Quantifying Protein-Fatty Acid Interactions using Electrospray Ionization Mass Spectrometry

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Abstract

The application of the direct electrospray ionization mass spectrometry (ES-MS) assay to quantify interactions between bovine β-lactoglobulin (Lg) and a series of fatty acids (FA), $CH_3(CH_2)_xCOOH$, where x = 6 (caprylic acid, CpA), 8 (capric acid, CA), 10 (lauric acid, LA), 12 (myristic acid, MA), 14 (palmitic acid, PA) and 16 (stearic acid, SA), is described. Control ES-MS binding measurements performed on the Lg-PA interaction revealed that both the protonated and deprotonated gas phase ions of the (Lg+PA) complex are prone to dissociate in the ion source, which leads to artificially small association constants (K_a). The addition of imidazole, a stabilizing solution additive, at high concentration (10 mM) increased the relative abundance of (Lg+PA) complex measured by ES-MS in both positive and negative ion modes. The K_a value measured in negative ion mode and using sampling conditions that minimize in-source dissociation is in good agreement with a value determined using a competitive fluorescence assay. The K_a values measured by ES-MS for the Lg interactions with MA and SA are also consistent with values expected based on the fluorescence measurements. However, the K_a values measured using optimal sampling conditions in positive ion mode are significantly lower than those measured in negative ion mode for all of the FAs investigated. It is concluded that the protonated gaseous ions of the (Lg+FA) complexes are kinetically less stable than the deprotonated ions. In-source dissociation was significant for the complexes of Lg with the shorter FAs (CpA, CA and LA) in both modes and, in the case of CpA, no binding could be detected by ES-MS. The affinities of Lg for CpA, CA and LA determined using the reference ligand ES-MS assay, a method

for quantifying labile protein-ligand complexes that are prone to in-source dissociation, were found to be in good agreement with reported values.

Introduction

The direct electrospray ionization mass spectrometry (ES-MS) assay has emerged as a useful technique for measuring the binding stoichiometry and affinity (K_a) of protein-ligand interactions, as well as other noncovalent biological complexes, in solution. The assay is based on the relative abundance of free and ligand-bound protein ions measured by ES-MS for a solution of known initial protein and ligand concentration.[1-3] The ES-MS assay is fast (measurements normally can be completed in less than ~1 min), consumes little sample (typically ~pmol per analysis when performed using nanoflow ES), provides direct insight into binding stoichiometry and can be used to measure multiple binding equilibria simultaneously. Furthermore, the assay is very versatile as there is no requirement for labeling or immobilization of analyte.

Absolute K_a values, in the 10^3 - 10^7 M^{-1} range, have been determined for a variety of protein-ligand complexes, including antibody-antigen, lectin-carbohydrate, enzyme-inhibitor complexes, and shown to be in reasonable agreement with values determined by other analytical methods.[4-10] To date, however, there have been few reported examples of the application of ES-MS to characterize protein-hydrophobic ligand binding [11-14] and, to our knowledge, no reports of absolute affinity measurements using the direct ES-MS assay. The two principal challenges to quantifying interactions between water soluble proteins and hydrophobic ligands using ES-MS are the low solubility of the ligand in aqueous solution, and the propensity of the desolvated complexes to dissociate during analysis. Low ligand solubility prevents the ES-MS assay from being applied to very weak interactions ($<10^4$ M^{-1}) because high ligand concentrations are needed to produce detectable levels of complex. Protein-ligand complexes stabilized predominantly by

nonpolar interactions also tend to have low kinetic stabilities in the gas phase and are prone to dissociate in the ion source (i.e., in-source dissociation).[11,12] In-source dissociation reduces the relative abundance of the ligand-bound protein ions and, consequently, leads to artificially low binding constants.[11,15-17] In extreme cases, in-source dissociation results in false negatives, wherein no ligand-bound protein ions are detected by ES-MS.[15] However, using gentle sampling conditions, along with stabilizing solution or gas phase additives, it is sometimes possible to preserve the labile protein-ligand complexes during ES-MS analysis and to quantify their association constants.[17,18]

Here, we describe the application of ES-MS to quantify the affinities of bovine β-lactoglobulin (Lg) for a series of linear, saturated fatty acids (FA), $CH_3(CH_2)_xCOOH$. Lg, a member of the lipocalin protein family, possesses a large hydrophobic cavity and is known to bind a variety of hydrophobic ligands, including FAs.[19,20] Association constants for the interaction between Lg and several FAs (x = 6 (caprylic acid), 10 (lauric acid) and 14 (palmitic acid)), at 25 °C and various pH values (6.2, 7.2 and 8.3) have been determined using a competitive fluorescence assay.[21] Notably, these measurements revealed that, at a given pH, the logarithm of K_a increases linearly with the length of the FA acyl chain. Based on this dependence, it is possible to estimate the K_a values for related FAs, *vide infra*. To test the reliability of the direct ES-MS assay for quantifying protein-hydrophobic ligand interactions, affinity measurements were carried out on Lg and a series of FAs (x = 6, 8, 10, 12, 14 and 16) at pH 8.5 and 25 °C. Importantly, it is shown that the K_a values for Lg interactions with the longer FAs (x = 12, 14 and 16) measured in negative ion mode and using gentle sampling conditions in conjunction with

a stabilizing solution additive are in good agreement with the reported values. In contrast, in-source dissociation of the gaseous ions of the complexes of Lg and the shorter FAs is extensive, which precludes the determination of reliable affinities using the direct ES-MS assay. However, the affinities determined for the shorter FAs using the reference ligand ES-MS assay [21], a method for quantifying labile protein-ligand complexes that are prone to in-source dissociation, are in good agreement with reported values.

Experimental

Protein and ligand

Bovine β-lactoglobulin (Lg, monomer MW 18281 Da), caprylic acid (CpA, 144.2 Da), capric acid (CA, 172.3 Da), lauric acid (LA, 200.3 Da), myristic acid (MA 228.4 Da), palmitic acid (PA, MW 256.4 Da) and stearic acid (SA, 284.8 Da) were purchased from Sigma-Aldrich Canada (Oakville, Canada). The Lg was dissolved and exchanged directly into Milli-Q water, using an Amicon microconcentrator with a molecular weight cutoff of 10 kDa, followed by filtration using an Amicon ultra centrifugal filter with a molecular weight cutoff of 100 kDa to remove Lg aggregates in solution. The concentration of the Lg solution was determined by lyophilizing a known volume of the filtrate and measuring the mass of the protein. The protein stock solution was stored at -20 °C. The ligand stock solutions were prepared by dissolving each FA into aqueous ammonium acetate (25 mM). The ES solutions were prepared from aqueous stock solutions of protein and ligand. Aqueous ammonium hydroxide was added to adjust the pH of the ES solution to 8.5. Where indicated, imidazole (10 mM) was also added in order to stabilize the (Lg+FA) complexes during ES-MS analysis.

Mass spectrometry

All experiments were performed on a 9.4 tesla Apex II Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker, Billerica, MA) equipped with a nanoflow ES ion source. NanoES was performed using borosilicate tubes (1.0 mm o.d., 0.68 mm i.d.), pulled to ~5 μm o.d. at one end using a P-2000 micropipette puller (Sutter Instruments, Novato, CA). Details of the instrumental parameters employed in positive ion mode are given below. For measurements in negative ion mode, the voltage polarity was switched. The electric field required to spray the solution in positive ion mode was established by applying a voltage of 800 V to a platinum wire inserted inside the glass tip. The solution flow rate was ~20 nL/min. The gaseous ions produced by nanoES were introduced into the mass spectrometer through a stainless steel capillary (i.d. 0.43 mm) maintained at an external temperature of 66 °C. The gas flow rate into the instrument was measured to be 0.6 L min^{-1}. The ions sampled by the capillary (50 V) were transmitted through a skimmer (0 V) and accumulated electrodynamically in an rf hexapole. Ions were then ejected from the hexapole and accelerated (-2700 V) into the superconducting magnet, decelerated, and introduced into the ion cell. The trapping plates of the cell were maintained at a constant potential of 1.4 V throughout the experiment. The typical base pressure for the instrument was ~$5x10^{-10}$ mbar. Data acquisition was controlled by an SGI R5000 computer running the Bruker Daltonics XMASS software, version 5.0. Mass spectra were obtained using standard experimental sequences with chirp broadband excitation. The time domain signal, consisting of the sum of 50 transients containing 128 K data points per transient, was subjected to one zero-fill prior to Fourier transformation.

Determining protein-ligand affinities using the direct ES-MS assay

The procedures used for the determination of protein-ligand binding constants (K_a) directly from ES mass spectra are described in detail elsewhere.[4] Briefly, the direct ES-MS assay is based on the quantification of the abundance (*Ab*) of ligand-bound and unbound protein ions in the gas phase, e.g., PL^{n+} and P^{n+}, respectively. The measured abundance ratio (*R*) is assumed to be equivalent to the equilibrium concentration ratio of ligand-bound and free protein in solution, eq 1:

$$\frac{[\mathrm{PL}]_{eq}}{[\mathrm{P}]_{eq}} = \frac{\sum_{\mathrm{n}} Ab(\mathrm{PL}^{\mathrm{n}+})}{\sum_{\mathrm{n}} Ab(\mathrm{P}^{\mathrm{n}+})} = R \qquad (1)$$

From the measured *R* value and initial concentrations of protein ($[P]_o$) and ligand ($[L]_o$), K_a can be calculated using eq 2:

$$\mathrm{K_a} = \frac{R}{[\mathrm{L}]_\mathrm{o} - \frac{R}{1+R}[\mathrm{P}]_\mathrm{o}} \qquad (2)$$

Determining protein-ligand affinities using the reference ligand ES-MS method

The *reference ligand ES-MS method* for determining protein-ligand affinities is based on the direct ES-MS assay and competitive ligand binding.[22] A reference ligand (L_{ref}), which binds specifically to the protein, at the same binding site as the ligand of interest, with known affinity and forms a stable protein-ligand complex in the gas phase, is added to the solution. The fraction of P bound to L_{ref}, which is determined directly from the ES mass spectrum, is sensitive to the fraction of P bound to L in solution and enables the affinity of P for L to be determined.

In the absence of in-source dissociation, the measured abundance ratios of ligand-bound to free P ions (*R* and R_{ref}) will reflect the concentration ratios in solution and K_a for the PL complex can be calculated using eq 3:

$$K_a = \frac{R}{[L]_o - \frac{R}{1+R+R_{ref}}[P]_o} \quad (3)$$

where R is given by eq 1 and R_{ref} by eq 4:

$$\frac{[PL_{ref}]_{eq}}{[P]_{eq}} = \frac{\sum_n Ab(PL_{ref}{}^{n+})}{\sum_n Ab(P^{n+})} = R_{ref} \quad (4)$$

If the gas phase ions of the PL complex are kinetically labile and undergo in-source dissociation, the relative abundance of ligand-bound and free protein ions measured by ES-MS will no longer reflect the original concentration ratios. Instead, the measured abundance ratio of L_{ref}-bound P to free P gas phase ions (i.e., $R_{ref,app}$) will depend on the concentration of both free P and PL originally present in solution, eq 5:

$$\frac{[PL_{ref}]_{eq}}{[P]_{eq} + \alpha[PL]_{eq}} = \frac{\sum_n Ab(PL_{ref}{}^{n+})}{\sum_n Ab(P^{n+})} = R_{ref,app} \quad (5)$$

where α is the fraction of PL that undergoes in-source dissociation.

In the case of complete dissociation of PL, i.e., $\alpha = 1$, R_{ref} (the "true" concentration ratio $[PL_{ref}]/[P]$) can be calculated from $R_{ref,app}$ and $K_{a,ref}$ using eq 6:

$$R_{ref} = K_{a,ref}([L_{ref}]_o - [P]_o \frac{R_{ref,app}}{R_{ref,app}+1}) \quad (6)$$

and R can be calculated with eq 7:

$$R = \frac{R_{ref}}{R_{ref,app}} - 1 \quad (7)$$

Once R and R_{ref} are known, the K_a value for the PL complex can be calculated using eq 3.

If the gas phase PL ions undergo partial dissociation in the source, i.e., $\alpha < 1$, the measured abundance ratio of L-bound P to free P gas phase ions (i.e., R_{app}) can be determined using eq 8:

$$\frac{(1-\alpha)[\mathrm{PL}]_{eq}}{[\mathrm{P}]_{eq}+\alpha[\mathrm{PL}]_{eq}} = \frac{\sum_{\mathrm{n}} Ab(\mathrm{PL}^{\mathrm{n}+})}{\sum_{\mathrm{n}} Ab(\mathrm{P}^{\mathrm{n}+})} = R_{app} \qquad (8)$$

Therefore, R_{ref} can be determined from the measured $R_{ref,app}$ and R_{app} values using eq 9:

$$R_{ref} = \mathrm{K}_{\mathrm{a},ref}([\mathrm{L}_{ref}]_{\mathrm{o}} - [\mathrm{P}]_{\mathrm{o}} \frac{R_{ref,app}}{R_{ref,app}+R_{app}+1}) \qquad (9)$$

and R can be found using eq 10:

$$R = \frac{(1+R_{app})}{R_{ref,app}} R_{ref} - 1 \qquad (10)$$

Once R and R_{ref} are known, $\mathrm{K_a}$ value for the PL complex can be calculated using eq 3.

Results and discussion

Association constant for the (Lg + PA) complex determined by the direct ES-MS assay

The (Lg+PA) complex served as a model protein-fatty acid complex for establishing appropriate experimental and instrumental conditions for the direct ES-MS binding measurements. Shown in Figure 1a is an ES mass spectrum acquired in positive ion mode for an aqueous solution of Lg (12 μM) and PA (10 μM) at pH 8.5 and 25 °C. At this pH and temperature, Lg is present only as monomer in solution.[23] Inspection of the mass spectrum reveals abundant signal corresponding to protonated Lg^{n+} ions, at n = 7 and 8, and protonated 1:1 complex, $(\mathrm{Lg+PA})^{n+}$, at the same charge states. Notably, the $\mathrm{K_a}$ value, 1.2×10^5 M^{-1}, which was calculated from the measured abundance ratio (R) of ligand-bound to unbound Lg ions, is roughly five-fold smaller than the reported

affinity.[21] As described previously, collisional heating of gaseous ions can occur during accumulation in the rf hexapole in the FT-ICR mass spectrometer used for the present measurements.[17] Indeed, it was found that *R* (and K_a) increased as the accumulation time decreased. For example, the mass spectrum shown in Figure 1a was acquired using an accumulation time of 0.8 s, while the spectrum in Figure 1b was acquired for the same solution but using an accumulation time of 0.4 s. The decrease in accumulation time led to an increase of 47% in the magnitude of *R*, which corresponds to an increase of 83% in the K_a value, 2.2×10^5 M^{-1}. The effect of accumulation time on the binding measurements is more clearly seen in Figure 2, where the average *R* values are plotted versus accumulation time (from 0.4 to 2.0 s). It can be seen that the magnitude of *R* decreases systematically with increasing accumulation time and is immeasurably small at times ≥2.0 s. These results indicate that, even using optimal instrumental conditions and the shortest accumulation time (0.4 s) that still provides sufficient protein ion signal, the relative abundance of the $(Lg+PA)^{n+}$ ions measured by ES-MS is affected (reduced) by in-source dissociation.

Recently, it was shown that the presence of imidazole, at high concentration (~10 mM), can protect protein-ligand complexes from in-source dissociation during ES-MS analysis.[17] It was proposed that the stabilizing effect is due, at least in part, to enhanced evaporative cooling, which arises from the loss of multiple nonspecifically bound imidazole molecules from the gaseous protein-ligand complexes in the ion source.[17] The stabilizing effect of imidazole is evident in the ES mass spectra shown in Figures 1c and 1d. These mass spectra were acquired under identical conditions as those shown in Figures 1a and 1b, respectively, but with the addition of 10 mM imidazole to the solution.

The presence of imidazole results in two obvious changes in the appearance of the mass spectra. The charge state distribution for the Lg^{n+} and $(Lg+PA)^{n+}$ ions broadens from 7–8 to 6–8. This change is due to the high gas phase basicity of imidazole (217 kcal mol^{-1})[24] and its ability to strip protons from multiply protonated protein ions in the gas phase.[25] Additionally, the relative abundance of the $(Lg+PA)^{n+}$ ions is larger than that measured in the absence of imidazole. In fact, the *R* values measured at 0.4 and 0.8 s hexapole accumulation times are nearly indistinguishable. However, the corresponding K_a value, $(2.7 \pm 0.2)\times10^5$ M^{-1}, is still a factor of two smaller than the reported value, suggesting the possibility that in-source dissociation was not completely eliminated under these conditions.

The ES-MS binding measurements were also carried out in negative ion mode. Shown in Figures 1e and 1f are mass spectra acquired in negative ion mode for a solution of Lg (12 μM) and PA (10 μM) using accumulation times of 0.8 and 0.4 s, respectively. Imidazole (10 mM) was also added to minimize the occurrence of in-source dissociation. Inspection of the mass spectra reveals signal corresponding to deprotonated Lg^{n-} and $(Lg+PA)^{n-}$ ions, at n = 6 to 8. Notably, the corresponding *R* values are significantly larger than those measured in positive ion mode under the same conditions (Figure 2). Furthermore, the average K_a value $(3.8\pm0.1)\times10^5$ M^{-1}, which was obtained from replicate measurements performed at four different concentrations, is in good agreement with the reported value of $(5.0\pm0.2)\times10^5$ M^{-1} (Table 1).[21]

The larger *R* values measured in negative ion mode compared to those measured in positive ion mode confirm that, even with the addition of imidazole to the solution, a fraction of the protonated $(Lg+PA)^{n+}$ ions are lost due to in-source dissociation. At

present, the origin of the differential stabilities of the protonated and deprotonated ions of the (Lg+PA) complex is not known but presumably reflects differences in the structure of the Lg ions arising from differences in the nature of the charges and their locations. Our laboratory is currently carrying out a comparative study, employing thermal dissociation rate constant measurements and molecular dynamics simulations, of the structures and stabilities of protonated and deprotonated (Lg+FA) ions. The results of this study will be reported in a forthcoming manuscript.

Association constants for the (Lg+FA) complexes determined by the direct ES-MS assay

Having established optimal experimental/instrumental conditions for quantifying the interaction between Lg and PA, the direct ES-MS assay was used to measure binding constants for the interactions between Lg and the other FAs (CpA, CA, LA, MA and SA). Shown in Figure 3 are illustrative ES mass spectra obtained in negative ion mode for aqueous solutions (pH 8.5) of Lg with one of the five other FAs, at ligand concentration ranging from 10 to 52 μM. Again, imidazole (10 mM) was added to the solutions to protect against in-source dissociation. Ion signal corresponding to the deprotonated (Lg+FA) complexes was detected for all of the FAs except for CpA, where no ions corresponding to the (Lg+CpA) complex were observed. Increasing the concentration of CpA from 52 to 100 μM (where 70% of Lg is expected to be bound to CpA, based on the reported affinity of $2.6\times10^4\ M^{-1}$)[21] gave similar results. The complete absence of signal corresponding to the (Lg+CpA) complex indicates that the gaseous ions are highly susceptible to in-source dissociation.

The average K_a values determined for CA, LA, MA and SA, from replicate measurements performed at multiple ligand concentrations, are listed in Table 1. In order

to more easily compare the K_a values measured by ES-MS with the values expected based on fluorescence measurements, the logarithm of K_a (determined by either ES-MS or competitive fluorescence) is plotted versus FA acyl chain length in Figure 4. It can be seen that the K_a values determined by ES-MS for the longer FAs, MA and SA, are in excellent agreement with the values expected based on the linear trend established from the fluorescence-derived K_a values.[21] In contrast, the K_a value measured for LA is only 25% of the reported value; the deviation is even more pronounced for CA, where the K_a is only 8% of the reported value. The discrepancy in the K_a values indicates that the deprotonated ions of (Lg+CA) and (Lg+LA) undergo in-source dissociation. The susceptibility of the deprotonated ions of the (Lg+FA) complexes composed of the shorter FAs to in-source dissociation can be explained, at least in part, by the results of recent time-resolved thermal dissociation experiments performed on deprotonated $(Lg+FA)^{7-}$ ions composed of LA, MA, PA and SA.[26] In this study it was found that the gaseous $(Lg+FA)^{7-}$ ions adopt one of two kinetically distinct structures, referred to as the *fast* and *slow* components. In the *fast* component, the FA ligand is stabilized predominantly by protein-lipid interactions, while for *slow* component, H-bonds between the ligand carboxyl group and Lg also contribute to the stability of the complex. Furthermore, over the temperature range investigated (26 to 66 ^{o}C), the kinetic stabilities of the *fast* components decreased with decreasing acyl chain length.[26] Based on these results it is reasonable to expect that the effect of in-source dissociation on the relative abundance of the $(Lg+FA)^{n-}$ ions will increase with decreasing acyl chaing length.

Association constants for the (Lg+FA) complexes determined by the reference ligand ES-MS assay

Recently, our laboratory developed a competitive ligand binding assay for the quantification of protein-ligand interactions that are labile in the gas phase and undergo partial or complete in-source dissociation during ES-MS analysis.[22] The method, referred to as the reference ligand ES-MS assay, was used in the present study to quantify the interactions between Lg and CpA, CA and LA. To implement the assay an appropriate L_{ref} is needed. As discussed above, the similarity in the binding constants measured for the (Lg+PA) complex directly by ES-MS in negative ion mode and by fluorescence suggests that in-source dissociation of the $(Lg+PA)^{n-}$ ions was negligible under the experimental conditions used. Therefore, PA was deemed a suitable L_{ref} for these measurements. Shown in Figure 5a is an ES mass spectrum acquired for a solution of Lg (12 μM), PA (10 μM) and imidazole (10 mM); the mass spectrum shown in Figure 5b was obtained for the same solution as above but with the addition of CpA (52 μM). Although ions corresponding to the (Lg+CpA) complex were not detected, the addition of CpA to the solution resulted in a decrease in the fraction of Lg bound to PA, indicating the presence of specific binding between Lg and CpA in solution. Plotted in Figure 6 is the fraction of bound (to PA) and unbound Lg determined by direct ES-MS measurements performed at fixed concentrations of Lg (12 μM) and PA (10 μM) and varying concentrations of CpA (0, 39, 52 and 65 μM). Notably, the fraction of Lg bound to PA decreased with increasing concentration of CpA. Analysis of the ES-MS data using the approach described above yields an average K_a value of $(2.3\pm0.3)\times10^4$ M^{-1} for the (Lg+CpA) complex (Table 1). Importantly, this value is indistinguishable, within experimental error, from the fluorescence-derived value of $(2.6\pm0.5)\times10^4$ M^{-1}.[21]

The reference ligand ES-MS method was also used to establish the binding constants for the (Lg+CA) and (Lg+LA) complexes. Again, PA was used as L_{ref} for these measurements. Shown in Figure S1 are illustrative ES mass spectra acquired for solutions of Lg and PA, in the absence and presence of either CA or LA. As outlined above, data analysis was carried out differently than was the case for the (Lg+CpA) complex because ions corresponding to the (Lg+CA) and (Lg+LA) complexes could still be detected.[22] The binding constant determined for (Lg+LA) complex using the reference ligand ES-MS assay, $(1.1\pm0.2)\times10^5$ M^{-1}, is in excellent agreement with the reported value, $(1.3\pm0.1)\times10^5$ M^{-1}. The value measured for (Lg+CA) complex, $(4.6\pm0.5)\times10^4$ M^{-1}, is also in reasonable agreement with the value (6×10^4 M^{-1}) estimated from the linear dependence of log K_a on acyl chain length (Figure 4).

Conclusions

In summary, the use of ES-MS to quantify protein-fatty acid complexes in aqueous solution is described. The interactions between Lg and a series of linear, saturated FAs (CpA, CA, LA, MA, PA and SA) were investigated using both the direct ES-MS assay and the reference ligand ES-MS method. The binding constant measured for PA at 25 °C and pH 8.5 using the direct ES-MS assay, performed in negative ion mode and utilizing a stabilizing solution additive (imidazole), is in good agreement with the value measured using a competitive fluorescence assay. The affinities measured directly for MA and SA are also consistent with values expected based on the linear dependence of the log K_a values with acyl chain length established from the fluorescence-derived binding data for related FAs. However, the K_a values measured directly for LA and CA were significantly smaller than the expected values. In the case of CpA, no

binding was detected by direct ES-MS measurement. These results indicate the occurrence of in-source dissociation, which reduces the relative abundance of the gaseous $(Lg+FA)^{n-}$ ions, for these shorter FAs. Notably, in-source dissociation was prevalent in the ES-MS binding measurements performed in positive ion mode for all of the (Lg+FA) complexes investigated. The reference ligand ES-MS assay was used to measure binding constants for the Lg interactions with the shorter FAs. Importantly, the measured K_a values for CpA and LA are in excellent agreement with values measured using the fluorescence assay.

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Figure captions

Figure 1. ES mass spectra obtained in positive ion mode for aqueous ammonium acetate (10 mM, pH 8.5) solutions of (a) Lg (12 μM), PA (10 μM), accumulation time 0.8 s; (b) Lg (12 μM), PA (10 μM), 0.4 s; (c) Lg (12 μM), PA (10 μM), imidazole (10 mM), 0.8 s; (d) Lg (12 μM), PA (10 μM), imidazole (10 mM), 0.4 s, and in negative ion mode for aqueous ammonium acetate (10 mM, pH 8.5) solutions of (e) Lg (12 μM), PA (10 μM), imidazole (10 mM), accumulation time 0.8 s, and (f) Lg (12 μM), PA (10 μM), imidazole (10 mM), 0.4 s. The solution temperature for all measurements was 25 ^{o}C.

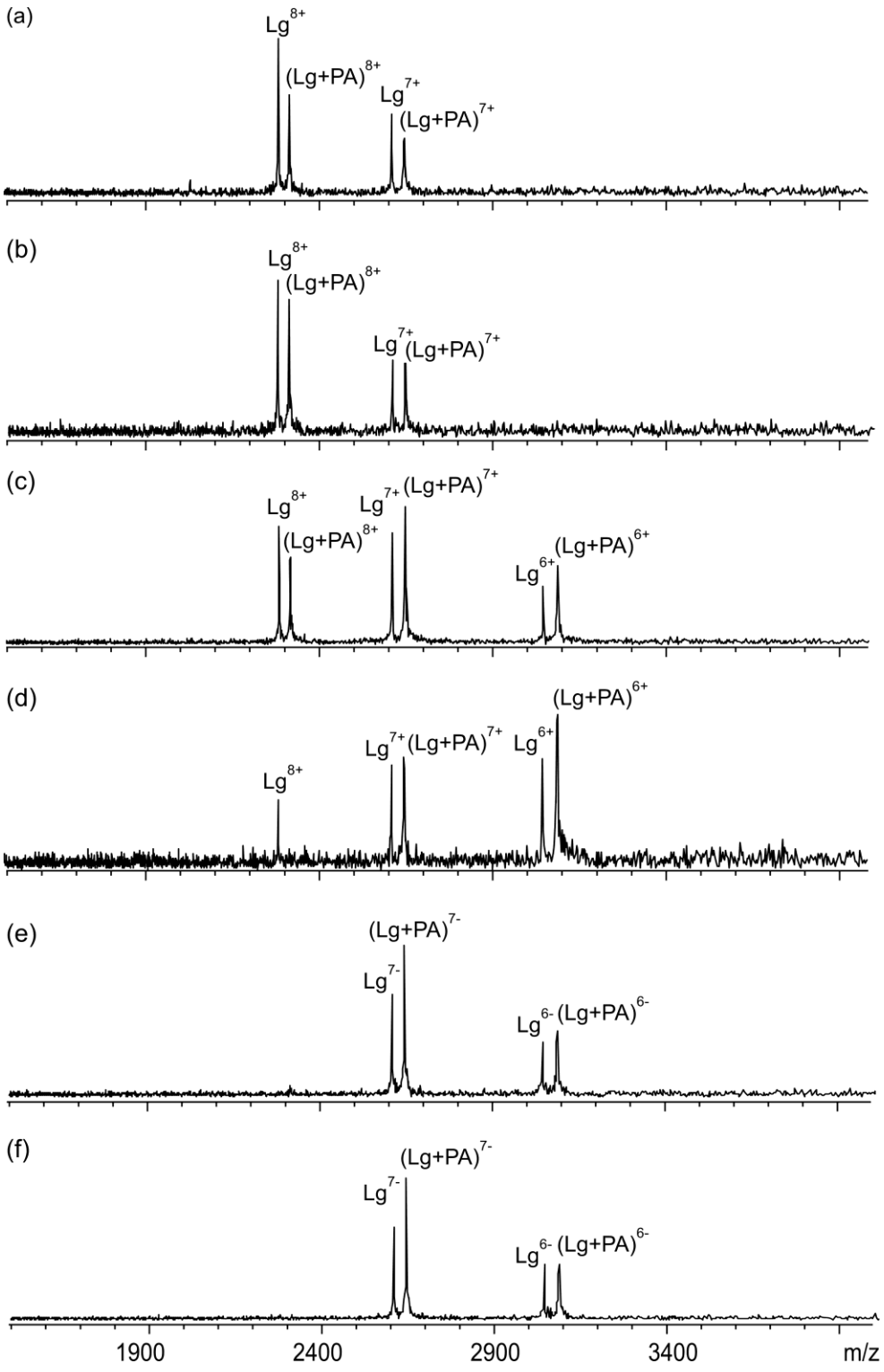
Figure 2. Influence of hexapole accumulation time on the *R* value determined by ES-MS in positive ion mode for aqueous ammonium acetate (10 mM, pH 8.5) solutions of Lg (12 μM) and PA (10 μM), (■); Lg (12 μM), PA (10 μM) and imidazole (10 mM), (▲); and in negative ion mode for an aqueous ammonium acetate (10 mM, pH 8.5) solution of Lg (12 μM), PA (10 μM), imidazole (10 mM), (●). The dashed line indicates the expected *R* value based on the K_a value reported in reference 21. The solution temperature for all measurements was 25 ^{o}C.

Figure 3. ES mass spectra acquired for aqueous ammonium acetate (10 mM, pH 8.5) solutions of (a) Lg (12 μM), CpA (52 μM), (b) Lg (12 μM), CA (51 μM), (c) Lg (10 μM), LA (30 μM), (d) Lg (10 μM), MA (22 μM), and (e) Lg (11 μM), SA (10 μM). Each solution contained 10 mM imidazole. The solution temperature for all measurements was 25 ^{o}C.

Figure 4. Plot of logarithm of K_a measured by direct ES-MS assay (▲), reference ligand ES-MS method (Δ) and competitive fluorescence assay (●) versus number of methylene groups (x) for the (Lg + $CH_3(CH_2)_xCOOH$) complexes. The dashed line corresponds to linear least squares fit of the log K_a values measured by fluorescence [21]. The ES-MS binding measurements were performed at pH 8.5 and 25 ^oC; the fluorescence data were determined at pH 8.3 and 25 ^oC.

Figure 5. ES mass spectra obtained in negative ion mode for aqueous ammonium acetate (10 mM, pH 8.5) solutions of (a) Lg (12 μM), PA (10 μM), and (b) Lg (12 μM), PA (10 μM), CpA (52 μM). Each solution contained 10 mM imidazole. The solution temperature for all measurements was 25 ^oC.

Figure 6. Distribution of the relative abundance of Lg (≡ P) and (Lg+PA) complex (≡ PL_{ref}) measured by ES-MS in negative ion mode for aqueous ammonium acetate (10 mM, pH 8.5) solutions of Lg (12 μM), PA (10 μM) and CpA at concentrations of 0, 39, 52 and 65 μM. Each solution contained 10 mM imidazole. The solution temperature for all measurements was 25 ^oC.


(a)
Lg8+
(Lg+PA)8+
Lg7+
(Lg+PA)7+
(b)
Lg8+
(Lg+PA)8+
Lg7+
(Lg+PA)7+
(c)
Lg8+
(Lg+PA)8+
Lg7+
(Lg+PA)7+
Lg6+
(Lg+PA)6+
(d)
Lg8+
Lg7+
(Lg+PA)7+
Lg6+
(Lg+PA)6+
(e)
Lg7-
(Lg+PA)7-
Lg6-
(Lg+PA)6-
(f)
Lg7-
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Lg6-
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1900
2400
2900
3400
m/z

Figure 1

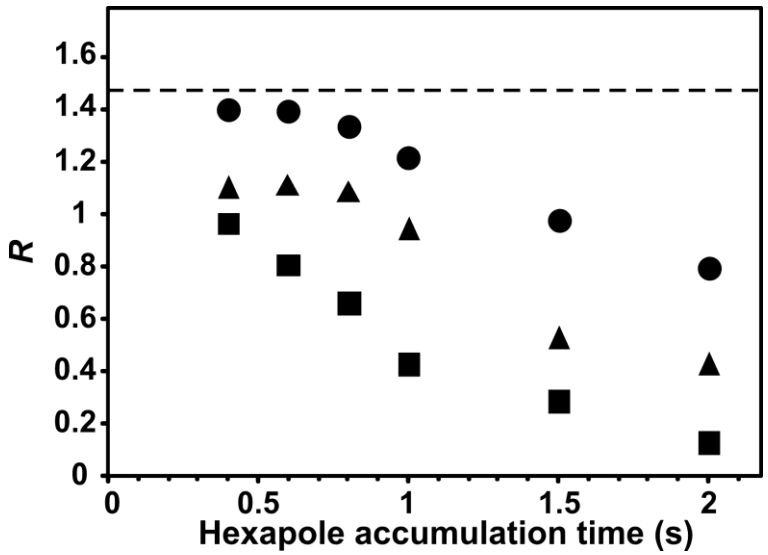
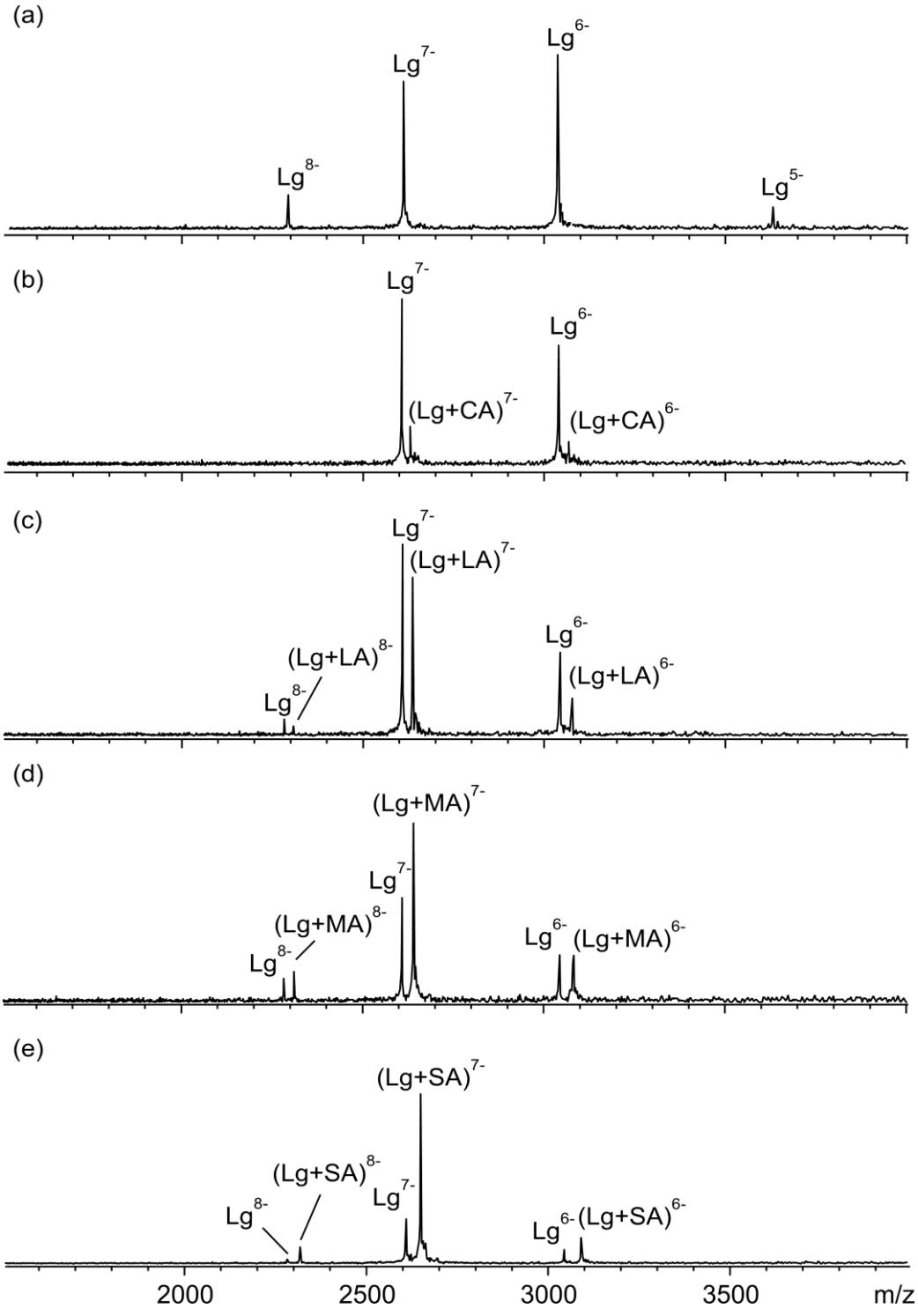


Figure 2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Lg7-
Lg6-
Lg5-
(b)
Lg7-
Lg6-
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(c)
Lg7-
(Lg+LA)7-
(Lg+LA)8-
Lg8-
Lg6-
(Lg+LA)6-
(d)
(Lg+MA)7-
Lg7-
(Lg+MA)8-
Lg8-
Lg6-
(Lg+MA)6-
(e)
(Lg+SA)7-
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2000
2500
3000
3500
m/z

Figure 3

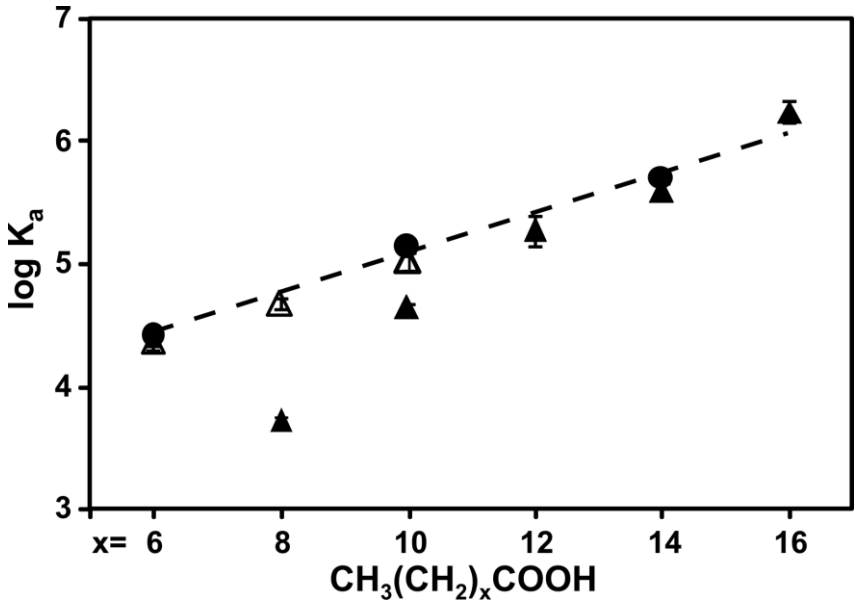
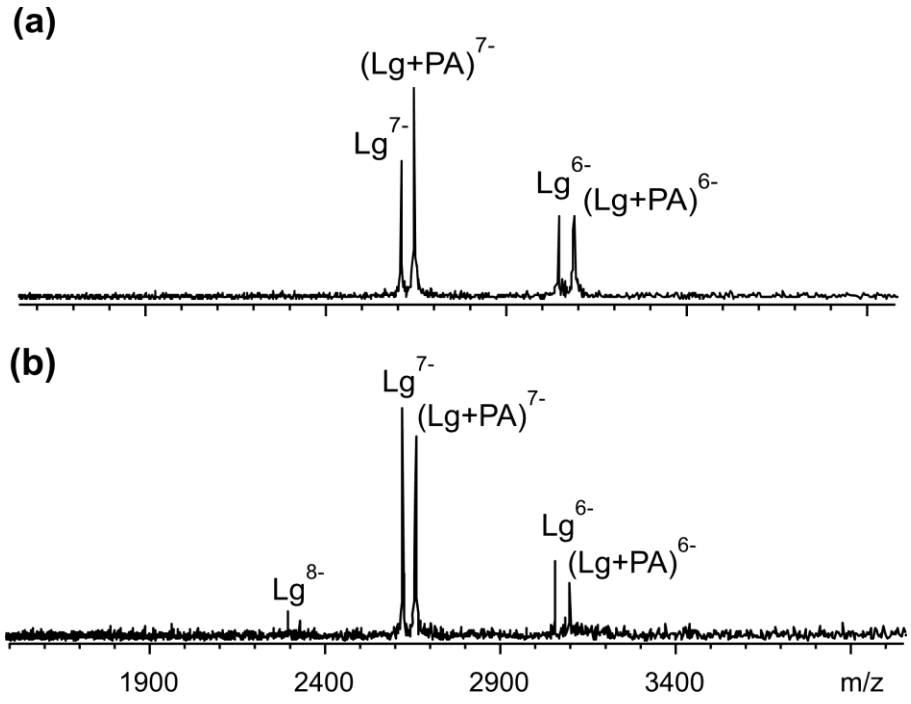


Figure 4


(a)
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Lg7-
Lg6-
(Lg+PA)6-
(b)
Lg7-
(Lg+PA)7-
Lg6-
(Lg+PA)6-
Lg8-
1900
2400
2900
3400
m/z

Figure 5

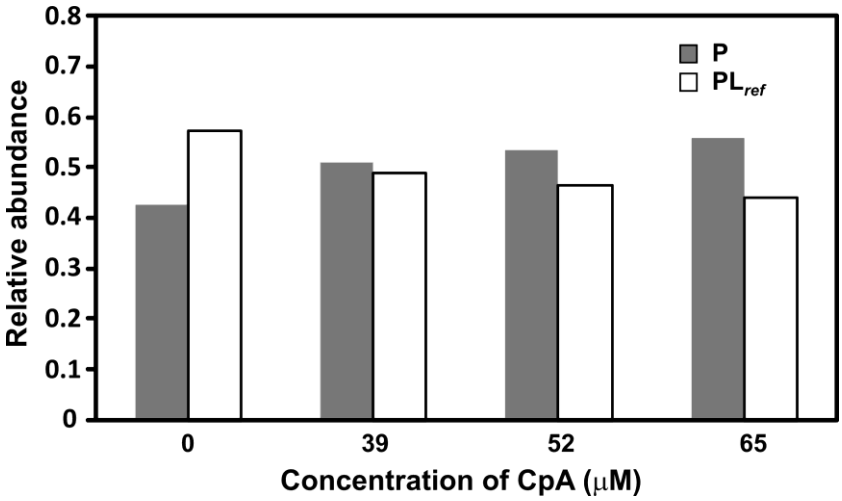
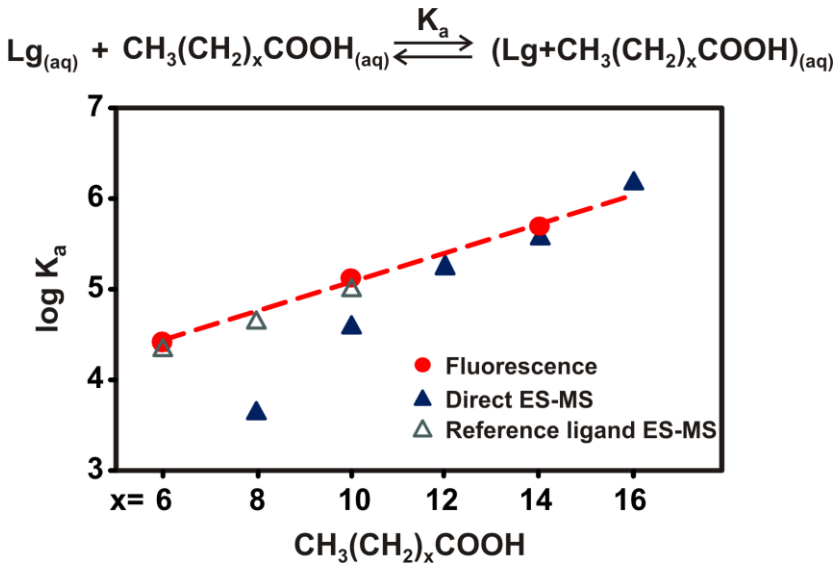

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative abundance
P
PL$_{ref}$
0
39
52
65
Concentration of CpA (μM)

Figure 6

Graphical Abstract



Synopsis: Affinities of protein-fatty acid complexes are quantified using the direct ES-MS assay and reference ligand ES-MS method.

Supplemental Information

Quantifying Protein-Fatty Acid Interactions using Electrospray Ionization Mass Spectrometry

Lan Liu, Elena N. Kitova and John S. Klassen

Table S1. Association constants (K_a) for Lg binding to a series of fatty acids (CpA, CA, LA, MA, PA and SA) determined by the direct ES-MS assay performed in negative ion mode on aqueous solutions at pH 8.5 and 25 ^{o}C and varying concentrations of Lg and fatty acids.[a]

[Lg] (μM)	[CpA] (μM)	[CA] (μM)	[LA] (μM)	[MA] (μM)	[PA] (μM)	[SA] (μM)		K_a (M^{-1})
12	39							NB[b]
12	52							NB[b]
12	65							NB[b]
12		19						$(4.3\pm0.1)\times10^3$
12		35						$(4.7\pm0.3)\times10^3$
12		51						$(4.6\pm0.3)\times10^3$
							Average K_a	$(4.5\pm0.1)\times10^3$
11			40					$(4.3\pm0.2)\times10^4$
11			30					$(4.0\pm0.2)\times10^4$
11			20					$(4.3\pm0.2)\times10^4$
13			34					$(3.8\pm0.1)\times10^4$
							Average K_a	$(4.1\pm0.1)\times10^4$
10				22				$(1.8\pm0.3)\times10^5$
12				12				$(1.6\pm0.1)\times10^5$
12				11				$(2.1\pm0.4)\times10^5$
11				15				$(1.9\pm0.2)\times10^5$
							Average K_a	$(1.9\pm0.1)\times10^5$
27					10			$(3.6\pm0.2)\times10^5$
12					10			$(3.8\pm0.1)\times10^5$
16					10			$(3.7\pm0.5)\times10^5$
10					10			$(4.1\pm0.1)\times10^5$
							Average K_a	$(3.8\pm0.1)\times10^5$

13		6	$(1.6\pm0.1)\times10^6$
16		6	$(1.7\pm0.3)\times10^6$
11		10	$(1.5\pm0.2)\times10^6$
		Average K_a	$(1.6\pm0.1)\times10^6$

a. Errors correspond to one standard deviation. b. NB ≡ No binding detected.

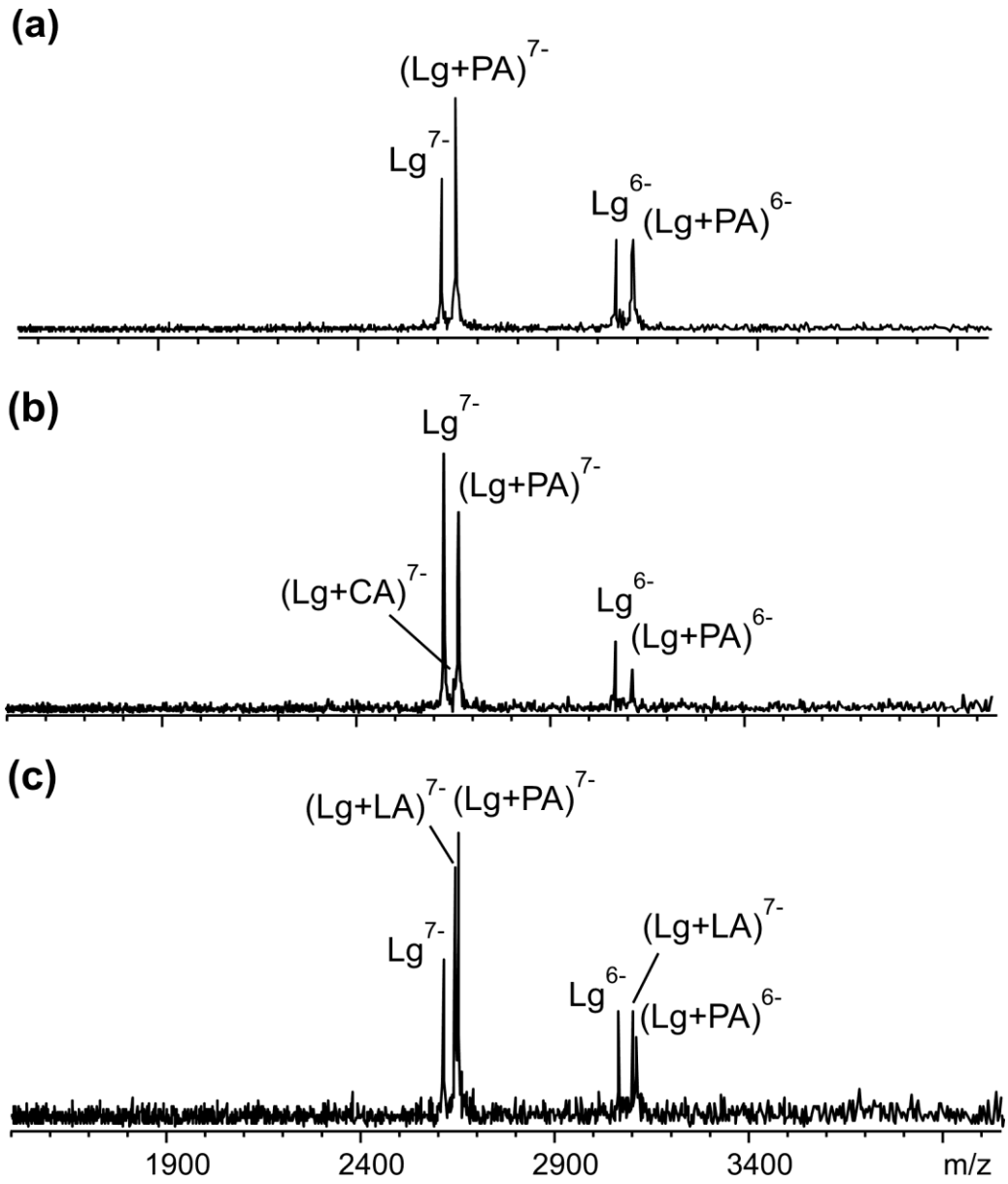


Figure S1. ES mass spectra acquired for aqueous solutions (pH 8.5) of (a) Lg (12 μM), PA (10 μM), (b) Lg (12 μM), PA (10 μM), CA (51 μM), and (c) Lg (12 μM), PA (10 μM), LA (30 μM). Each solution contained 10 mM ammonium acetate and 10 mM imidazole.