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# Development of an ESI-MS screening method for evaluating binding affinity between integrin fragments and RGD-based peptides

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**Abstract**

Electrospray ionization mass spectrometry (ESI-MS) is used to evaluate binding affinities between integrin fragments and RGD-based peptide ligands. The integrin fragment peptides synthesized and studied were selected based on crystallographic and literature data which highlighted the specific binding pockets for the RGD motif in intact integrin proteins. Relative binding constants were determined by fitting the data obtained from ESI-MS titration experiments to a quadratic equation based on a 1:1 association model. Frontal analysis capillary electrophoresis (FACE) was used as a complementary solution phase technique to measure the absolute binding constants for the same host–guest systems and the results from both techniques were compared. Gas phase collision activated dissociation threshold measurements in an ion trap mass spectrometer also were carried out on the complexes to further validate binding constant measurements. Better correlation was observed between the MS/MS and CE results, than for ESI-MS titration. The ESI-MS titration results showed some correlation; however, it is apparent that there are important methodological shortcomings which need to be addressed. Several factors related to the titration method, ionization efficiencies, enthalpy/entropy contributions during phase transfer, and solvent effects are discussed. This work introduces a first approach to development of a high throughput ESI-MS-based method suitable for screening potential peptide-based drug compounds that target integrins on cancerous cells. It also serves as a cautionary tale to those interested in performing similar types of analyses.


*Keywords:* Integrin; RGD motif; Collision threshold; Binding constant; Titration

## 1. Introduction

Integrins are a group of ubiquitous transmembrane proteins that are known to mediate a number of cellular activities in biological systems. Many of the cellular processes mediated by integrins are fostered by noncovalent interactions between integrins and extracellular matrix proteins (ECM) containing the RGD (Arg-Gly-Asp) recognition motif [1,2]. This characteristic has made the integrin–RGD recognition system a very important one for studying integrin involvement in cellular processes such as adhesion, signaling, differentiation, and apoptosis. In addition, because of the roles of integrins in diseases, the integrin–RGD recognition system has become a major target of interest for developing peptidomimetic drugs and pharmaceuticals.

Many studies have been carried out which investigate the modality by which RGD-based peptides interact with integrins; with the main goal being inhibition of unfavorable integrin interactions believed to be detrimental to normal cell life. For example, both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been found to play major roles in angiogenesis, a process through which tumor growth is effected via the formation of new blood vessels [3,4]. Peptide inhibition studies have shown that monoclonal antibodies and other small molecules interact with these receptors and consequently inhibit the angiogenic process. A good example is the methylated cyclic RGD peptide, Cilengitide (cyclo(-RGDf[NMe]V-)), which has now entered phase II clinical trials [5,6].

Structural information on integrin–ligand interactions have typically been obtained from traditional techniques like X-ray diffraction (XRD) and nuclear magnetic resonance (NMR) spectroscopy; as well as by electron microscopy and molecular modeling [7]. Also included on the list are

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traditional biochemical techniques like immuno-affinity labeling, tagging, and site-directed mutagenesis. Due to the fact that three-dimensional structures of most of the integrin subtypes are still not available, it remains a challenging problem to determine what factors control specificity between different integrin/ligand pairs [8]. More techniques are thus needed to gain a better understanding in this area. In this work, we explore the use of molecular recognition mass spectrometry; employing an electrospray ionization source interfaced to a quadruple ion trap as a complementary tool for investigating integrin–ligand interactions and for extracting relative quantitative binding information for the noncovalent complexes between selected integrin fragments and RGD-containing peptides.

ESI-MS offers some advantages over other techniques like NMR and XRD. One obvious advantage is its ability to measure the molecular weights of molecules in their bound and unbound forms. In addition, ESI-MS offers greater speed, sensitivity, and flexibility compared to traditional solution phase techniques. Other inherent capabilities of MS, like its amenability to studying gas phase behavior of complex ions, including their decomposition through collision-induced dissociation, make it possible to obtain useful information about structure, stoichiometry and solvation that are not generally available using other methods.

A number of solution phase and gas phase-based techniques have been introduced for molecular recognition studies by soft ionization mass spectrometry. Many of these techniques have been detailed in recent review articles [9–11]. One of the most useful methods for obtaining quantitative binding information through ESI-MS is the titration method. In this approach, the gas phase ion abundances of a noncovalent host–guest complex and free (unbound) host, resulting from the variation of guest concentration in solution, can be fit to algorithms based from solution phase host–guest association models in order to extract binding constants. Values are determined using graphical methods such as the well-known Scatchard analysis [12] and similar semi-logarithmic plots [13,14].

In this work, we have used the titration method to evaluate binding affinities between selected integrin fragments, obtained through standard peptide synthesis methods, and selected RGD-based peptides. We report a data treatment method that fits the gas phase ion abundance data obtained from ESI-MS to a quadratic equation based on 1:1 association between the host and guest peptides. Dissociation constants are then obtained from the fit of this quadratic equation to experimental data. This particular method was first used by Schug et al. where it was shown that relative affinity and selectivity values in small-molecule chiral recognition systems correlated well with that determined by microcalorimetric methods and chiral liquid chromatography [14]. Lacking chromatographic or calorimetric data for the RGD–integrin system studied here, a complementary solution phase-based capillary electrophoresis binding assay is used to correlate MS results.

Capillary electrophoresis is a popular solution phase technique for evaluating molecular associations in many host–guest systems. It has many advantages over other solution phase techniques, including short analysis time and small sample consumption. In addition, there are at least six different experimental approaches that can be explored using CE for quantitative measurement of binding affinities [15].

Gas phase collision threshold measurements are also employed to evaluate the gas phase binding affinity of the integrin fragments and the RGD-based peptides. By varying the collisional excitation in small increments, the voltage required to dissociate 50% of a selected complex ion ($V_{50}$) was determined [16]. This value measures the stability of the complex in the absence of solvation. As such, relative binding affinities obtained by this method should be viewed with caution when compared to those taken strictly from solution phase methods.

The goal of this work is to extend the use of molecular recognition mass spectrometry techniques to studying biochemically relevant peptide–peptide interactions. This work represents a first approach for studying the viability of assembling the specificity of protein–ligand interactions through the analysis of peptide-based contacts in integrin–RGD recognition systems by mass spectrometry. Specific integrin fragment peptides were chosen based on crystallographic and literature data which highlighted the specific binding pockets in integrin subunit proteins [17–19]. Commercially available and synthetic RGD-based peptides were screened against the integrin fragments to study changes in selectivity and affinity resulting from sequential variation of the RGD peptide ligands. Significant variation is shown when single residues adjacent to the RGD motif are permuted. This fundamental, yet application oriented, approach is expected to be useful in related future high throughput combinatorial approaches to studying protein–ligand interactions on a smaller scale. Relevant points, including specific advantages and disadvantages are discussed.

## 2. Experimental

### 2.1. Materials

All synthetic peptides used (Table 1) were prepared by a solid-phase method with $N^{\alpha}$-Fmoc/$^{t}$Bu chemistry. $N^{\alpha}$-Fmoc protected amino acids and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink amide resin) were purchased from EMD Biosciences (San Diego, CA), Advanced Chemtech (Louisville, KY), Peptides International (Louisville, KY), and Senn Chemicals (La Jolla, CA). Other chemicals and solvents were purchased from the following sources: trifluoroacetic acid (TFA; VWR); *N*,*N*-diisopropylethylamine (DIEA; Advanced ChemTech), anisole, 1,2-ethanedithiol, dimethyl sulfide, triisopropylsilane, piperidine, acetic anhydride, dichloromethane (Aldrich and Fisher); *N*,*N*-dimethylformamide (DMF), ethyl ether (EMD Biosciences); 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt; Advanced ChemTech); HPLC grade acetonitrile (EMD Biosciences). Glacial acetic acid, ammonium acetate, and LC–MS grade water were supplied by J.T. Baker (Phillipsburg, NJ); and LC–MS grade methanol from EMD (Canada). The peptides, GRGDsP, RGD, and Glycoprotein IIb fragment 206-306 (TDVNGDGRHDL), were obtained commercially from Bachem California Inc. (Torrance, CA).

Table 1
Primary sequences of the synthesized peptides

| Peptide | Sequence | Mass |
|---|---|---|
| Fibronectin III segment ($Fib_{III}f$) | Ac-Ala-Val-Thr-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-$NH_2$ | 1144.6[a] (1144.6[b]) |
| Integrin $\beta_3$ segment 1 [$\beta_3$f1 (110–131)] | Ac-Met-Asp-Leu-Ser-Tyr-Ser-Met-Lys-Asp-Asp-Leu-Trp-Ser-Ile-$NH_2$ | 1743.6 (1743.8) |
| Integrin $\beta_3$ segment 2 [$\beta_3$f2 (211–220)] | Ac-Ser-Val-Ser-Arg-Asn-Arg-Asp-Ala-Pro-Glu-$NH_2$ | 1170.5 (1170.6) |
| Integrin $\alpha_{IIb}$ segment [$\alpha_{IIb}$f (184–193)] | Ac-Gly-Asp-Pro-Gly-Gly-Tyr-Tyr-Phe-Leu-Gly-$NH_2$ | 1041.4 (1041.5) |
| Integrin $\alpha_v$ segment [$\alpha_v$f (213–220)] | Ac-Ala-Gln-Ala-Ile-Phe-Asp-Asp-Ser-Tyr-Leu-Gly-$NH_2$ | 1239.3 (1239.6) |

[a] Mass observed by ESI.
[b] Calculated mass. Letter "f" in the above nomenclature indicates that these peptides are fragments of the respective integrin subunits.

GRGDSP was obtained from Bachem Bioscience Inc. (King of Prussia, PA) and GRGDNP was purchased from Biomol (Plymouth Meeting, PA). Sodium phosphate, acetic acid, dimethyl sulfoxide (DMSO), sodium hydroxide, and 85% phosphoric acid were all purchased from Aldrich (Milwaukee, WI). Capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). All amino acids were of the L-configuration unless stated otherwise. The purity of the peptides was checked by analytical reverse-phase high-pressure liquid chromatography (HPLC) using a VYDAC 218TP104 column (4.6 mm × 250 mm), and the structures of the purified peptides were characterized by electrospray mass spectrometry. The purification of the peptides was achieved using an Agilent Technology 1100 series HPLC instrument with a C4 or C18-bonded silica columns (VYDAC, 10 mm × 250 mm, 10 μm, 300 Å, semi-preparative columns, Cat. Nos. 214TP1010 and 218TP1010, respectively). The peptides were eluted with a linear gradient of acetonitrile in 0.1% aqueous TFA at a flow rate of 3.0 mL/min.

### 2.2. Peptide synthesis and purification

Many peptides used in this study were synthesized using standard solid-phase peptide chemistry with $N^{\alpha}$-Fmoc/$^{t}$Bu protection group strategy. A solid support, Rink amide resin [20], was used to prepare peptides with C-terminal amide. After removal of $N^{\alpha}$-Fmoc protection group on the resin with 20% piperidine in DMF, the released free amine was coupled to a $N^{\alpha}$-Fmoc-protected amino acid which was activated with HBTU. These steps were repeated to complete sequences, and each coupling reaction was monitored by Kaiser ninhydrin [21], 2,4,6-trinitrobenzenesulfonyl acid (TNBS) [22], and chloranil tests [23]. The resulting peptides were cleaved from the resin, deprotected with TFA, and purified with reverse-phase HPLC.

The peptides were synthesized by manual shaking in room temperature or by microwave-assisted peptide synthesis. For the synthesis by manual shaking, Rink amide resin (substitution 0.75 mmol/g) was washed with DMF (3× 1 min), and $N^{\alpha}$-Fmoc protection group was removed with 20% piperidine in DMF (1× 5 min and 1× 30 min). Then, the resin was washed with DMF (3× 1 min), and the first amino acid was coupled to the free amine on the resin using pre-activated $N^{\alpha}$-Fmoc amino acid in DMF over 2 h or until the Kaiser ninhydrin and the 2,4,6-trinitrobenzenesulfonyl acid (TNBS) tests became negative. For the pre-activation, $N^{\alpha}$-Fmoc amino acid (4 equiv.), HBTU (4 equiv), and HOBt (4 equiv.) in DMF were stirred over 30 min with DIEA (8 equiv.). In case of incomplete reaction indicated by positive test results, the resin was washed with DMF (3× 1 min), and the coupling reaction with fresh pre-activated $N^{\alpha}$-Fmoc amino acid (2 equiv.) was repeated. If double coupling did not result in negative Kaiser ninhydrin and TNBS tests, the resin was washed with DMF (3× 1 min), and the unreacted amine was capped with acetic anhydride (20 equiv.) in DMF over 10 min. To check the presence of unreacted secondary amine group of proline, a chloranil test was used. When the coupling reaction was completed, the resin was washed with DMF (3× 1 min), and the Fmoc-removal and amino acid-coupling steps were repeated until all amino acids in the sequence were coupled. After the construction of the peptide was done, the resin was washed with DCM (5× 1 min) and dried under vacuum.

On the other hand, microwave-assisted peptide synthesis was accomplished using a Discover SPS manual microwave peptide synthesizer (CEM Corporation, Matthews, NC) with open vessel mode and fiber optic to monitor temperature of reaction mixture. Rink amide resin (substitution 0.75 mmol/g) was washed with DMF (3× 1 min), and $N^{\alpha}$-Fmoc was removed with 20% piperidine in DMF containing 0.1 M HOBt over 5 min with maximum microwave power and temperature set as 50 W and 75 °C, respectively. Then, the resin was washed with DMF (3× 1 min), and the first amino acid was coupled to the free amine on the resin using pre-activated $N^{\alpha}$-Fmoc amino acid in DMF over 5–10 min with maximum microwave power and temperature set as 25 W and 75 °C, respectively.

The dried resin was treated under vacuum with a cleavage mixture consisting of trifluoroacetic acid (18.0 mL), dimethyl sulfide (0.5 mL), 1,2-ethanedithiol (0.5 mL), and anisole (1.0 mL) for 90 min in a disposable 50 mL polystyrene tube. Then, the resin was filtered and the TFA solution was concentrated with a gentle stream of nitrogen to a volume of ca. 3 mL. The peptide was precipitated by adding cold ethyl ether (40 mL) and centrifuged to remove the ether solution. The peptide was washed with another aliquot of cold ethyl ether (40 mL) and dried under vacuum.

To purify the peptides the crude mixture was dissolved in 50% aqueous acetic acid, and insoluble materials were centrifuged out. The peptide was purified with HPLC using a semi-preparative VYDAC reverse phase (C4- or C18-bonded) HPLC column with gradient elution at a flow rate of 3.0 mL/min. Approximately 5 mg of the crude peptide was injected to the column each time, and the fraction containing the purified peptide was collected, followed by lyophilization. The purity of the purified peptide was checked by analytical HPLC, using an analytical VYDAC C18-bonded column (4.6 mm × 250 mm) at a

flow rate of 1.0 mL/min with gradients, such as 10–90% acetonitrile in 0.1% aqueous TFA over 40 min; 20–60% over 20 min; and 0–50% over 25 min.

### 2.3. Mass spectrometry

Stock solutions of the peptides were prepared by dissolving known amounts of the peptides in 50/50 methanol/water solutions. Different concentrations used for the titrations were prepared from the stock solutions by dilution with the same solvent mixture. Other modifiers, such as ammonium acetate and acetic acid, were likewise prepared from stock solutions and incorporated directly into the sample mixtures for analysis.

After initial screening to determine which host–guest systems exhibit binding, titration experiments were carried out for corresponding combinations of host ($\alpha_{IIb}$f, $\alpha_v$f) and guest (GRGDNP, GRGDSP or GRGDsP) peptides. For each titration experiment, the concentration of host peptide was held constant at 10 μM while that of the guest was varied between 0.5 and 50 μM. Each titration point was measured in triplicate with each measurement taken as the average of 50 sequential scans. All experiments were carried out in the presence of 100 μM ammonium acetate and 0.5% acetic acid. Analyses were carried out using a LCQ Deca XP Plus ESI-ion trap mass spectrometer from Thermo Electron Corporation (West Palm Beach, FL). The mass spectrometer was equipped with a Thermo Surveyor LC-5 Autosampler and a Surveyor MS Pump. The ESI source was operated at a spray voltage of 4.5 kV in positive ionization mode with a $N_2$ nebulizer flow of 20 arbitrary units. Sample mixtures (20 μL injection) were introduced into the ESI source at a flow rate of 10 μL/min.

### 2.4. Capillary electrophoresis

Experiments were carried out in a buffer condition containing 100 μM $NH_4OAc$, and 0.5% (v/v) HOAc in a water–methanol mixture (50:50) using capillary electrophoresis (CE). The frontal analysis method was used to determine the association constants between receptors and ligands [24]. A series of sample mixtures with fixed receptor concentration (50 μM) and varying ligand concentrations (0.5–10 mM) were injected. DMSO was used as an electro-osmotic flow marker. The capillaries were conditioned before their first use by rinsing with 1 M sodium hydroxide for 5 min, water for 5 min, sodium hydroxide for 1 min, and finally, water for 1 min. The receptors and ligands were premixed for sufficient time (45 min) to ensure the formation of an equilibrium complex. Between each run, the capillary was rinsed (1 min each) with 1 M phosphoric acid, 1 M sodium hydroxide, water, and run buffer. Samples were injected for 5 s via hydrodynamic pressure (0.5 psi). The capillary was maintained at a temperature of 25 °C. Separations were performed in the normal polarity mode with an applied voltage of 15 kV. CE measurements were performed on a Beckman P/ACE MDQ with a 50 μm i.d. × 358 μm o.d. capillary, 40 cm in length (30 cm to the detector). Detection was accomplished by UV absorbance at 200 nm. Data analysis was done with Beckman System Gold software.

## 3. Results and discussion

### 3.1. Integrin fragment–RGD peptide binding by ESI-MS

One of the goals of this study is to demonstrate the applicability of ESI-MS as a screening tool for potential peptide-based anti-tumor agents that will target integrins which are overexpressed in tumor cells. Our results have shown that binding specificity and selectivity between integrin fragments and RGD-containing peptides can be evaluated using ESI-MS. The integrin fragment peptides were chosen because they represent the specific binding pockets in integrin subunit proteins as pointed out by crystallographic and literature data [17–19]. As shown in Fig. 1a–c, the ion abundances corresponding to the host and the guest are easily distinguishable from each other, as well as from those corresponding to the complex in the ESI-MS spectra; making it possible to evaluate binding affinities by monitoring the intensities of the free host and the host–guest complex ions at different host–guest concentrations. The spectra are normalized to the intensity of the ion with the highest response; therefore, it should be emphasized that the ionization responses of the different hosts and guests employed are not always the same. This aspect contributes to further discussion below.

### 3.2. Determination of binding constants

A titration approach was adopted to evaluate dissociation constants from ion abundance data obtained using ESI-MS. A range of guest peptide concentrations (0.5, 1, 2, 5, 10, 20, and 50 μM) were titrated against the host peptide (held fixed at 10 μM concentration). The guest concentration range was chosen so as to cover at least two orders of magnitude while taking into consideration the limits of linearity (at high concentration) and limits of detection (at low concentration) for the instrument. The ion abundances measured from these titration experiments can be assumed to relate to the equilibrium concentrations of the different species in solution according to their respective response factors. Given the equilibrium expression for the host (H)–guest (G) complexation as shown in Eq. (1), we can write an expression for the dissociation constant, $K_d$ (Eq. (2)):

$$H + G \Leftrightarrow HG \tag{1}$$

$$K_d = \frac{[H][G]}{[HG]} \tag{2}$$

This relationship is then used to derive a model for measurement of $K_d$ based on host–guest complex and free host ion abundances:

$$\text{Let } [G] = C_{iG} - [HG] \tag{3}$$

where $C_{iG}$ is the initial concentration of guest. Substituting Eq. (3) into Eq. (2) and further manipulations yield the new expression for $K_d$ in Eq. (4):

$$K_d = \frac{[H](C_{iG} - [HG])}{[HG]} \tag{4}$$

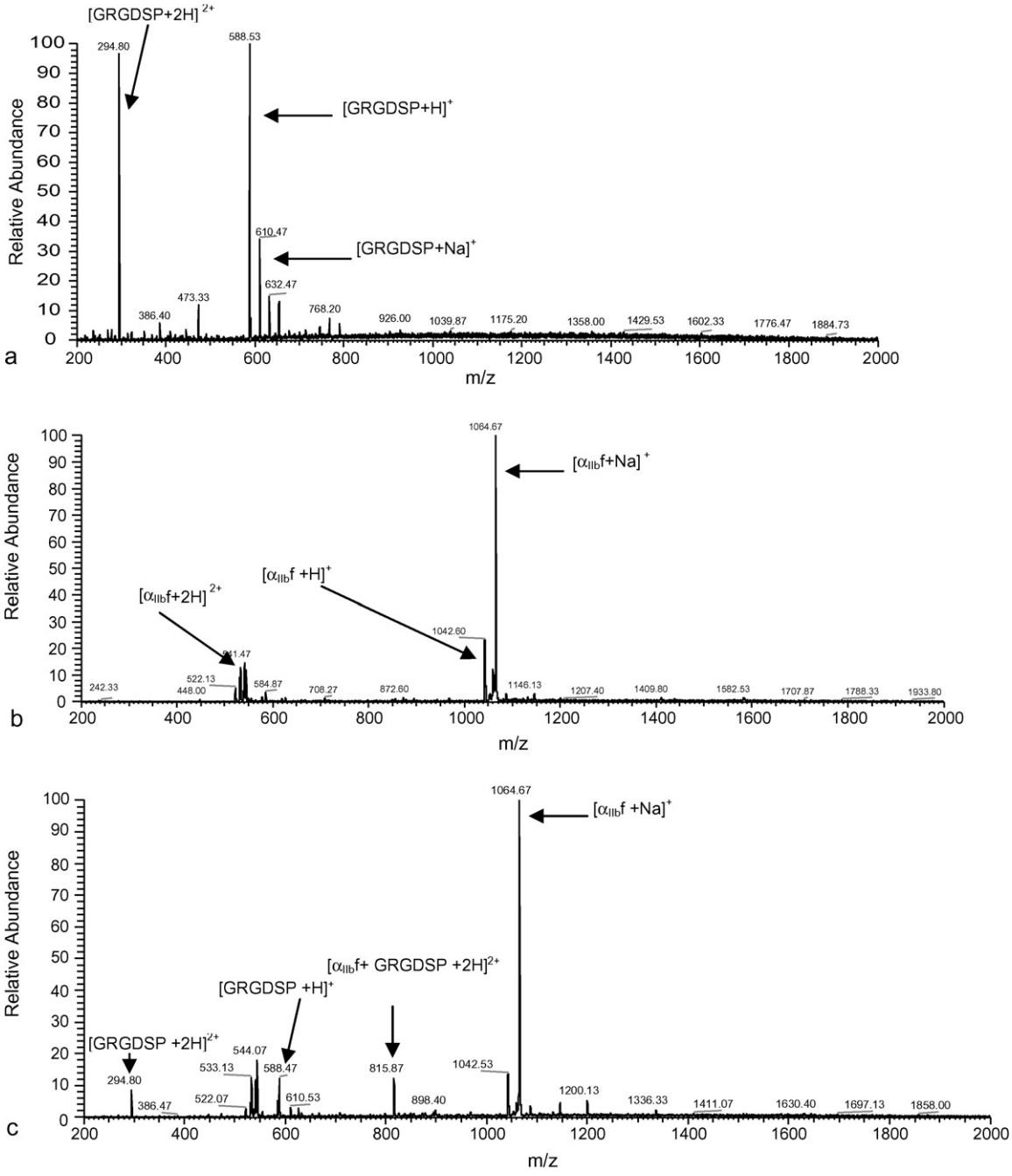


Fig. 1. ESI mass spectra of (a) GRGDSP at 10 μM; (b) $\alpha_{IIb}$f at 20 μM; and (c) mixture of $\alpha_{IIb}$f and GRGDSP each at 10 μM.

If we define degree of association as [HG]/([H] + [HG]), then

$$[\mathrm{HG}] = C_{\mathrm{iH}}\left(\frac{[\mathrm{HG}]}{[\mathrm{H}]+[\mathrm{HG}]}\right) \tag{5}$$

and

$$K_{\mathrm{d}} = \frac{[\mathrm{H}]}{[\mathrm{HG}]}\left(C_{\mathrm{iG}} - \left(C_{\mathrm{iH}}\left(\frac{[\mathrm{HG}]}{[\mathrm{H}]+[\mathrm{HG}]}\right)\right)\right) \tag{6}$$

We can also define the response factor relating the ion intensity of a species to its solution concentration as $f_x = i_x/[\mathrm{X}]$ (where [X] is the equilibrium concentration of species $x$ and $i_x$ is its absolute ion intensity taken from the mass spectrum). Substituting this expression for the different species, followed by subsequent manipulation will yield the expressions:

$$\frac{[\mathrm{H}]}{[\mathrm{HG}]} = \frac{i_{\mathrm{H}}/f_{\mathrm{H}}}{i_{\mathrm{HG}}/f_{\mathrm{HG}}} = \frac{i_{\mathrm{H}}}{i_{\mathrm{HG}}}\left(\frac{f_{\mathrm{HG}}}{f_{\mathrm{H}}}\right) = IF \tag{7}$$

Eq. (7) can be substituted in Eq. (6) to obtain the quadratic expression:

$$(IF)^2 C_{\mathrm{iG}} + IF(C_{\mathrm{iG}} - C_{\mathrm{iH}} - K_{\mathrm{d}}) - K_{\mathrm{d}} = 0 \tag{8}$$

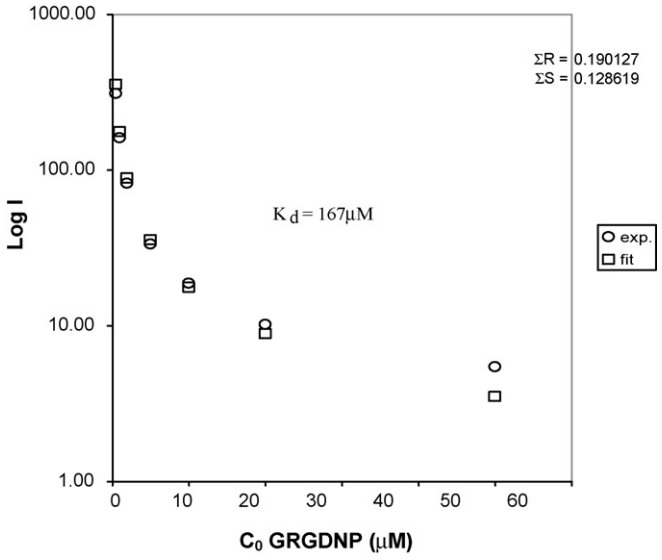


Fig. 2. ESI-MS titration plot for $\alpha_{IIb}$f-GRGDNP. ΣR and ΣS represent, respectively, point-to-point and slope-to-slope weighted difference between the experimental and best model fit data points.

where $C_{iH}$ is the initial concentration of the host and the other variables are defined as above. Eq. (8) represents the model algorithm for 1:1 binding to which the ESI-MS experimental data is fit to obtain $K_d$.

The ratio of ion intensities ($I$) can be obtained from the spectrum for each concentration point. If we assume that the response factor of the host and that of the complex are similar, then $F$ can be approximated to unity. Although this assumption may not be strictly valid, there does not yet exist a suitable method whereby the solution phase equilibrium complex concentration can be determined (and thus the response factor of the complex) without first knowing the association constant of the interaction. This point represents perhaps the biggest disadvantage in the use of a MS-based titration approach for studying molecular recognition by small molecules. Even so, this approach has been previously shown to provide a good estimation of relative solution phase binding strengths [14].

By iteratively solving the quadratic equation (Eq. (8)) with various values of $K_d$ in the range 1–1000 μM; and fitting the curves generated therefrom to our experimental data, the $K_d$ value can be obtained as the one that confers the best fit between the experimental curve and the model quadratic curve [14]. An example of an ESI-MS titration plot for $\alpha_{IIb}$f host peptide with GRGDNP guest peptide is shown in Fig. 2. The $K_d$ values obtained by ESI-MS titration for each of the integrin fragment–RGD peptide host–guest pairs are shown in Table 2.

### 3.3. Correlation of binding constants

The approach described above is the first attempt to delineate integrin protein–ligand specificities using integrin peptide fragments and RGD peptide ligands by ESI-MS analysis. As such, there exists no published data for the binding constants, either in the solution or the gas phase, for this system. In order to validate our approach, we measured the solution phase binding constants for these peptide–peptide interactions using frontal analysis capillary electrophoresis (FACE) [15]. Binding measurements made by FACE and ESI-MS titration were performed using identical solution phase conditions (50/50 water/methanol with 0.5% HOAc and 100 μM $NH_4OAc$). Physiological conditions could not be employed due to the poor ionization response under 100% aqueous conditions in ESI-MS, the limited amounts of peptide materials, and the inability for FACE to measure some of the peptide binding systems which do not carry a net charge (no net electrophoretic mobility). In general, electrostatic interactions which dominate integrin–RGD interactions are expected to be strengthened in a 50/50 water/MeOH versus 100% aqueous systems due to the decreased dielectric constant of the medium.

Table 2
Comparison of association constants ($K$) obtained from ESI-MS to those obtained from CE

| Host | Ligand | $K$ (ESI-MS) ($M^{-1}$) | $K$ (CE) ($M^{-1}$) | $V_{50}$ (V) |
|---|---|---|---|---|
| $\alpha_{IIb}$f | GRGDSP | 3800 | 91 | 0.935 |
| $\alpha_{IIb}$f | GRGDNP | 6000 | 67 | 0.940 |
| $\alpha_{IIb}$f | GRGDsP | 2100 | 39 | 0.910 |
| $\alpha_v$f | GRGDSP | 6100 | 480 | 0.980 |
| $\alpha_v$f | GRGDNP | 4700 | 2300 | 0.985 |
| $\alpha_v$f | GRGDsP | 1500 | 480 | 0.960 |

Also shown is the trend of the threshold collision-induced dissociation measurements.

The binding constants obtained from both ESI-MS titration and FACE for the various combinations of integrin fragments and RGD ligands are shown in Table 2. An example titration plot obtained from CE measurements is shown in Fig. 3 for $\alpha_{IIb}$f-GRGDSP and the collision threshold measurements for $\alpha_{IIb}$f-GRGDSP and $\alpha_v$f-GRGDSP are shown in Fig. 4. The $V_{50}$ values (the voltage required to dissociate 50% of the precursor complex ion after its isolation in the ion trap) are also displayed in Table 2. The absolute binding strengths obtained by ESI-MS titration are in all cases higher than those obtained from capillary electrophoresis; but a similar (relative) trend can be drawn for some of the systems studied using both methods. The higher values obtained from ESI-MS titration are not unexpected; and can be explained based on the dynamic (phase transfer) nature of the ESI process and the methodological assumptions incorporated into MS titration procedure.

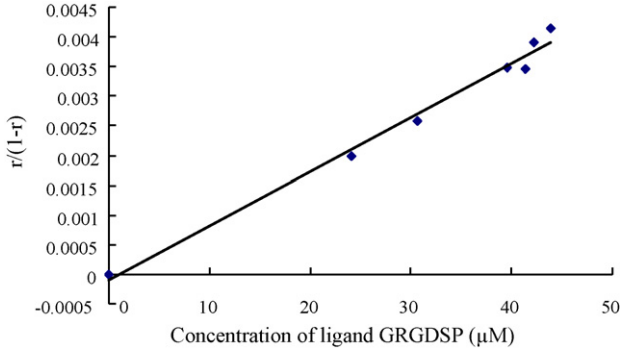


Fig. 3. CE frontal analysis (FACE) plot for GRGDSP and $\alpha_{IIb}$f (where $r$ is the fraction of ligand bound per $\alpha_{IIb}$f).

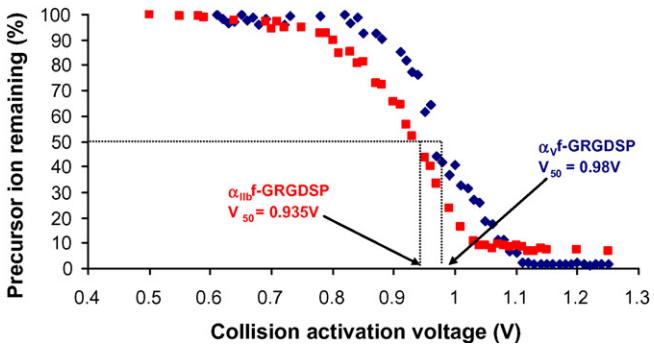


Fig. 4. Collision threshold measurements for $\alpha_{IIb}$f-GRGDSP and $\alpha_v$f-GRGDSP complexes. $V_{50}$ is the voltage required to fragment 50% of the precursor complex ion.

Three major factors can be responsible for the relative, rather than absolute, nature of the binding constants determined by ESI-MS titration. First, electrostatic forces are strengthened upon transfer of the noncovalent complex from solution phase to gas phase. This is an important factor in the case of integrin–RGD complexes because the complex formation is believed to be driven mainly by electrostatic forces (by virtue of the strong basicity of the guanidine moiety on arginine and the carboxylic acid residue on aspartate) [25]. Upon transfer from a high dielectric to a low dielectric medium, these forces are strengthened. In addition, the complex experiences loss of hydrophobic or solvophobic interactions upon moving from the solution phase to the gas phase. This is due to loss of solvent as a result of evaporation. This increases the degree of motion of the complex. Previously locked regions of the complex (due to solvophobic interactions) are now exposed and free to move. This is often referred to as the "entropic contribution" to the overall binding. Thus, the enhancement of electrostatic forces of attraction ("enthalpic contribution") in the gas phase, coupled with the reduction in hydrophobic interactions ("entropic contribution") is partly responsible for the discrepancy between ESI-MS titration and FACE measurements of binding constants. Secondly, as previously mentioned, if the response factors of the complexes, for different permutations of host and guest, do not scale in a similar manner relative to that of the free host, this could introduce further error in the reported binding constants determined by ESI-MS. Thirdly, we must consider that the transfer of species between the solution phase and the gas phase by ESI is a highly dynamic process. As the charged droplets shrink to emit gas phase ions, an increase in concentration of the species contained therein may alter the initial host–guest equilibrium. A method which provides a quantitative description of this effect on host–guest equilibria inside the droplets has not been reported.

### 3.4. Binding specificity

We have shown in this work that it is possible to evaluate specific binding between integrin and RGD peptide fragments. Of the five different integrin fragments studied ($\alpha_{IIb}$f, $\alpha_v$f, $\beta_3$f1, $\beta_3$f2, and Glycoprotein IIb), only $\alpha_{IIb}$f, and $\alpha_v$f showed specific binding with some of the RGD peptides. For ESI-MS, $\alpha_{IIb}$f showed greatest binding with GRGDNP compared to the other RGD peptides tested. The order of binding strength observed for the RGD peptides tested with $\alpha_{IIb}$f is GRGDNP > GRGDSP > GRGDsP. For $\alpha_v$f, the trend is reversed between GRGDNP and GRGDSP. In the case of FACE measurements, $\alpha_{IIb}$f showed greater solution phase binding for GRGDSP than for GRGDNP while $\alpha_v$f showed greater binding for GRGDNP than for GRGDSP. Both ESI-MS and FACE measurements of binding affinities reveal that GRGDsP has the weakest binding of all three RGD peptides studied.

Threshold CID measurements also support some of the trends observed in the binding constants. For example, the threshold CID measurements show that $\alpha_v$f binds more tightly to the RGD ligands than $\alpha_{IIb}$f; which is similar to that observed from solution phase FACE. On the other hand, the trends observed in threshold CID measurements compared to ESI-MS binding constants are similar only in a few cases. The discrepancy between the trends observed in threshold CID measurements and the ESI-MS binding constants can again be partially attributed to our inability to accurately determine the response factor of the host–guest complex. In principle, positive correlation between such measurements is not necessarily expected since the titration approach measures solution phase association and threshold CID measures gas phase dissociation in the absence of solvation. The fact that the trend of threshold CID measurements correlates better with FACE measurements, compared to ESI-MS titration measurements, highlights the importance of utilizing complementary strategies to evaluate binding in new systems. Such a result indicates that threshold CID may provide an adequate indication of relative binding strength in this system. For further consideration of these systems in a high throughput setup, this is a key point. CID measurements can be performed very quickly (ms) and are particularly amenable to a high throughput setting.

The other host peptides ($\beta_3$f1, $\beta_3$f2, and Glycoprotein IIb) studied did not show significant binding with the RGD peptides studied. Even though $\beta_3$ fragments were chosen because they contain putative ligand binding sites, it is possible that the linear RGD peptides studied do not possess the requisite degree of conformational rigidity to form tight complexes with these integrin fragments. Also, these $\beta_3$ fragments contain mainly metal ion-directed binding sites and may only be indirectly involved (for conformational purposes) in ligand binding for the intact protein [17]. The RGD-containing segment of human fibronectin protein, as well as the tripeptide RGD ligands studied did not show significant binding with any of the integrin fragments. This observation further demonstrates the specificity of these peptide–peptide interactions.

### 3.5. Evolution of the titration method

There is no doubt that ESI-MS-based techniques for studying noncovalent complexes have significant advantages (such as speed, sensitivity, and exact stoichiometric measurements) compared to traditional solution phase approaches. However, the manner in which mass spectrometric data are treated for the purpose of obtaining binding constants raises some questions. The most commonly asked questions are: (1) how do the response

factors of the different species in solution change in the presence of the other species in solution? and (2) do the binding constants measured through MS techniques correlate well with those measured via other solution phase techniques? In many cases, favorable correlations have been established between ESI-MS-based determinations of binding constants and those obtained from solution phase techniques [26–29].

Several different approaches abound for calculating values of association or dissociation constants from data acquired using mass spectrometric methods [9]. The titration method employed in this work is both simple and straightforward. Like many other ESI-MS titration methods that involve just the host and the guest and no reference complex, the issues of response factors of the host and the complex (which both appear in the quadratic equation) still have to be considered. The assumption that the response factors of the host and the complex are similar was necessarily made in order to simplify the approach; mainly because experimental determination of response factors for noncovalent complexes is still an elusive task. Until the issue of response factors is properly resolved, $K_d$ values obtained via this method should be used with caution, especially in the absence of rigorously established data from other solution phase techniques for similar systems. From a practical standpoint, the $K_d$ values obtained by MS can most often be used to determine relative binding affinities (selectivities) rather than absolute values. Nonetheless, our response factor assumption may be valid for systems in which the host and the complex are not too different in size (as in the case of a large host binding a small guest), have similar solvation energies, and the complex remains in the same charge state as the host being considered. Work is ongoing in our group towards developing new methods for the determination of the ESI-MS response factors for noncovalent complexes between small molecules with similar sizes.

## 4. Conclusion

The binding constants obtained by ESI-MS titration for complexes formed between integrin fragments and RGD peptides show little agreement with values obtained by frontal analysis CE. The discrepancy in the data obtained from both methods may be attributed to three major factors. These are: (1) the strengthening of electrostatic forces upon transfer of the noncovalent complex from solution phase to gas phase; (2) the inability to accurately determine the response factors of the complexes; and (3) the possible alteration of host–guest equilibria due to concentration gradients in shrinking droplets. Better correlation is observed between gas phase CID and FACE measurements.

To the best of our knowledge, this is the first application of ESI-MS and MS/MS methods for studying noncovalent interactions between integrin fragment and RGD peptides. The result of this work and subsequent studies will allow proof of concept as well as help establish some fundamentals of ESI-MS application for this particular biological system. Further work is needed to address the methodological shortcomings of ESI-MS titration methods. Improved titration models would aid the development of rapid and high throughput screening methods for targeting synthetic peptide and peptidomimetic drug molecules for binding various substrates.

With respect to the workability of this approach in real biological systems, the magnitude of binding constants reported here is likely higher than would be expected for a completely aqueous environment. To employ more physiologically relevant conditions in ESI-MS, though challenging, may lend greater merit to the use of ESI-MS for obtaining affinity values relevant to a physiological environment. For this system, however, the difficulties with respect to ionization efficiency by ESI-MS, sample quantity, and FACE methodology limit such an approach.

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