RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2009; **23**: 1249–1254
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.3993

RCM

Matrix/analyte ratio influencing polymer molecular weight distribution in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Gitta Schlosser[1,4,†], Annamária Jakab[1,†], Gabriella Pocsfalvi[2], Károly Vékey[3], Ferenc Hudecz[1,4] and Gábor Mező[1]*

[1]Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, Budapest, Hungary
[2]Proteomic and Biomolecular Mass Spectrometry Centre, Institute of Food Science and Technology, CNR, Avellino, Italy
[3]Chemical Research Center, Hungarian Academy of Sciences, Budapest, Hungary
[4]Department of Organic Chemistry, Eötvös L. University, Budapest, Hungary

Received 30 December 2008; Revised 18 February 2009; Accepted 19 February 2009

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been used to characterize poly(L-lysine) polymers and unique oligomer peptides, like 10-, 15- and 25-mer $[Lys]_n$ oligolysine peptides. Several matrices have been tried in order to find optimal conditions, but only α-cyano-4-hydroxycinnamic acid gave analytically useful spectra. The synthetic oligomers and their mixtures gave good quality spectra, showing protonated and cationized molecules, including doubly charged species. The polymers, analogously, gave a wide distribution of single- and double-cationized peak series. The polymer distributions observed indicate the presence of significant suppression effects. The concentration (matrix/analyte ratio) was found to influence the results significantly; distributions shifting to higher masses when higher polymer concentrations were used. This effect was studied in detail using the synthetic ('monodisperse') oligolysine peptides. It was found that the relative intensities change by over an order of magnitude in the 0.1–10 pmol/μL concentration range (typical for most proteomic analyses). The results indicate that concentration effects need to be considered when MALDI-MS is used for quantitative purposes.

Synthetic, biodegradable poly-amino acids and their derivatives have been used in various fields of research for a long time, including medical, biological and biochemical research. Polylysine and polylysine-based branched polypeptides are employed especially in the field of drug discovery, due to their advantageous physicochemical and biological properties. Polylysine-based bioconjugates are effective synthetic antigens for immunization and monoclonal antibody production. They are also used as macromolecular carriers for target-specific drug delivery and are applied in cancer research.[1–5] Cationic poly-amino acids are used as non-viral gene delivery systems, because these polyelectrolytes form non-covalent complexes with DNA.[6,7] Polylysine, produced by microbial fermentation, is widely used as a natural food preservative due to its ability to inhibit the growth of various microorganisms. As variations in the structure of a given polymer can change its physicochemical properties significantly, the structural characterization of such polymers is highly important. Application of synthetic polymers in human therapy is greatly restricted by the fact that their structures cannot be characterized in sufficient detail using currently available analytical techniques. The molecular weight and the molecular weight distribution of synthetic polymers can be estimated using various analytical methods, e.g. gel chromatography, viscosimetry, analytical ultracenrifugation and light scattering. However, these techniques need suitable calibration standards, and may give inaccurate results in the case of polymers with high adsorption properties or with a tendency to form aggregates in solution. Matrix-assisted laser desorption/ionization (MALDI)[8] and electrospray ionization (ESI)[9] mass spectrometry (MS) have been used in the analysis of synthetic polymers with increasing success.[10–13] These techniques produce intact protonated or cationized molecules from high molecular weight compounds and are able not only to measure molecular weight distributions, but also to determine the structures of the polymers without the need for polymer standards. The advantages of MALDI time of flight (TOF)-MS are the high mass range together with the formation of predominantly singly charged ions without fragmentation,

**Correspondence to*: G. Mező, Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, P.O. Box 32, Budapest 112, H-1518 Hungary.
E-mail: gmezo@elte.hu
†These authors contributed equally to this work.
Contract/grant sponsor: Hungarian Research Fund (OTKA); contract/grant number: K68285.
Contract/grant sponsor: National Office for Research and Technology, Hungary; contract/grant number: 3.2.1-2004-04-0005/3.0 and 3.2.1-2004-04-0352/3.0.
Contract/grant sponsor: Magyary Zoltán Higher Educational Public Foundation, Hungary.

which facilitates the evaluation and interpretation of the spectra. MALDI-TOF-MS has been shown to be a powerful method for determining the molecular weight distribution and structure of synthetic polymers of various chemical types. It is assumed that the molecular weight distribution obtained by MALDI-TOF-MS measurements does not depend on the structure and conformation of the polymer in contrast to conventional analytical methods where the results may be influenced by the conformation and the hydrodynamic volume of the given polymer. When single polymer ions are resolved (i.e. the mass difference in the repeating units is sufficiently large), the composition of the repeating units and the end groups can be determined, based on the masses of the individual polymer ions and the differences in m/z units between the successive ions. The recent development of new sample preparation techniques, matrices and additives allows the investigation of both polar and apolar synthetic polymers. However, the disadvantage of MALDI-MS analysis is that the results may depend on the sample preparation method applied. In addition, in the case of polydisperse polymers, high-mass components may be under-represented compared with lower mass components, due to mass discrimination effects.[14–17]

Polylysine polymers are most often characterized by gel chromatography and by sedimentation equilibrium measurements. While these techniques yield useful information, their accuracy is not always sufficient, and conflicting results are often obtained. However, the application of polylysine-based bioconjugates in human therapy or clinical diagnosis requires proper characterization of the chemical and biological properties. It has been shown that the cytotoxicity of poly(L-lysine) polymers depends on the molecular weight. Due to the increasing demand to obtain more specific information on the structure of polylysine polymers, we have investigated the feasibility of obtaining mass and structural information using MALDI-MS.

The principal aim of our experiments was to optimize the effects of matrix and sample preparation for the MALDI-TOF-MS analysis of poly(L-lysine) polymers. Results obtained on two different instruments are compared to give a better insight into instrumental dependence.

EXPERIMENTAL

Materials

Poly(L-lysine) polymers with various molecular weights were obtained from Fluka (Buchs, Switzerland). Molecular weight distributions were indicated by the supplier as follows: 5–10, 10–20, 20–30, 30–70 kDa. The MALDI matrices were from Sigma-Aldrich (Budapest, Hungary). Some polymers (as mentioned in the text) were synthesized in our laboratory as described earlier.[18]

Fmoc-Lys(Boc)-OH was purchased from NovaBiochem (Läufelfingen, Switzerland) and Cl-trityl resin was purchased from Reanal (Budapest, Hungary). *N,N′*-Diisopropylcarbodiimide (DIC), benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate (BOP), diisopropylethylamine (DIEA), 1-hydroxybenzotriazole (HOBt) and trifluoroacetic acid (TFA) were from Fluka. Dichloromethane (DCM) solvent for synthesis was purchased from Reanal. HPLC grade acetonitrile (ACN) was from Sigma-Aldrich. Other reagents and solvents were of analytical grade or the highest available purity.

Peptide synthesis

Lysine oligomers were built up using 5-mer blocks of side-chain-protected lysine. These building blocks were synthesized on a Cl-trityl resin that allows the fully protected pentalysine peptides to be cleaved from the resin using a mild acidic cleavage mixture. The peptides were synthesized manually in a funnel equipped with a glass filter.

Coupling of the first Lys residue was performed on 1 g of Cl-trityl chloride resin. The resin was swollen in DCM, then 940 mg (2 mmol) of Fmoc-Lys(Boc)-OH and 1.37 mL DIEA were added, and the solution was reacted for 100 min. The remaining active groups were capped with 0.8 mL of methanol in DCM (10 min). The resin was washed twice each with DCM, *i*-propanol, methanol, and diethyl ether. The resin was dried in air and Fmoc quantification followed.[19] The next four lysine residues were introduced using a three-fold excess of the protected amino acid, Fmoc-Lys(Boc)-OH, in the presence of coupling reagents DIC and HOBt, corresponding to the calculated 0.78 mmol/g capacity of the resin. The coupling efficiency was monitored by the ninhydrin test.[20]

After the stepwise synthesis, the resin was washed several times with DCM and methanol, and then dried in air. Half of the amount was cleaved using a cleavage mixture of acetic acid, methanol and DCM (2:2:6 v/v) during 3 h at room temperature. This cleavage mixture selectively removes the peptide from the resin but protecting groups remain intact. The solvents were filtered off and the resin was washed twice with fresh cleavage mixture. The solvent was removed in vacuum, and the remaining oil was precipitated by *n*-hexane. The product was washed with 5% $NaHCO_3$ solution (pH 7.5) and water in order to remove traces of the acetic acid. The final product was then dried in air. Mass spectrometric measurement and analytical high-performance liquid chromatography (HPLC) showed sufficient purity for the pentapeptide to be used without further purification steps.

The peptide synthesis was continued by coupling the protected pentapeptide to the oligomer peptide on resin using a 1:2:1 ratio of BOP/DIEA/HOBt coupling reagents, in at least 1.5-fold excess. The reaction times were approx. 2–3 h, slightly increasing with the size of the growing peptide. After each coupling an aliquot of the peptidyl-resin was taken thus giving a series of 10-, 15-, 20- and 25-mer peptides up to the 50-mer peptide. The peptide was cleaved from the resin and the protecting groups were removed by a final cleavage with a mixture of TFA and water (95:5, v/v); the cleavage time was 2 h. After the cleavage reaction, the cleavage cocktail was filtered into cold diethyl ether. The resin was washed twice with TFA and DCM. The precipitated peptides were centrifuged at 3500 rpm for 10 min, and washed three times with cold diethyl ether. The peptide was dissolved in 10% acetic acid and freeze-dried. To remove traces of the acetic acid the products were lyophilized twice from 1 M HCl.

DOI: 10.1002/rcm

Matrix-assisted laser desorption/ionization mass spectrometry

MALDI-TOF mass spectra were recorded on a Bruker Biflex mass spectrometer (Bruker Daltonics, Bremen, Germany) and a Voyager DE-Pro instrument (Applied Biosystems, Framingham, MA, USA), both equipped with a nitrogen laser ($\lambda = 337$ nm).

The matrices used in this study were α-cyano-4-hydroxycinnamic acid (CCA), sinapinic acid (SA) and 2,5-dihydroxybenzoic acid (DHB). The matrices were dissolved in an ACN/water mixture (1:1, v/v), containing 0.1% TFA in a concentration of 10 mg/mL. The samples were dissolved in water in appropriate concentrations. Prior to analysis, 0.5 μL of sample was mixed with 0.5 μL of matrix, spotted onto the target, and allowed to dry in air. Mass calibration was performed externally using a peptide mixture standard. Spectra were acquired by randomly scanning the sample surface. At least 500 spectra were averaged. When needed, the samples were purified by ZipTips (Millipore, Billerica, MA, USA), which were filled with C_{18} material and used according to the manufacturer's instructions.

RESULTS AND DISCUSSION

In these experiments, we optimized the experimental conditions for studying polymeric poly(L-lysine), using two different MALDI-TOF instruments in linear mode. We tested three different matrices for sample preparation: CCA, SA and DHB. These matrices are generally used for the analysis of peptides and proteins in a broad *m*/*z* range. We observed that in the case of DHB, ions corresponding to protonated molecules of the polymers were detected only at the low *m*/*z* range, approximately up to *m*/*z* 2000 (data not shown). In the case of the SA matrix no signal could be detected (data not shown). On the other hand, using the CCA matrix, we detected abundant $[M+H]^+$ ions of the intact polylysine molecules, and the polymers gave well-defined distributions in the linear MALDI spectra. Figures 1(A)–1(C) show the MALDI-TOF mass spectra of polylysine polymers with different molecular weight distributions using the CCA matrix, with the higher molecular weight polymers showing ions at higher *m*/*z* values. Formation of doubly charged ions was observed giving a separated second ion distribution at lower *m*/*z* values. The relative signal intensities of the doubly protonated molecules compared with those of the singly charged species increase with the molecular weight of the polymer (Figs. 1(A)–1(C)). The abundances of the doubly charged ions are significantly different on the two instruments, with the Voyager DE-Pro mass spectrometer producing many more doubly charged species. Signals in the linear MALDI spectra were observed up to approximately *m*/*z* 15000–18000. In the case of low molecular weight polymers, the detected molecular weight distribution is similar to the mass range indicated by the supplier; however, on increasing the molecular weight, the MALDI measurements result in significantly lower molecular weight distributions.

In addition to commercially available polylysine polymers, polymer samples synthesized in our laboratory were also analyzed in order to identify possible by-products and to apply MALDI-TOF-MS as a quality control method in the synthesis of poly(L-lysine) polymers. In general, the polymers show a well-defined *m*/*z* distribution, with well-resolved ions. Fragmentation of the polypeptide ions was not observed. By using reflectron mode mass analysis, the resolution of the measurements can be improved, and baseline-resolved isotopic peaks can be obtained. This is particularly useful because the composition of the repeating units and the end groups can be determined based on the

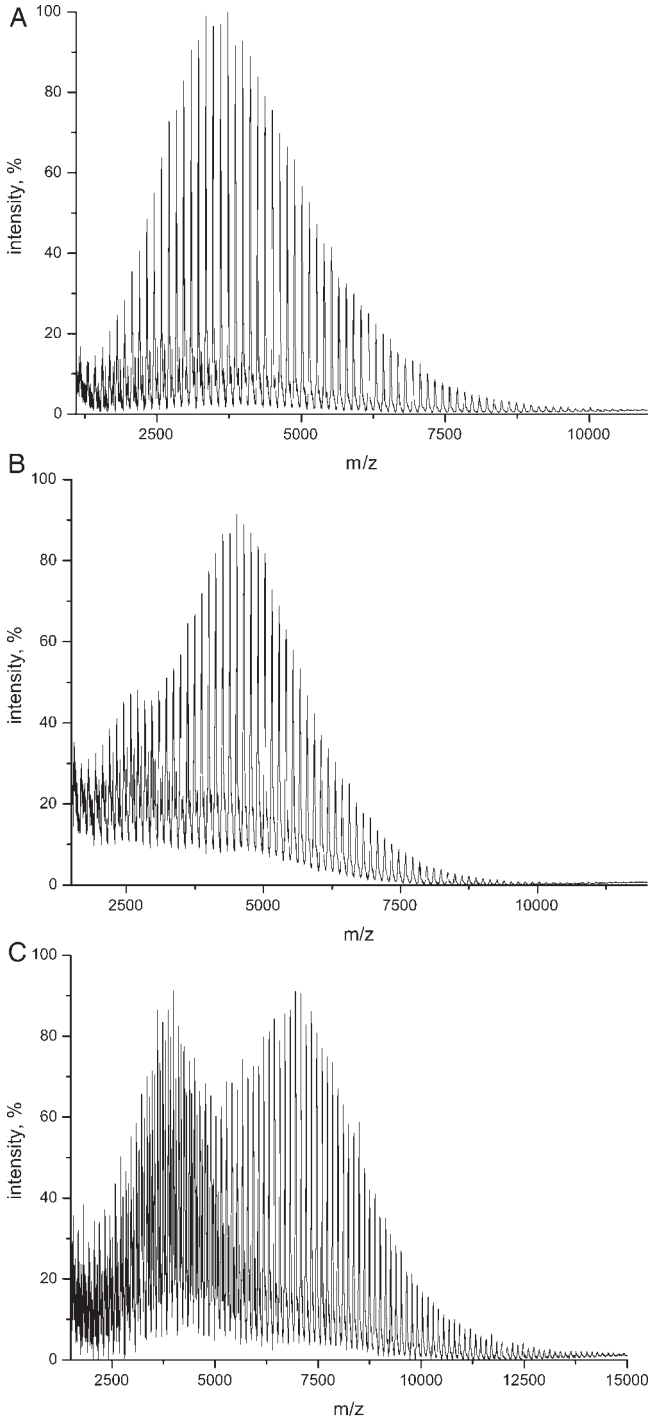

Figure 1. MALDI-TOF mass spectra of polylysine polymers with different molecular weights: (A) 5–10 kDa, (B) 10–20 kDa, and (C) 20–30 kDa (linear mode, CCA matrix, instrument: Voyager DE-Pro, polymer concentration: 0.1 mg/mL).

DOI: 10.1002/rcm

RCM

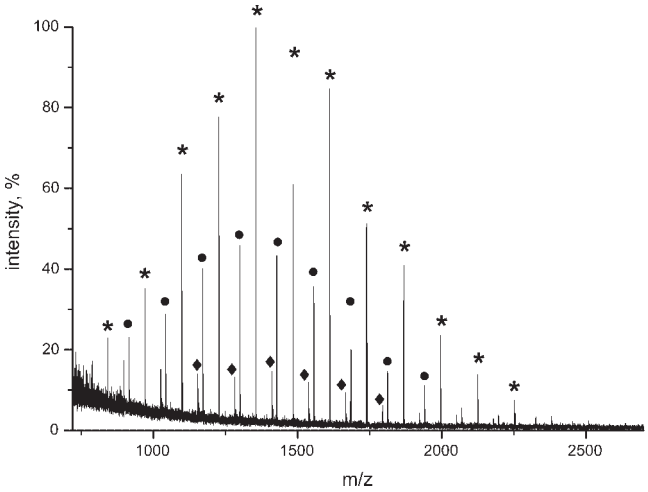

Figure 2. By-products identified in the MALDI-TOF spectrum of a low molecular weight polylysine polymer (reflectron mode, CCA matrix, instrument: Voyager DE-Pro). Ion labeling: poly(L-lysine) molecules with C-terminal diethylamide group (∗), free carboxyl group (•); and cyclic molecules (◆).

measured accurate mass values. Figure 2 shows the MALDI-TOF mass spectrum of a low molecular weight polylysine polymer in reflectron mode. In this case we detected three independent series of well-resolved ions. The first one corresponds to polylysine molecules with a C-terminal diethylamide group (diethylamine was used as the initiator during the synthesis of the polymer). The two additional ion series with lower signal intensities correspond to by-products of the polymeric reaction. Based on the detected masses, the first by-product is a polymer with a free carboxyl group at the C-terminal. Formation of such a compound can be explained by the presence of traces of water in the reaction mixture, which also acts as an initiator during polymerization. The third ion series with the lowest intensity corresponds to cyclized polylysine molecules.

Interestingly, we have also observed that the signal distributions of MALDI mass spectra depend on the concentration of the sample (i.e. on the analyte/matrix ratio). Figure 3(A) shows the linear MALDI mass spectra of a polylysine polymer at three different concentrations (0.01, 0.1 and 1 mg/mL) measured on the Bruker MALDI-TOF instrument. We observed that the distribution of the ions is significantly broader and is shifted to higher *m*/*z* values on increasing the concentration of the sample. The ion intensities at lower masses are decreased, while the intensity of the higher mass ions increased to a large extent, resulting in a broader mass distribution, and a very remarkable shift of the maximum to higher *m*/*z* values. A similar effect was observed on the Voyager DE-Pro MALDI-TOF instrument; however, the shift of the distribution maximum to higher *m*/*z* values was not as prominent (Fig. 3(B)). These results suggest that such anomalies may influence the detected mass distributions to a great extent. Similar results were observed for all the polymers studied.

In order to study the influence of concentration on the mass distributions observed in MALDI spectra, we have synthesized a series of oligolysine peptides with different

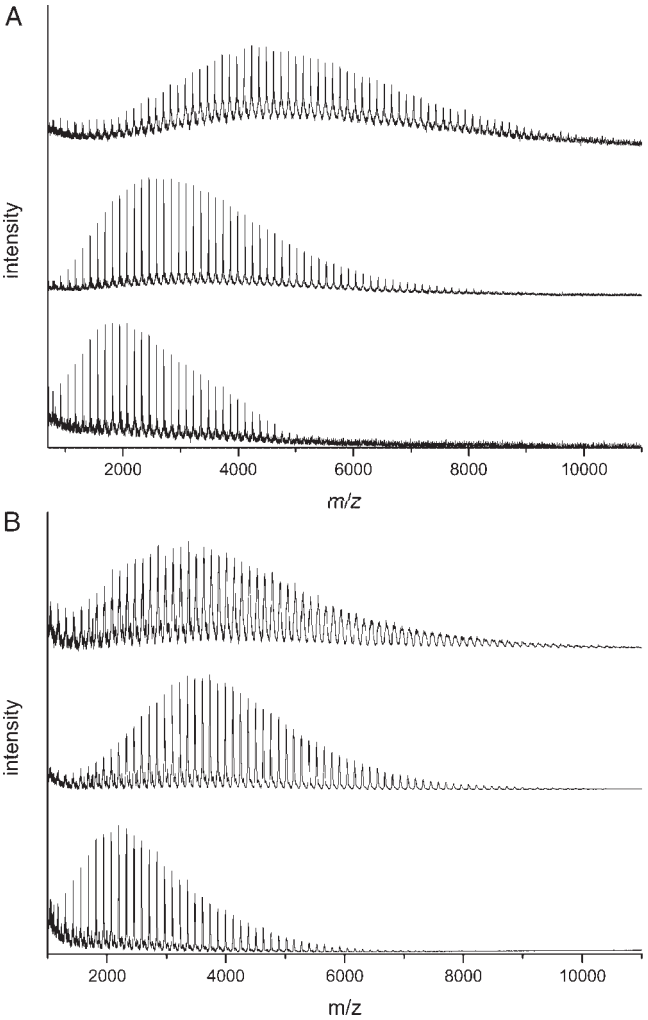

Figure 3. MALDI-TOF mass spectra of a polylysine polymer of 5–10 kDa molecular weight acquired in different concentrations (0.01, 0.1 and 1 mg/mL from bottom to top, respectively). Spectra in (A) were acquired on a Bruker Biflex mass spectrometer; spectra in (B) were acquired on a Voyager DE-Pro instrument (linear mode, CCA matrix).

chain lengths $[Lys]_n$, n = 10, 15, 20, 25, etc. Oligolysine peptides of different lengths were mixed in an equimolar ratio, and the mixtures were analyzed by linear MALDI-TOF-MS in different concentrations. Figures 4(A) and 4(B) show the mass spectra of the equimolar mixture of 15-, 20- and 25-mer oligolysine peptides in two different concentrations. We observed that, under these experimental conditions, the relative signal intensities of the peptides do not match the real molar ratios and a change in the peptide concentration causes a dramatic rearrangement in their relative intensities. At the lowest concentration range (below 1 pmol/μL each) the low molecular weight peptides have higher intensities (Fig. 4(A)). However, on increasing the concentration of the sample, the intensities of the higher mass peptides increase more significantly. Above a certain concentration, higher molecular weight peptides have much higher intensities than the peptides with lower molecular weight and their intensity ratio reverses (Fig. 4(B)). A similar effect was observed using both MALDI-TOF mass spec-

DOI: 10.1002/rcm

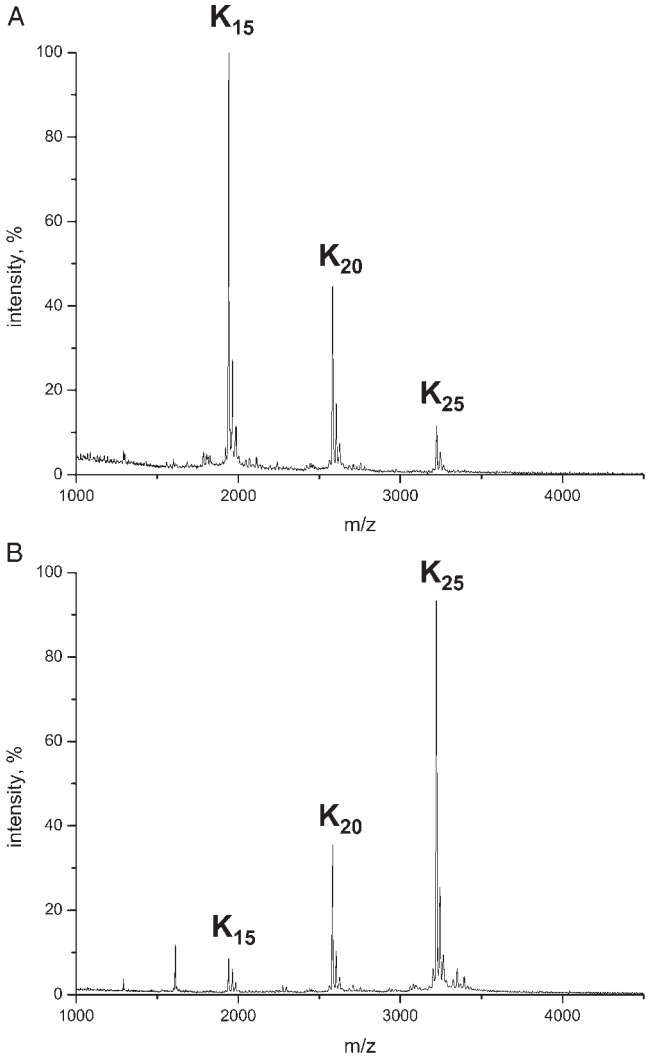

Figure 4. MALDI-TOF mass spectra of an equimolar mixture of oligolysine peptides of 15, 20 and 25 amino acid residues. Concentration of the peptides were (A) 0.2 pmol/μL and (B) 13 pmol/μL (linear mode, CCA matrix, instrument: Bruker Biflex).

trometers; however, in the case of the AB Voyager instrument, the change in the relative intensity of the peptides was not as prominent. We observed that the change in relative intensities in the function of sample concentration is independent of the size and number of the oligolysine peptides present in the mixtures. It is important to note that the effect of concentration on relative intensities is particularly prominent in the concentration range generally used for the analysis of peptides and proteins (0.1–10 pmol/μL) (Fig. 5).

The results show that MALDI-TOF-MS is a useful technique in the quality control analysis of poly(L-lysine) polymers. Based on MALDI-TOF measurements, the composition and structure of the polymer can be determined and possible by-products can be identified. It is also useful for the investigation of mass distribution. However, it was also observed that the mass distribution obtained by MALDI-TOF-MS depends on the matrix/analyte ratio (concentration

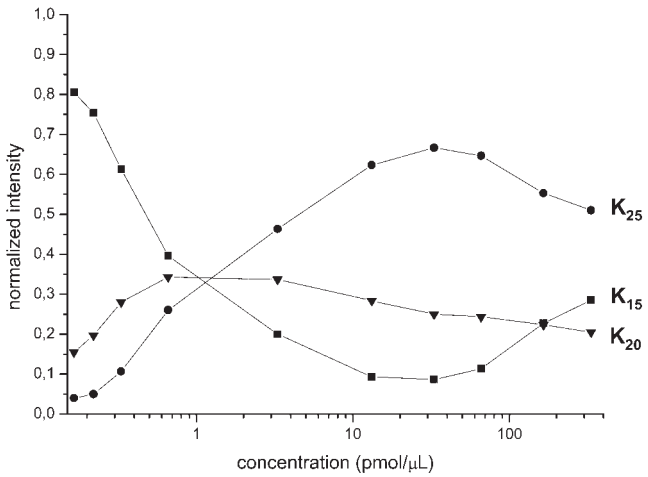

Figure 5. Normalized intensities of oligolysine peptides of 15, 20 and 25 amino acid residues, as a function of the concentration (linear mode, CCA matrix, instrument: Bruker Biflex).

of the sample), which represents a limitation in the MALDI analysis of such polymers.

CONCLUSIONS

In the present work, very strong dependence of MALDI mass spectra on the matrix/analyte ratio has been observed. The results show that even small changes in the concentration of the analyte may have a large effect on the ratio of the ion intensities in the spectra both for polymers and for peptide mixtures. A tentative explanation of the observed concentration effect may be that at low analyte/matrix ratio all molecules could be ionized; but the low-mass compounds have a higher sensitivity possibly due to instrumental discrimination effects. At high analyte concentration, higher mass compounds have an increased intensity; possibly due to saturation effect and/or ion-molecule reactions occurring in the MALDI plume, favoring higher mass (higher proton affinity or higher cation affinity) oligomers.

This may question the validity of using MALDI for quantitative analysis, and may cause problems for determining polymer distributions. At present the results have to be considered preliminary – it needs to be established whether these are particular to polylysine polymers and analogous oligopeptides, or if they represent a general phenomenon also influencing peptide/protein analysis and other polymers.

Acknowledgements

This work was supported by grants from the Hungarian Research Fund (OTKA) Nr. K68285, and from the National Office for Research and Technology, Hungary (3.2.1-2004-04-0005/3.0 and 3.2.1-2004-04-0352/3.0). G. Schlosser acknowledges financial support from the Magyary Zoltán Higher Educational Public Foundation, Hungary.

DOI: 10.1002/rcm

REFERENCES

1. Clegg JA, Hudecz F, Mező G, Pimm MV, Szekerke M, Baldwin RW. *Bioconjugate Chem.* 1990; **1**: 425.
2. Hudecz F, Clegg JA, Kajtár J, Embleton MJ, Szekerke M, Baldwin RW. *Bioconjugate Chem.* 1992; **3**: 49.
3. Hudecz F, Clegg JA, Kajtár J, Embleton MJ, Pimm MV, Szekerke M, Baldwin RW. *Bioconjugate Chem.* 1993; **4**: 25.
4. Mező G, Mező I, Pimm MV, Kajtár J, Seprődi J, Teplán I, Kovács M, Vincze B, Pályi I, Idei M, Szekerke M, Hudecz F. *Bioconjugate Chem.* 1996; **7**: 642.
5. Ryser HJ-P, Shen W-C. *Proc. Natl. Acad. Sci. USA* 1978; **75**: 3867.
6. Segura T, Shea LD. *Bioconjugate Chem.* 2002; **13**: 621.
7. Wagner E, Zatloukal K, Cotten M, Kirlappos H, Mechtler K, Curiel DT, Birnstiel ML. *Proc. Natl. Acad. Sci. USA* 1992; **89**: 6099.
8. Karas M, Bahr U, Ingendoh A, Nordhoff E, Stahl B, Strupat K, Hillenkamp F. *Anal. Chim. Acta* 1990; **241**: 175.
9. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. *Science* 1989; **246**: 64.
10. Bahr U, Deppe A, Karas M, Hillenkamp F, Giessmann U. *Anal. Chem.* 1992; **64**: 2866.
11. Hanton SD. *Chem. Rev.* 2001; **101**: 527.
12. Nielen MWF. *Mass Spectrom. Rev.* 1999; **18**: 309.
13. Scrivens JH, Jackson AT. *Int. J. Mass Spectrom.* 2000; **200**.
14. Dogruel D, Nelson RW, Williams P. *Rapid. Commun. Mass Spectrom.* 1996; **10**: 801.
15. Schriemer DC, Li L. *Anal. Chem.* 1997; **69**: 4169.
16. Schriemer DC, Li L. *Anal. Chem.* 1997; **69**: 4176.
17. Guo B, Chen H, Rashidzadeh H, Liu X. *Rapid. Commun. Mass Spectrom.* 1997; **11**: 781.
18. Mező G, Reményi J, Kajtár J, Barna K, Gaál G, Hudecz F. *J. Controlled Release* 2000; **63**: 81.
19. Gude M, Ryf J, White PD. *Lett. Peptide Sci.* 2002; **9**: 203.
20. Kaiser E, Colescott RL, Bossinger CD, Cook PI. *Anal. Biochem.* 1970; **34**: 595.

DOI: 10.1002/rcm