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**Microchip-ESI-MS Determination of Dissociation Constant:
Lysozyme-NAG3 Complex**

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Keywords:	non-covalent interaction, enzyme kinetics, microfluidic chip, nano-ESI-MS, enzymatic inhibition



1 Microchip-ESI-MS Determination of Dissociation 2 Constant of the Lysozyme-NAG₃ Complex

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15 Aleš Svatoš^{1*}
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49 **Abbreviations:** HEWL, hen egg white lysozyme; K_D , dissociation constant; *N*-
50 acetyl glucosamines (NAGs); PMMA, polymethyl metacrylate
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55 **Keywords:** non-covalent interaction, enzymatic inhibition, enzyme kinetics,
56 nano-ESI-MS, microfluidic chip
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1 Abstract

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8 An on-line microfluidic system for determination of dissociation constants of
9 enzyme/substrate weak interactions by nanoelectrospray ionization mass
10 spectrometry (nanoESI-MS) is introduced. The microchip was designed to
11 permit the enzyme and the substrate to mix by molecular diffusion in a
12 pressure-driven laminar flow. Introduction of reagent solutions into the chip
13 was an optimized combination of a micropump and an autosampler to enable
14 automation of the measurements. The system performance was tested for
15 monitoring of non-covalent interactions of hen egg white lysozyme (HEWL)
16 with *N*-acetyl glucosamine oligomers (NAG_n). Dissociation constant (K_D) of the
17 HEWL-NAG₃ complex was determined by non-linear regression analysis, and
18 the range of K_D values ($39 \pm 6 \times 10^{-6}$ M and $19.6 \pm 8 \times 10^{-6}$ M for manual and
19 automated infusions, respectively) confirms the previously reported values.
20 Such miniaturization of a continuous-flow enzyme assay system to a
21 microfluidic format can maximize the capabilities of mass spectrometric
22 detection; reduce the sample size and analysis time required, as well as the
23 associated costs for on-line enzymatic kinetic studies.
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1. Introduction

Microfluidic devices (planar platforms with microchannel networks) make it possible to direct low amounts of reagents/substrates for analytical or synthetic purposes. These devices enable integration of several preparation and analytical steps, including sample introduction, pretreatment or separation, on an area of few square centimeters, or even their direct integration with various detection methods. In recent years many such devices have been introduced into various fields of analytical chemistry, especially in off chip combination with mass spectrometry.¹

The first coupling of a glass microfabricated device with a mass spectrometer was carried out in 1997 by Karger and coworkers.² These fluidic chips have been used as electrospray ionization interfaces, yielding micro or nanoelectrospray.³ Currently, polymeric microfabricated electrospray chips are being frequently used due to several advantages such as their tunable surface (and the possibility to suppress the adsorption of biochemical analytes or induce specific surface interactions)⁴, the ease with which they can be mass-produced, their low cost, disposability, high flexibility and/or high-throughput capacity.⁵ The first application of microfluidic chips to enzyme assays was described by Ramsey *et al.* for a β -galactosidase assay using resorufin β -D-galactopyranoside, a substrate that was hydrolyzed to resorufin, a fluorescent product.⁶ Enzyme reactions have also been used to determine other substrates, e.g. for glucose detection⁷ and to continuously analyze a DNA restriction enzyme digestion.⁸ Recently, an electrospray micromixer chip was used in combination with on-line electrochemical labeling of cysteins to enhance protein determination.⁹ Furthermore, miniaturization was used¹⁰ for studying a protease continuous-flow mixing assay; the assay's analytical performance for enzymatic studies

1 was fully comparable to the assay performed by classical fluid mixing.¹¹
2 Recently, chips interfaced to electrospray mass spectrometer (ESI-MS) were
3 used to observe non-covalent interactions and to make enzymatic kinetic
4 measurements, leading to a major advance in the study of enzymatic reactions
5 in small volumes directed by fast diffusion.^{12,13}

6 Hen egg white lysozyme, a well-known bacteriolytic agent in hen egg
7 white, catalyzes the hydrolysis of β -1,4-glycosidic linkages in certain gram-
8 positive bacterial cell walls and chitin was used to illustrate the concept. The
9 active site of HEWL (denoted A to F) is a cleft, which is able to bind up to six
10 saccharide residues. The catalytic centre is located between sites D and E.¹⁴ The
11 substrate NAG₆ is cleaved into NAG₄ (substrate of subsequent transformations)
12 and NAG₂ as major products, and into two molecules of NAG₃ (product
13 inhibitor) as minor products (Fig.1).^{15,16} Normally, an assay of such enzymatic
14 reactions require relatively high amounts of enzyme as well as substrate.
15 Determining the kinetic constants is often either time consuming or inaccurate
16 (due to slow diffusion in large volumes and long introduction times into the
17 mass spectrometer).

18 In the following sections we describe a semi-automated microfluidic
19 system based on a polymeric nanoESI-MS chip¹⁷ for studying enzyme kinetics;
20 our system obviates the disadvantages of classical macroscopic set-ups¹¹.

2. Experimental

2.1 Reagents

Hen egg white lysozyme (HEWL, MW 14.7 kDa), the *N*-acetyl glucosamine substrate NAG₃ (MW 627.6 Da), NAG₆ (MW 1237.2 Da), ammonium acetate, and leucine enkephalin were purchased from Sigma Aldrich (St. Louis, MO, USA). The stock solutions (1mM) were prepared by dissolving the corresponding material in a 10 mM ammonium acetate (pH 5.2) buffer. All stock solutions were kept at 4°C prior analysis. Methyl methacrylate (stabilized with 5 ppm hydroquinone inhibitor), benzoin methyl ether (α -methoxy- α -phenylacetophenon) and alumina (Grade CG20) were obtained from Polysciences (Warrington, PA, USA). The aluminium plates used for fabricating the molding spacer were prepared at a local machine shop. Two components, fast setting epoxy (Araldit) and Teflon tape (12×0.08 mm), were from Carl Roth (Karlsruhe, Germany).

2.2 Microfluidic chip layout and fabrication

The fabrication of the microchips followed a modified procedure we reported recently.¹⁷ Briefly, a silicon master was created by soft photolithography/wet chemical etching protocols and used as an in-situ molding tool. The atmospheric molding by radical polymerization was completed in 1h under UV light (365 nm lamp, 8 W, Carl-Roth, 10 cm distance). The polymer chip was demolded by sonication of the casted mold for 10 min in distilled water at 40°C. The edges of the above prepared structured polymethyl metacrylate chip were cut off with a sharp blade and smoothed with a fine wetted polishing paper (P1000 grade, FAS Union, Bielsko-Biala, Poland). A laboratory drill drilled 1.6 mm diameter access ports (Proxxon, model 28510/N, from Carl-Roth) to the arm of mixing

1 chamber. Chips were then cleaned by sonication in propan-2-ol for 1 min and
2 dried under a stream of nitrogen. A two-cm-long, 20 μm ID, 90 μm OD, fused
3 silica capillary serving as a nanoelectrospray emitter was inserted into the chip
4 channel (ca 60 μm diameter) outlet (with 1 cm protruding from the chip
5 channel, 1 cm lying in it). The PMMA chips with inserted emitters were then
6 sealed with 125 μm thick PMMA cover sheets (Goodfellow, Huntington, UK).
7 Chips and the sheets were sandwiched between two glass plates, clamped
8 together and annealed in a convection oven for 10 min at 108°C. The sealed
9 chips were removed from the clamps after slow cooling to laboratory
10 temperature. Three 2.5 cm long elastic Tygon tube pieces (2 mm OD, 250 μm
11 ID, Carl-Roth, Karlsruhe, Germany) were placed into the drilled access ports to
12 create the interface between the inlet capillaries and the microfluidic network
13 (Fig.2). Epoxy was used to seal the outside of the Tygon tubes to the upper
14 surface of the chip. The elasticity of the tubes together with the selected
15 dimensions ensured a reliable internal seal and minimal dead volume, when the
16 silica capillaries were inserted down the bottom of the chip. A 2-cm-long, 300
17 μm OD copper wire was used to establish high voltage contact to the system
18 using the third access port (Fig.2 C).

20 **2.3 Microchip-ESI-MS Coupling**

21 HEWL (10 μM) in ammonium acetate buffer (10 mM, pH 5.2) was injected
22 through syringe pump 1 (Infusion pump 22, Harvard Apparatus, Holliston, MA,
23 USA) at a flow rate of 0.5 $\mu\text{L}/\text{min}$ to reservoir A (Fig.2a,b). Various
24 concentrations of NAG₃ (10, 20, 40, 80, 160, 250, and 300 μM) in ammonium
25 acetate buffer (10 mM, pH 5.2) containing the internal standard (leucine
26 enkephalin, 5.4 μM) were injected at the flow rate of 0.5 $\mu\text{L}/\text{min}$ to reservoir B
27 through syringe pump 2. Electrospray voltage was applied through reservoir C

1 (Fig.2). For automated infusions, an Agilent 1100 μ -Well plate autosampler and
2 1100 capillary pump were used (Agilent, Palo Alto, CA, USA). HEWL (15 μ M)
3 and in ammonium acetate buffer (10 mM, pH 5.2) were pumped at a flow rate
4 of 0.3 μ L/min through channel A. Various concentrations of NAG₃ [7.5, 15, 30,
5 45, 60, and 120 μ M in 10 mM ammonium acetate buffer, pH 5.2 containing
6 leucine enkephalin internal standard (7.5 μ M)] were injected by the autosampler
7 pumped by part of the capillary LC setup (Capillary pump 1100, Agilent) at 0.6
8 μ L/min to channel B (Fig.2c). An LTQ linear trap mass spectrometer (Thermo
9 Fisher Scientific, San Jose, CA, USA) was used to acquire mass spectra in
10 positive enhanced full scan mode in the range 400-4000 Da. The spray voltage
11 was set to 3.8 kV or 1.8 kV for manual and automated infusions, respectively,
12 the tube lens was 250 V, and the capillary temperature was adjusted to 160 °C
13 with an ion injection capillary voltage of 4 V.

14 15 **2.4 Enzyme-substrate Mixing**

16 The mixing of enzyme with the substrate solutions was studied by fluorescence
17 microscopy. Twenty μ M rhodamine B (Sigma) in an aqueous solution of 10
18 mM ammonium acetate, pH 5.2, and a blank buffer were infused from the
19 channel inlets (Fig.2a and b) simultaneously at a flow rate of 0.5 μ l/min. The
20 channel inlet for the high voltage (C) was blocked by a thin metal wire during
21 the experiment. The images (325 x 25 μ m) were taken from one half channel
22 width parallel to the flow and saved in a TIFF format. An upright microscope
23 equipped with 43HE DS red filter (Axioskop 40, Carl Zeiss, Jena, Germany) set
24 to 550 nm excitation and 605 nm emission wavelengths, respectively, was used
25 to evaluate the fluorescence signal. The intensity of pixels was normalized over
26 the 0-255 range using the Image J software (<http://www.nist.gov>) and plotted
27 against the distance from the mixing channel inlet (Fig. 4).

2.5 Determination of Dissociation Constants (K_D)

The K_D values were determined from the ratio of the integrated peak areas of mass spectral signals for free protein and protein complex was plotted against initial ligand concentration, and the titration curves obtained were then fitted using recently introduced model.¹⁸ The non-linear regression analysis was performed using the model described by Jecklin and co-workers.¹⁹ The samples were measured in triplicate. Peak areas were integrated using OriginPro (version 8.0) and non-linear regression analysis was performed using SPSS (version 13.0).

3. Results and discussion

Our microchip was first used for qualitative investigation of HEWL-NAG complexes as a nanoESI-MS interface of a linear trap mass spectrometer. The ESI source assembly was slightly modified to accommodate the microfluidic chip in front of the cone and to obtain constant sample spraying during analyses. The 2.5 cm long Tygon tube pieces provided a very good coupling of fused silica capillaries to the chip body due to their elasticity providing minimal dead volumes and accommodating capillaries of different outer diameters. Such elastic coupling has also minimized spatial distortions to the chip in the ESI assembly caused by usual manipulation of system components.

Initially, samples were infused by two syringe pumps through the microchannel ports (Fig.2). The artificial substrate NAG₆ was infused and subsequently cleaved into different NAG oligomers. As a result of the enzymatic reaction, NAG₂ up to NAG₅ protonated ions were observed under native measurement conditions, together with intense peaks of several forms of

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3 1 non-covalent complexes of NAG oligomers formed with HEWL. The mass
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5 2 signal of the free (unbound) 8-times protonated enzyme was detected at m/z
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7 3 1788.0 in the higher mass region of the spectrum. The highest intensities were
8
9 4 obtained for 8-times protonated molecule of lysozyme with NAG tetramer,
10
11 5 trimer, pentamer and the hexamer substrate, respectively (Fig.3, inset). These
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13 6 signals (m/z 1867.2, 1892.2, 1917.5, 1942.7, respectively) arising in the mass
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15 7 spectrum after the interaction of HEWL and NAG oligomers confirm data
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17 8 reported by Denhart and Letzel¹⁵ who analyzed HEWL-NAG complexes after
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19 9 mixing solutions in a simple syringe reactor, and the first experiments with a
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21 10 continuous-flow mixing assay published by Clark and Konermann.²⁰ Our results
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23 11 confirm the findings of these studies, however, the new procedure provides real
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25 12 time measurements with minimal sample consumption (hundreds nanoliters of
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27 13 sample) and highly reproducible data.

30 14 The proof of the concept is demonstrated by quantification of the binding
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32 15 constant of the enzyme-inhibitor (HEWL-NAG₃) non-covalent complex. Since
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34 16 the different enzyme and substrate solutions were hydrodynamically pumped
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36 17 into the chip, it was important to characterize the completeness of mixing within
37
38 18 the time of measurement. Complete mixing must occur before the binding
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40 19 strength of the two binding partners can be quantified.¹⁰ Rhodamine B was used
41
42 20 as fluorescent marker and mixed with sample buffer in the same experimental
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44 21 set-up. The decrease of fluorescence that occurs by diffusion is shown in Fig.4.
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46 22 The total channel length necessary to decrease the fluorescence signal by 50%
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48 23 (i.e. full mixing of both flows) was 2.6 mm, i.e. 5% of the mixing channel
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50 24 length (5.2 cm) ensuring complete mixing of an enzyme and a substrate on the
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52 25 chip.

55 26 Titration experiments of HEWL with different NAG₃ concentrations were
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57 27 carried out for determination of the dissociation constant (K_D) of the HEWL-

1 NAG₃ complex. Various concentrations of NAG₃ containing the internal
2 standard were mixed on-chip with HEWL in ammonium acetate buffer (10 mM,
3 pH 5.2) with the flow delivered from pumps 1 and 2, respectively, providing 5,
4 10, 20, 40, 80, 125, and 150 μM NAG₃; 2.7 μM leucine enkephalin; 5 μM
5 HEWL on chip concentration. The resulting solutions were electrosprayed to
6 the MS inlet.

7 However, syringe pumps may not be optimal for infusing the samples
8 into a microchip, as the performance of a stable nanoelectrospray may suffer
9 upon occurrence of air bubbles in the channels during solvent and sample
10 replacement. Although the microchip was primed with the buffer prior to each
11 experiment to remove any air bubbles trapped inside the system, it was
12 impossible to avoid introducing small air bubbles. Therefore, we have added an
13 automation by using a microflow autosampler to deliver samples onto the chip
14 (Fig.2c). The titration experiments mentioned above were carried out with such
15 an autosampler at various concentrations of NAG₃ (5, 10, 20, 30, 40, 80 μM) for
16 comparison. The concentration of internal standard was kept at 2.5 μM. Each
17 sample was run in triplicate for 35 min. The elution time of the NAG₃ flow
18 injection peak was between 15-16 min (Fig.5a, bold line). A mass spectrum of
19 NAG₃ recorded in the peak maximum is shown in Fig.5b. To demonstrate
20 minimal dead volume of the microchip enzymatic system, a micro tee-piece was
21 employed in place of the microchip and 30 μM of NAG₃ was injected. The
22 chromatogram obtained (shown in Fig.5a; dashed line) is very similar to the
23 elution profile obtained with the microchip. The identical elution times of both
24 peaks prove minimal dead volume of the microfluidic network itself. The
25 relatively broad width of the elution peak can be explained by no chromato- or
26 electrofocusing effect (no stationary phase or electrophoretic separation used to
27 maintain simplicity). A typical mass spectrum from the titration experiments

1 using an automated setup is shown in Fig.6a. Here HEWL solution (5 μM) was
 2 titrated against NAG₃ solution (80 μM) in the presence of an internal standard
 3 and the interaction of the enzyme with the substrate was monitored. Complexes
 4 of the enzyme with 1 and 2 molecules of NAG₃ were observed along with the
 5 peaks of unbound enzyme and the free substrate. Extracted mass
 6 chromatograms corresponding to the protein as well as the complex were then
 7 generated and divided into 1 min. elution zones and integrated. Three most
 8 intense slots were averaged for the final data analysis in each case. The obtained
 9 data were processed using the non-linear regression analysis (Equation 1) where
 10 $A(P) / A(PL)$ is the ratio of integrated peak areas of free protein over protein
 11 complex, $[P]_0$ is the initial protein concentration, $[L]_0$ is the initial ligand
 12 concentration and K_D is the dissociation constant and the only fitting
 13 parameter.¹⁹

14
 15 Equation (1)

$$\frac{A(P)}{A(PL)} = \frac{-K_D + [L]_0 - [P]_0 - \sqrt{4K_D[L]_0 + (K_D - [L]_0 + [P]_0)^2}}{2[L]_0}$$

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 17
 18 $[P]_0$ was kept constant at a value of 5 μM in all experiments. The determined K_D
 19 was slightly lower ($19.6 \pm 8 \times 10^{-6}$ M) than when a syringe pump was used. The
 20 K_D values for both set-ups are summarized in Table 1. The kinetics of the
 21 enzyme - NAG₃ interaction (Fig.6b) is characterized by a hyperbolic decrease
 22 of the free enzyme concentration upon titration with increasing concentration of
 23 the ligand (graphically expressed as concentrations of free enzyme $[E_{\text{free}}]$ /bound
 24 enzyme $[C_{\text{px}}]$ vs. the increasing total NAG₃ concentration $[NAG]_{\text{TOT}}$).

1 4. Conclusions

2 The microfluidic system for determination of kinetic constants of enzymes
3 consumes a small amount of protein and substrate and can be linked to an
4 autosampler to screen ligand libraries of enzymatic substrates or inhibitors. The
5 chip was prepared from PMMA, only the sprayer was realized using 20 μm (ID)
6 fused silica capillary. The full-polymeric chip did not provided stable spray
7 signal, presumably due to close to neutral pH of the reagent solutions in water
8 with no organic solvent added. The system will be further optimized for other
9 enzymes and inhibitor types as are proteases, and kinases. Another viable
10 application could well be hydrogen/deuterium exchange reactions, where fast
11 mixing followed by mass analysis is needed. All those application can gain
12 from a possibility to vary the reagent flow and to shorten/extend the channel
13 length in the chip. The non-linear regression analysis seems to be the method of
14 choice for K_D determination using titration ESI-MS as obtained values are
15 within already described dissociation constants.

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14 6 *The authors declare no conflict of financial interest.*
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For Peer Review

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3 **List of Tables**
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8 **Table 1.** Comparison of K_D values for the HEWL oligosaccharide NAG₃
9
10 complex obtained by the automated ESI-MS chip system with data available
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12 from literature.
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Data	K_D (M)
present study	$39.0 \pm 6.4 \times 10^{-6}$ (syringe pump) $19.6 \pm 8 \times 10^{-6}$ (autosampler)
Jecklin <i>et al.</i> ¹⁹	$38.3 \pm 2.1 \times 10^{-6}$ (NanoESI-MS)
Veros <i>et al.</i> ¹⁶	9.1×10^{-6} (ESI-MS)

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1 **Figure Captions**

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7 **Fig.1.** HEWL converts its substrate NAG₆ into NAG₂ and NAG₄ as major
8 products and 2 molecules of NAG₃ as minor products. Structure of the chitin
9 molecule, showing β -1,4-linked *N*-acetylglucosamine (NAG) monomeric unit.
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14 **Fig.2.** General scheme of the microfluidic set-up. Syringe pumps a and b
15 delivered the HEWL (A) and its substrate NAG₃ (B) at a flow rate of 0.5
16 μ L/min and the electrospray high-voltage was supply from HV source to the
17 port C. Alternatively, the sample was injected by autosampler (AS) into a flow
18 of the LC pump (c). The sample is mixed by diffusion in the mixing channel
19 prior to electrospraying through 20 μ m ID fused silica capillary emitter to the
20 MS orifice.
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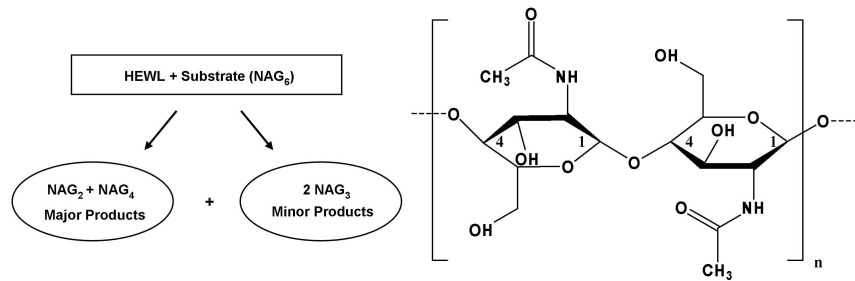
31 **Fig.3.** Spectra of HEWL-NAG complexes monitored on-line using chip-ESI-
32 MS. HEWL (5 μ M) and NAG₆ solutions (50 μ M) in 10 mM ammonium acetate
33 buffer (pH 5.2) infused from channel inlets A and B at a flow rate of 0.5 μ L/min
34 through pumps 1 and 2, respectively. E represents HEWL.
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41 **Fig.4.** Fast diffusion of rhodamine B in the mixing and enzymatic reaction
42 channel shown as the integrated pixel density of channel zones observed with a
43 fluorescence microscope. Magnification 50 \times , increments of the microscoped
44 zones shown in the inset, window size 325 \times 25 μ m.
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51 **Fig.5.** (a, bold) TIC trace of a single run NAG₃ (30 μ M in ammonium acetate
52 buffer 10 mM, pH 5.2) injected into the plastic chip by an Agilent autosampler.
53 (a, dashed) is a TIC trace of a single run NAG₃ (30 μ M in ammonium acetate
54 buffer 10 mM, pH 5.2), blank sample (without chip); T_R represents retention
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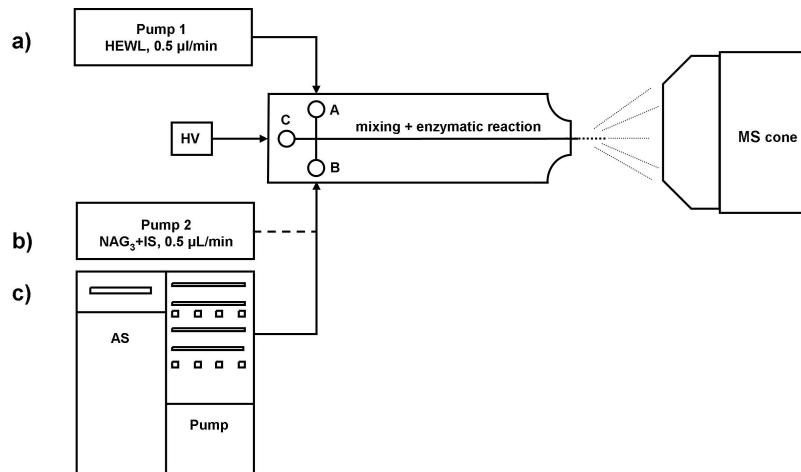
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4 1 time. (b) Mass spectrum acquired during 15-16 min of the run during the
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6 2 analysis of NAG₃.

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8 3
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10 4 **Fig.6.** (a) ESI-MS spectrum of the enzyme reaction between HEWL (5 μM) and
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12 5 NAG₃ (80 μM) in the presence of an internal standard (leucine enkephalin, 2.5
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14 6 μM) in ammonium acetate buffer (10 mM, pH 5.2). (b) titration experiment of
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16 7 HEWL by NAG₃ and its the non-linear regression curve fit. [E_{free}] represents the
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18 8 molar concentration of free enzyme, [C_{px}] concentration of bound enzyme, and
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20 9 [NAG₃]_{TOT} starting inhibitor concentration in the assay.
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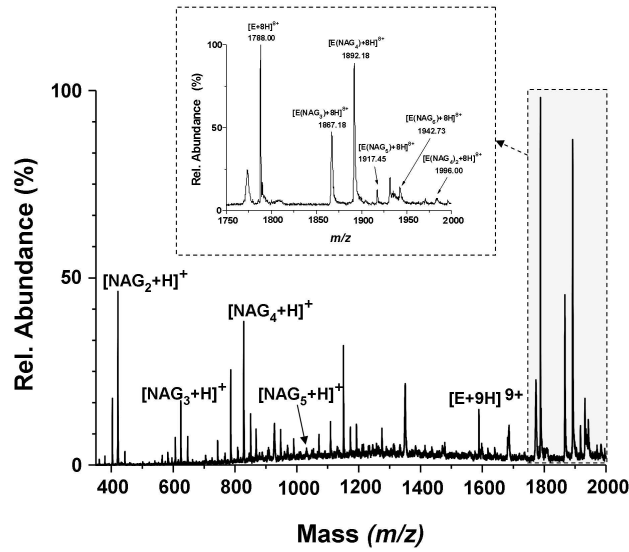


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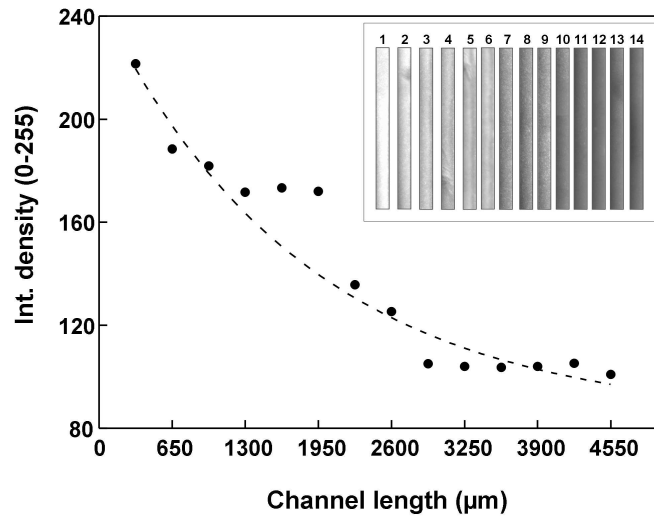
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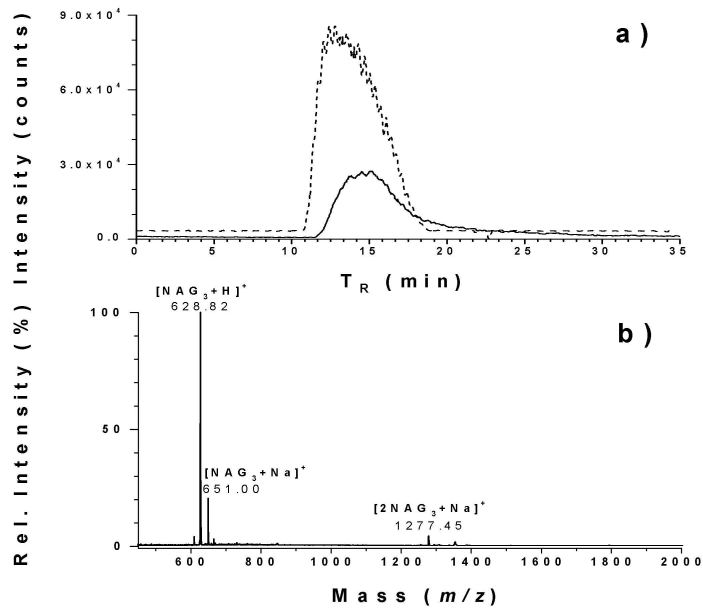
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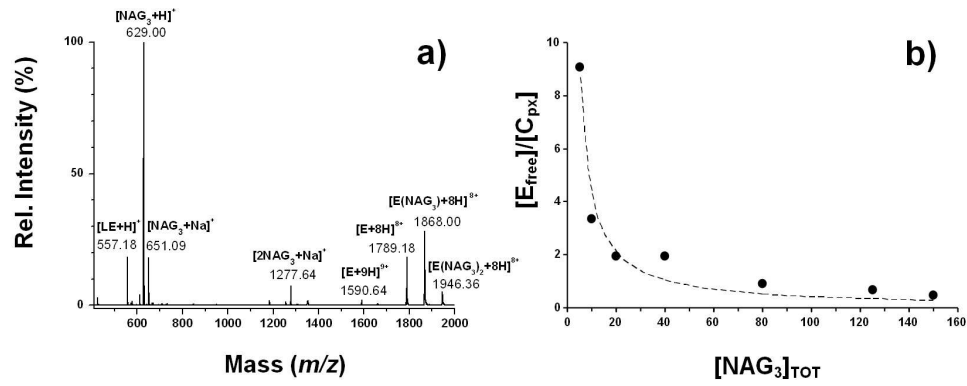
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254x190mm (300 x 300 DPI)



190x254mm (400 x 225 DPI)



451x206mm (96 x 96 DPI)