z* 896.3591 $[M+Na]^+$ and 902.3328 $[M+K]^+$). The most intense peak (*m*/*z* 524.2518) was assigned as a fragment ion (**11'**) resulting from *N*–*O* bond cleavage. This fragment could also have been generated through fragmentation of the hydroxyl amine-functionalized LALDI tag **6** (*N*–*O* bond fragmentation was observed during LALDI-MS analysis of PyU-OEG-ONH_2 **6**; Figure S7), but it should be noted that the amount of **6** added to the milk sample was less than the estimated amount of lactose present, and that ions corresponding to intact **6** were not detected in this LALDI-MS experiment. Different LALDI tags and in situ labelling conditions need to be explored in future studies to reduce in-source fragmentation and increase signal intensity (see Discussion), and to develop the technology for the detection of low-abundance carbohydrates. However, these results highlight the potential of LALDI tags to be used for the labelling and analysis of reducing carbohydrates directly in complex biological samples. Furthermore, it is important to note that detection of only pyrene-containing molecules indicates that, at the concentrations tested, the LDI enhancers did not act as a matrix for the many other non-carbohydrate species present in the samples.

Discussion

We have developed a set of novel LDI enhancers, exhibiting properties including compatibility with aqueous media, low

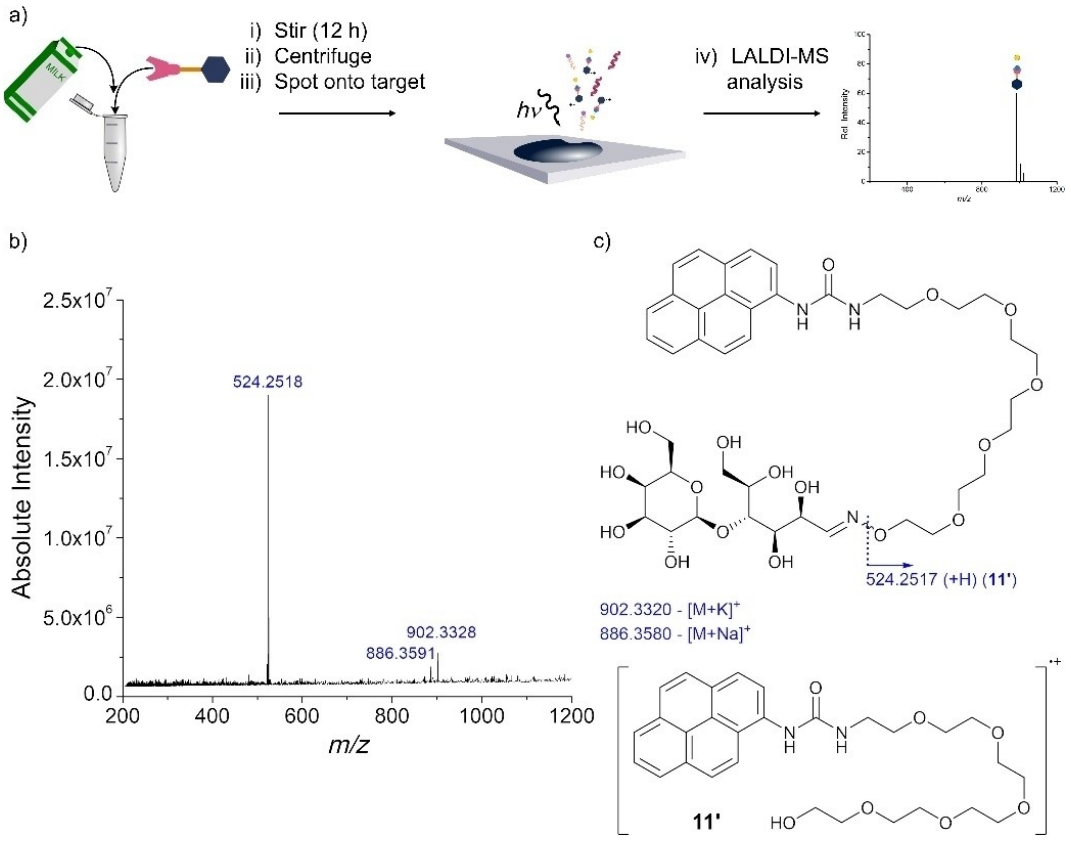


Figure 4. LALDI-MS analysis of milk following glycan labelling with LALDI tag **6**. a) Schematic representation of the general workflow for in situ labelling of lactose in milk and subsequent LALDI-MS analysis. b) LALDI mass spectrum obtained following direct LALDI-MS analysis of the labelling reaction mixture; identified fragments and molecular species are annotated with their observed *m/z* values. c) Chemical structures for LALDI tag-labelled lactose **11** and proposed major fragment ion **11'**. Proposed assignments for fragments and molecular species observed in the LALDI mass spectrum in (b) are annotated in the structure of **11**, accompanied by their corresponding calculated monoisotopic masses.

limits of detection, good stability, and predictable MS fragmentation. We introduced two new LDI enhancers, 1-APy and PyU, derivatives of which can be readily synthesised from commercially available 1-aminopyrene. By studying the behaviour of samples on different target plates, we optimised both limits of detection and reproducibility of LALDI-MS measurements. 1-APy **3** displayed lower limits of detection than PyBA derivative **1**, and higher stability than 6-APy derivative **2** in LALDI-MS experiments. In contrast, PyU **4** showed no in-source fragmentation but had higher limits of detection. By combining pyrene-based LDI enhancers with handles for tagging reducing carbohydrates, we were able to develop a range of LALDI reagents capable of labelling and detecting reducing carbohydrates (in this work: lactose) by LALDI-MS. We have demonstrated that LALDI tagging can be performed in aqueous media (including cow's milk), and that pyrene-tagged lactose derivatives can be detected by LALDI-MS in complex (biological) matrices (HBSS and cow's milk) without the need for further purification, chemical modification, or additional MALDI matrices.

The correlation between increased UV absorption at 355 nm and (slightly) improved limit of detection in LALDI-MS experiments displayed by 6-APy **2** and 1-APy **3** compared to PyBA **1** is consistent with results reported by Yoneda et al. However, the improvement in limits of detection in our experiments was modest. Moreover, PyU **4** was found to have a higher limit of detection than PyBA **1**, despite displaying a similar bathochromic shift to **2** and **3**. These results emphasise that factors additional to optical properties of the pyrene tag (e.g., ease of ionisation, stability of molecular ion and cationised molecules, stability to in-source fragmentation) are also likely to influence limits of detection in LALDI-MS. It should also be noted that, in LALDI-MS, samples are analysed from a dried spot, not as solutions. Sharma et al. demonstrated that, in solution, the UV absorption profile of pyrene is identical to that of PyBA derivative **1**, while solid pyrene exhibits a broader and red-shifted UV absorption profile with much higher absorbance at 355 nm.[36] They proposed that the observed change in absorbance was due to increased π-stacking interactions in solid pyrene. Therefore, while information gained on the solution phase UV absorbance of **1**–**4** was useful for comparison of our compounds to previously reported pyrene-based LDI enhancers, it may not accurately represent the absorbance of an LDI enhancer during a LALDI-MS experiment. Indeed, Kigoshi, Kita et al. recently demonstrated that LDI enhancers based on differently substituted pyrenes (including PyBA, 6-APy

and a new, highly sensitive *N,N*-dimethylaminopyrene) all displayed a broad absorbance band <391 nm when UV/vis was performed on solid samples.[22] The authors observed that the sensitivity of three pyrene derivatives in LALDI-MS was inversely correlated with their fluorescence quantum yield, suggesting that performance of LDI enhancers is related to their heat emission. This observation should be taken into account in the design of future generations of LALDI tags with increased LALDI-MS sensitivity.

LALDI-MS was preceded by the development of fluorophore-assisted laser desorption/ionisation mass spectrometry (FALDI-MS),[58] in which fluorescent dyes acted as the LDI enhancers. In addition, West et al. demonstrated that 2-aminobenzamide, a common fluorescent label for reducing carbohydrates, could be used for the FALDI-MS detection of the oligosaccharide maltoheptaose.[59] However, the resulting spectrum featured unidentified higher mass signals and analysis required a relatively large amount of sample (~157 nmol). In addition, the fluorophores appeared to act as weak matrices, ionising impurities and species other than the carbohydrate analytes, thereby generating more complicated spectra than was the goal. In contrast, our results suggest that pyrene derivatives do not act as a general MALDI matrix under the conditions tested in this work, and result in ionisation of only covalently labelled species even when analysed against complex (biological) backgrounds.

Our first generation LALDI tags for the labelling and detection of reducing carbohydrates incorporate reactive handles that are commonly used for carbohydrate derivatisation. LALDI tags with a hydroxylamine or 2-AB reactive handle were successfully used to label and detect lactose. The selection of LALDI tags for specific (biological) sample application will depend both on the compatibility of the samples with labelling conditions (e.g., solvent and pH) and the required stability: the 2-AB linked products displayed higher in-source stability than oxime-linked products, with the latter undergoing *N–O* fragmentation. The successful in situ labelling and detection of lactose in cow's milk suggests that LALDI tags can indeed be used for detection of carbohydrates in biological matrices, but it should be noted that carbohydrate ligation conditions need to be optimised further for the specific biological matrix. In addition, enrichment may be needed for the LALDI-MS analysis of less abundant carbohydrates. Interestingly, recent reports suggest that the affinity of pyrene derivatives for materials such as polystyrene[23] and TSK-G3000S gel (styrene-divinylbenzene copolymer)[21,22] might allow specific affinity enrichment of LALDI-tagged carbohydrates from complex mixtures before LALDI-MS analysis, which could be used to significantly improve the limit of detection.

Through this investigation we have gained a better understanding of how the structure and chemical functionality of LDI enhancers, linkers, and reactive handles affect the limits of detection and in-source stability of reagents analysed by LALDI-MS. Our findings highlight LALDI tags as promising reagents for the detection/analysis of carbohydrates as well as other biomolecules from complex biological environments. Important for glycomics and activity-based profiling approaches, the reproducibility and concentration-dependence of the LALDI-MS signal intensity may additionally allow (semi-)quantitative measurements, for example through careful external calibration or internal calibration using isotopically labelled internal standards.

Experimental Section

Synthesis and characterization

Synthetic procedures, compound characterisation and relevant spectra of final compounds are available in the *Supporting Information*.

UV/vis analysis

UV/vis absorption was measured using an Agilent Technologies Cary 100 UV-Vis Spectrophotometer. All samples were analysed as solutions in a 10 mm Hellma Analytics High Precision Quarts Suprasil cell. Samples for UV/vis analysis were prepared from a 1 mM methanolic solution of the purified analytes, then adjusted to the desired concentration by serial dilution. Absorption maxima (λ_{max} [nm]) are given to the nearest whole nanometre, with the corresponding molar extinction coefficient (ε) given in $M^{-1}\,cm^{-1}$.

LALDI-MS analysis

LALDI-MS was carried out on a Bruker Daltonics solariX XR FTMS 9.4T mass spectrometer with an Apollo II dual ESI/MALDI ion source. All optimised analysis was performed using a Bruker Daltonics MTP AnchorChip Target 384. Ionisation/desorption of the samples was achieved using a fixed wavelength (355 nm) Bruker Smartbeam II (Nd:YAG) laser. *m/z* values are reported to four decimal places to reflect the mass accuracies obtained using external mass calibration with phosphorus red. LALDI-MS fragmentation assignment was carried out with the assistance of a web application developed by ChemCalc that provides a list of possible molecular formulae from a given monoisotopic mass.[60]

Samples for LALDI-MS analysis were prepared from 1 mM solutions of the purified analytes in methanol, which were then adjusted to the desired concentration with either methanol or Hanks' balanced salt solution. A known volume (1 μL) of each analytical sample was spotted onto the target plate and the solvent allowed to evaporate under ambient conditions. Evaporation of aqueous solutions was assisted using reduced pressure. LALDI-MS analysis was then performed directly on each sample (without addition of further reagents such as MALDI matrix or SALDI surface material) with 1600 laser shots per acquisition (8 scans, 200 shots, 200 Hz, medium laser focal size). Each sample was analysed in triplicate (three separate aliquots on separate targets) and the average values of the peak intensities are reported. Mass spectra were acquired using ftmsControl software version 2.1 (Bruker Daltonics) and processed using flexAnalysis software version 3.0 (Bruker Daltonics).

In situ labelling of lactose from milk with hydroxylamine LALDI tag 5

3,5-Diaminobenzoic acid (3 mg, 0.02 mmol) was added to a stirred solution of 3-[17-(aminooxy)-3,6,9,12,15-pentaoxaheptadecan-1-yl]-1-(pyren-1-yl)urea **5** (10 mg, 0.02 mmol) in a 1:1 mixture of cow's milk: 0.1 M citrate buffer pH 3 (0.5 mL) and stirred (12 h). The mixture was then diluted tenfold with methanol, subjected to

centrifugation (10000 *g*, 30 s), and 1 μL of the supernatant directly spotted onto an AnchorChip target plate, dried, and analysed by LALDI-MS.

Author contributions

J.R.H. carried out synthesis and characterisation of reagents, and UV/vis and LALDI mass spectrometry experiments. E.T.B. contributed to LALDI mass spectrometry experiments. A.N.K. performed electron microscopy experiments. J.R.H, S.L.W., J.T.-O. and R.S.B. designed experiments. J.R.H. and A.N.K. analysed data. J.R.H. made figures. S.L.W., J.T.-O. and R.S.B. provided project supervision. R.S.B. conceived the project. J.R.H., J.T-O. and R.S.B. wrote the manuscript. All authors commented on the manuscript.

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Conflict of Interests

The authors declare no conflicts of interests.

Data Availability Statement

The authors declare that the data supporting the findings of this study are available within the article and *Supporting Information* file, or from the corresponding author upon reasonable request.

Keywords: carbohydrates · ionisation enhancers · LALDI-MS · mass spectrometry · pyrenes

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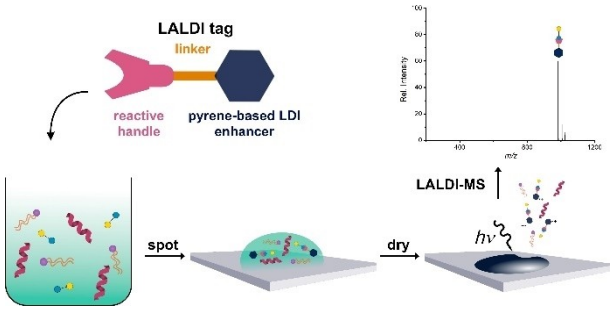

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Pyrene Tags for the Detection of Carbohydrates by Label-Assisted Laser Desorption/Ionisation Mass Spectrometry

Gie it LALDI! The derivatisation of (bio)molecules with laser desorption/ionisation (LDI) enhancers allows their selective detection by label-assisted laser desorption/ionisation mass spectrometry (LALDI-MS). We have developed and characterised pyrene-based LDI enhancers (LALDI tags) for the labelling and LALDI-MS analysis of reducing carbohydrates, highlighting the compatibility of LALDI-MS with complex biological samples.