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Kinetic Size-Exclusion Chromatography with Mass Spectrometry Detection: An Approach for Solution-Based Label-Free Kinetic Analysis of Protein–Small Molecule Interactions

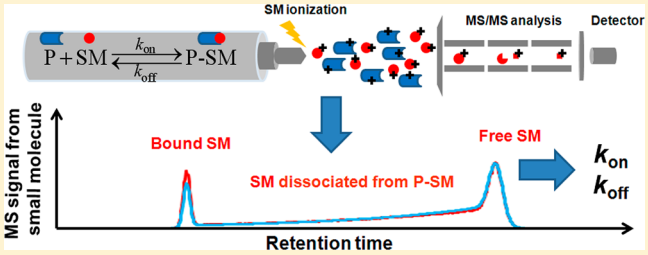
Jiayin Bao,[†] Svetlana M. Krylova,[†] Leonid T. Cherney,[†] J. C. Yves LeBlanc,[‡] Patrick Pribil,[‡] Philip E. Johnson,[†] Derek J. Wilson,[†] and Sergey N. Krylov*[,†]

[†]Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto, Ontario M3J 1P3, Canada

[‡]AB Sciex, 71 Four Valley Drive, Vaughan, Ontario L4K 4V8, Canada

Supporting Information

ABSTRACT: Studying the kinetics of reversible protein–small molecule binding is a major challenge. The available approaches require that either the small molecule or the protein be modified by labeling or immobilization on a surface. Not only can such modifications be difficult to do but also they can drastically affect the kinetic parameters of the interaction. To solve this problem, we present kinetic size-exclusion chromatography with mass spectrometry detection (KSEC-MS), a solution-based label-free approach. KSEC-MS utilizes the ability of size-exclusion chromatography (SEC) to separate any small molecule from any protein–small molecule complex without immobilization and the ability of mass spectrometry (MS) to detect a small molecule without a label. The rate constants of complex formation and dissociation are deconvoluted from the temporal pattern of small molecule elution measured with MS at the exit from the SEC column. This work describes the concept of KSEC-MS and proves it in principle by measuring the rate constants of interaction between carbonic anhydrase and acetazolamide.



Reversible binding between small molecules and proteins plays an important role in the regulation of various cellular processes.[1] Additionally, such interactions are important in modern drug discovery as small molecule drugs are designed to alter protein functions upon binding.[2−4] Understanding the dynamics of both cellular regulation by small molecules and the action of small molecule drugs requires knowledge of the kinetics of formation and dissociation of protein–small molecule complexes.[5−7] Thus, it is important to determine the rate constants, k_{on} and k_{off}, of the following reaction:

$$\mathrm{P} + \mathrm{SM} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \mathrm{P{-}SM} \qquad (1)$$

where P is a protein, SM is a small molecule, and P–SM is a protein–small molecule complex. Complex stability is typically characterized by an equilibrium dissociation constant $K_d = k_{off}/k_{on}$ (smaller K_d values correspond to more stable complexes), and determining any two of the three constants will define the third one.

All current methods used for practical measurements of k_{on} and k_{off} in reaction 1 are either surface-based or label-based. Surface-based methods, such as surface plasmon resonance (SPR)[8,9] and biolayer interferometry,[10,11] require the immobilization of either P or SM on the surface of a sensor. Label-based methods, such as stopped flow spectroscopy,[12,13] require the modification of either P or SM with a spectroscopically detectable label, typically a fluorophore. Moreover, it is preferable that SM, rather than P, is immobilized or labeled in order to maximize the sensitivity of detection.[14,15] However, modifications of SM are difficult to achieve without drastically affecting its ability to bind P. Therefore, a solution-based label-free approach would be ideal for simple and accurate measurements of k_{on} and k_{off}. Here, we propose such an approach, termed kinetic size-exclusion chromatography with mass spectrometry detection (KSEC-MS). Size-exclusion chromatography (SEC) allows generic separation of SM from P–SM without the immobilization of SM or P. Mass spectrometry (MS), in turn, allows the generic detection of SM without labeling it. Instrumentation-wise, SEC is easily integrated with MS, and this combination has been extensively used to study proteins, antibodies, and peptides.[16−18] In KSEC-MS, the migration pattern of SM through the column depends on k_{on} and k_{off}. The rate constants can, thus, be deconvoluted from the temporal pattern of SM elution at the exit of the SEC column.

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Here, we present an implementation of KSEC-MS, in which short plugs of SM and P are injected sequentially into a SEC column without the need to premix P and SM outside the column. We call this implementation plug−plug KSEC-MS (ppKSEC-MS) in analogy with plug−plug kinetic capillary electrophoresis.[19−21] Figure 1A depicts migration of the species

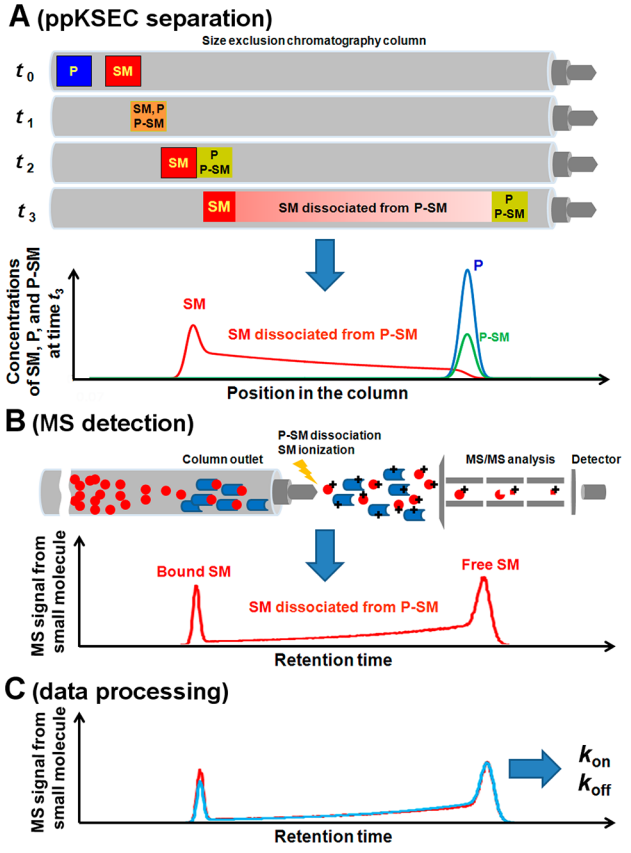

Figure 1. Conceptual depiction of ppKSEC-MS. (A) The small molecule, SM, and protein, P, are sequentially injected into a SEC column separated by a spacer of the buffer (t_0). P moves faster than SM, and then, P meets SM; they can bind each other forming complex P−SM (t_1). After P and P−SM leave the zone of SM (t_2), P−SM starts dissociating. The released SM is continuously separated from P−SM and creates a bridge between the zones of P/P−SM and SM (t_3). The graph illustrates the corresponding concentrations of P, SM, and P−SM at time t_3. (B) The eluate from the column is continuously sampled into the ion source of a tandem MS/MS mass spectrometer, which is tuned to detect SM. The blue rods represent P while the red dots represent SM. SM dissociates to form P−SM which facilitates indirect detection of intact P−SM exiting the column. The resulting ppKSEC-MS chromatogram contains two peaks and a bridge between them. (C) The experimental ppKSEC-MS chromatogram is numerically fitted with a computer-simulated one. The values of k_{on} and k_{off} are used as fitting parameters, and the best fit corresponds to the sought correct values of k_{on} and k_{off}.

in a SEC column. In the beginning, a short plug of SM is injected into the column followed by injection of a short plug of P, with a small volume of buffer in between, as a spacer, to prevent mixing during the injection. The chromatographic migration is immediately started after injecting P. The molecular size of P is much larger than that of the SM and thus P moves faster than the SM. The P plug passes through the SM plug allowing for P to bind SM and form P−SM, which has a molecular size similar to that of P and thus comigrates with P. When the P/P−SM plug overtakes the SM plug, P−SM starts dissociating into P and SM. The latter is continuously separated from P and P−SM creating a trail of SM behind the P/P−SM plug. The resulting migration pattern is the following. The zone containing P and intact P−SM migrates first. The zone of SM that has not bound P (during the passage of the P plug through the SM plug) migrates the last. The trail of SM that dissociated from P−SM lies between these two zones. Figure 1B schematically illustrates the detection step. P and P−SM elute first followed by SM that dissociated from P−SM and finally by SM that has not bound P. The eluate is sampled into an MS ionization source; the typical ionization methods are atmospheric-pressure chemical ionization (APCI) and electrospray ionization (ESI). The ionized SM is fragmented and detected by MS/MS, which offers high specificity and signal-to-noise ratio. All intact complexes are destroyed during the ionization so that SM is released from P−SM and also quantitated by MS/MS. In general, the time-dependence of signal from SM (a chromatogram) contains three features merging into one another: (i) a peak corresponding to SM originating from the decay of P−SM during ionization, (ii) a peak of SM that has not bound P, and (iii) a "bridge" between the two peaks that corresponds to SM that dissociated from P−SM during migration in a column (Figure 1B). The shape of the chromatogram is defined by k_{on} and k_{off} so that, in the final step of analysis, their values are found by fitting the experimental chromatogram with a computer-simulated one while varying k_{on} and k_{off} (Figure 1C). The best fit of a single chromatogram reveals the values of k_{on} and k_{off}.

To experimentally prove the suggested concept of ppKSEC-MS, we used carbonic anhydrase II (CAII) as P and acetazolamide (ACZ) as SM. CAII catalyzes the interconversion between carbon dioxide and bicarbonate, which is a critical reaction in regulating cellular respiration;[22] ACZ is a known CAII inhibitor.[23] A series of ppKSEC-MS experiments were performed at constant [SM] but varying [P]. The resulting chromatograms are shown by red traces in Figure 2. The general shape of the chromatograms corresponds to the expected one with two peaks and a bridge between them (Figure 1B). Increasing [P] led to an anticipated increase of both the leftmost peak, corresponding to intact P−SM eluting from the column, and the bridge, corresponding to SM dissociated from P−SM during its migration through the column. At the same time, the rightmost peak, which corresponds to SM that had not bound P, predictably decreased with increasing [P]. The integral area under the chromatogram, which is proportional to the total amount of SM exiting the column, did not change with increasing [P]. This finding indicates that the P−SM was completely destroyed during the ionization process (which was desirable) and that all SM was accounted for.

The values of k_{on} and k_{off} are convoluted into the shape of chromatograms. An analytical solution for their deconvolution does not exist, leaving us with a single option: numerical solution of an inverse problem. In essence, an experimental chromatogram should be fitted with a simulated chromatogram computed using a 1-dimensional mathematical model describing both reaction 1 and mass transfer in a SEC column.

There are many theoretical works on the separation of polymers by SEC that consider the thermodynamics of distribution of polymer fractions between the mobile and stationary phases.[24−29] They mainly study separation principles and often use fairly complicated mathematics and detailed

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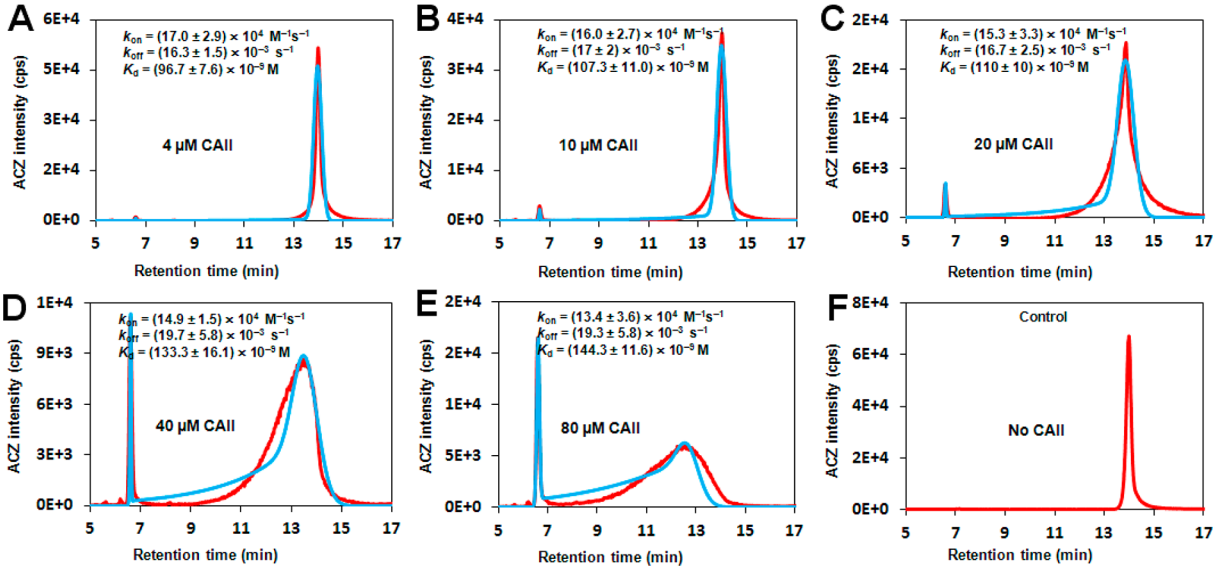

Figure 2. Experimental (red) and simulated (blue) ppKSEC-MS chromatograms for kinetic analysis of reversible binding between CAII and ACZ. The concentration of ACZ was 20 μM, and the concentration of CAII varied from 4 μM (A) to 80 μM (E). The control (F) corresponds to a run with a zero concentration of CAII. The ACZ signal was recorded in negative MRM mode for 221/83 (Q1/Q3) m/z. Simulated chromatograms were generated from modeling the processes involved in ppKSEC by using COMSOL multiphysics software. The binding parameters were determined from the best fit of the experimental chromatogram by the simulated one and were calculated on the basis of the averages and standard deviations of results obtained in triplicates.

process descriptions. Two-dimensional hydrodynamic models have also been suggested to describe SEC columns.[30−32] These models employ additional differential equations to take into account diffusion of solutes within the bead pores. On the other hand, a simple one-dimensional hydrodynamic model can be used in our case if this model takes into account the basic features of the described experiments (Figure 1).

We developed such a model in which a long and narrow cylindrical chromatography column is coaxial with the x coordinate. A SEC column is packed with beads that have pores; the solution inside the pores constitutes the stationary phase. We assume that the pores are large enough for SM to enter and reside inside for a significant time and too small for the protein or the protein−small molecule complex to penetrate and be significantly retarded. This is a typical assumption that is confirmed by a significant difference in retention times between SM and P−SM (Figure 2). We also assume fast re-equilibration between the mobile phase (solution outside the beads) and stationary phase. This is also a typical assumption that is confirmed by narrowness of peaks of P−SM and SM in Figure 2. In ppKSEC-MS, a short plug of SM is injected followed by injection of a considerably longer buffer spacer and finally a short plug of P. The injection times, τ, for SM and P are short and equal while the injection time, t_{spc}, for the spacer is much longer, $t_{spc} \gg \tau$, thus eliminating the possibility of mixing between SM and P prior to the start of separation. Since P cannot enter the pores, reaction 1 can only proceed outside the beads in the so-called free volume. In addition, a hydrodynamic flow of the solution exists only outside the beads. Therefore, we assume that the buffer velocity as well as [P] and [P−SM] are averaged across the column over the area lying outside the beads. Moreover, [SM] outside the beads and inside the beads is averaged across the column over the total area lying outside the beads and inside the pores. Interactions between the species and their mass transfer are described by the following equations:

$$(\partial_t + \nu_{SM}\partial_x - D_{SM}\partial_x^2)[SM] = \alpha(k_{off}[P{-}SM] - k_{on}[SM][P]) \quad (2)$$

$$(\partial_t + \nu\partial_x - D_P\partial_x^2)[P] = k_{off}[P{-}SM] - k_{on}[SM][P] \quad (3)$$

$$(\partial_t + \nu\partial_x - D_P\partial_x^2)[P{-}SM] = k_{on}[SM][P] - k_{off}[P{-}SM] \quad (4)$$

$$\nu_{SM} = \alpha\nu, \quad \alpha = \frac{\phi_{out}}{\phi_{out} + \phi_{in}},$$
$$D_{SM} = \frac{\phi_{out}D_{out} + \phi_{in}D_{in}}{\phi_{out} + \phi_{in}} + \frac{\phi_{out}^2\phi_{in}^2\nu^2}{k(\phi_{out} + \phi_{in})^3} \quad (5)$$

Here, ν is the average velocity of the hydrodynamic flow in the column; ν_{SM} is the average velocity of SM in the column; D_{out} and D_{in} are diffusion coefficients of SM outside the beads and inside their pores, respectively; D_P is the diffusion coefficient of P and P−SM (we consider it to be the same as SM binding P does not significantly affect the molecular size of P); ϕ_{out} and ϕ_{in} are relative volumes (i.e., fractions of the column volume) located outside beads and inside pores, respectively; $k \sim D_{in}/R_{in}^2$ is the kinetic rate constant for a diffusion relaxation between concentrations of small molecules outside the beads and inside their pores; R_{in} is the characteristic size of beads. The average concentrations of SM outside the beads, $[SM]_{out}$, and inside the pores, $[SM]_{in}$, can be considered to be similar due to fast diffusion equilibration between the pores and the outside-the-beads volume ($[SM]_{out} = [SM]_{in} = [SM]$). Indeed, for a characteristic time, t_{in}, of the diffusion relaxation between SM outside the beads and inside their pores, we have $t_{in} \sim R_{in}^2/D_{in} \sim 0.01$ s for typical values of $R_{in} \sim 3$ μm and $D_{in} \sim 10^{-5}$ cm^2/s. Thus, $t_{in} \ll t_{sep} = W/(\nu - \nu_{SM})$, where t_{sep} is the separation time which is usually in the order of a few seconds (W is the plug length). It should be noted that a coefficient α depends only on the ratio ϕ_{out}/ϕ_{in} that coincides with the ratio of actual (not relative) volumes located outside beads and inside pores.

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In ppKSEC-MS, nonequilibrium boundary conditions at $x = 0$ were used. Such boundary conditions for eqs 2−5 can be formulated as follows:

$$[\mathrm{SM}] = [\mathrm{SM}]_0 \quad (x = 0, \quad 0 < t < \tau)$$
$$[\mathrm{P}] = [\mathrm{P}]_0 \quad (x = 0, \quad t_{spc} < t < t_{spc} + \tau) \tag{6}$$

where $[\mathrm{SM}]_0$ and $[\mathrm{P}]_0$ are initial concentrations of SM and P injected in the column inlet, τ is the injection time of SM and P, and t_{spc} is the time interval between injections of SM and P. Concentrations at $x = 0$ are assumed to be zero for other time intervals. Relations 2−6 were used to obtain a numerical solution of the problem and to simulate signal $S(t)$ generated by SM. We assume that all intact P−SM that reaches the end of the column dissociates during ionization and SM released from this dissociation is detected. As a result, $S(t)$ is proportional to the total concentration of SM (both unbound and bound to P) at the column exit and g is a proportionality coefficient:

$$S(t) = g([\mathrm{SM}](t) + [\mathrm{P{-}SM}](t)) \tag{7}$$

The described model was implemented in COMSOL Multiphysics 4.3a (commercial software). The Transport of Diluted Species module of COMSOL was used in simulations of eqs 2−6. The program generated simulated ppKSEC-MS chromatograms, $S(t)$. Nonlinear regression was used to find best fits of the experimental ppKSEC-MS chromatograms (red traces) by the simulated ones (blue traces) while varying the values of k_{on} and k_{off} (Figure 2). It should be noted that parameters v_{SM}, D_{SM}, and g can be determined by fitting the experimental chromatogram obtained for injecting SM alone (i.e., in the absence of the protein). Similarly, parameters v and D_P can be found by fitting the experimental chromatogram obtained for injecting P without SM. Provided that v_{SM} and v are determined, parameter α can be calculated using the first relation in eq 5. As a result, only parameters k_{on} and k_{off} have to be varied in the fitting procedure involving experimental ppKSEC-MS data obtained for injecting both SM and P.

We varied the concentration of the protein to test if the solutions for k_{on} and k_{off} were stable. When the protein concentration increased 20-fold, the values of k_{on} and k_{off} remained stable: $k_{on} = (15.4 \pm 2.2) \times 10^4\ \mathrm{M^{-1}\ s^{-1}}$ and $k_{off} = (17.8 \pm 2.0) \times 10^{-3}\ \mathrm{s^{-1}}$ (rules of error propagation were used to find the errors of k_{on} and k_{off}). There was a noticeable trend of monotonic increase in k_{on} and less monotonic increase in k_{off}. This trend indicates that there is a small systematic error in the calculations. The error is most likely due to some minor phenomena in the separation and/or molecular interactions that are not taken into account by the simple mathematical model used to fit the experimental chromatograms. The value of the equilibrium dissociation constant was calculated as k_{off}/k_{on}: $K_d = (117 \pm 16) \times 10^{-9}$ M. To validate our results, we used another solution-based label-free method, isothermal titration calorimetry (ITC). ITC can only determine K_d, but we used this validation method since there is no other label-free kinetic method available for such a validation. ITC experiments revealed $K_d = (76 \pm 5) \times 10^{-9}$ M (Figure 3). K_d values obtained with ppKSEC-MS (~120 nM) and ITC (~80 nM) are in reasonable agreement considering that the temperatures in the ppKSEC-MS and ITC are difficult to make equal and conceptually different methods can lead to up to several-fold differences in measured equilibrium constnats.[33] This agreement indicates that K_d calculated as k_{off}/k_{on} for k_{on} and k_{off} obtained with ppKSEC-MS is correct. Even though there is still

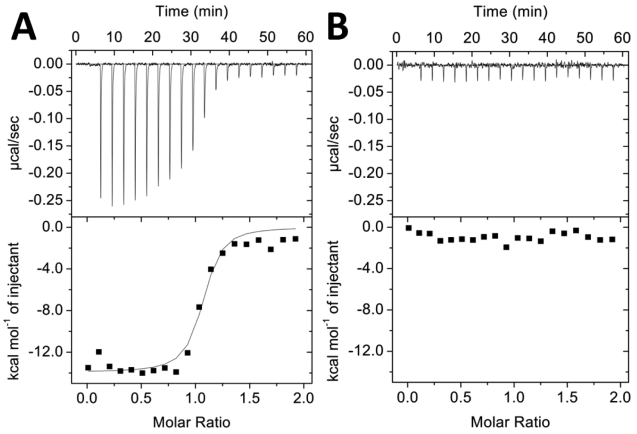

Figure 3. Thermograms of binding analysis between CAII and ACZ by ITC. Panel (A) corresponds to titrating CAII with ACZ while panel (B) corresponds to titrating CAII with buffer only. The upper graphs show the raw ITC data in real time, and the lower graphs show the corresponding integrated total heat per injection with respect to molar ratio. The fitting was generated by using Origin software with a single-binding-site model.

a possibility that k_{on} and k_{off} were determined with a similar systematic error which was canceled upon division of k_{off} over k_{on}, such an error appears to be extremely unlikely. Therefore, we can conclude that ppKSEC-MS correctly determined k_{on} and k_{off} for reversible binding of CAII and ACZ.

In conclusion, we outline major features of ppKSEC-MS in application to kinetic studies of protein−small molecule interactions. The method relies on generic separates of SM and P−SM by SEC without immobilization and generic quantitative detection of SM by MS without labeling. Any pair of P and SM can be separated by SEC assuming that neither of the molecules adsorbs on the beads material. MS, in turn, can detect any small molecule assuming that suitable ionization conditions are found and the major ion products are known. Advantageously, ppKSEC-MS requires no detection of intact P−SM, which is a very challenging task.[34] Moreover, data processing becomes simpler if the intact P−SM completely dissociates during ionization, which is easy to achieve in APCI.[35] ppKSEC-MS is a kinetic method that does not require equilibrium to be reached in reaction 1. As a result, $[\mathrm{P}]_0$ and $[\mathrm{SM}]_0$ that significantly differ from K_d[20] can be used, thus relaxing the requirements for the limit of detection in MS.[36,37] In ppKSEC-MS, SM and P are injected separately and reacted inside the column, thus minimizing sample consumption and making the process easily suitable for automation without the use of sophisticated liquid handlers. Overall, our results suggest that ppKSEC-MS has a potential to become a generic solution-based label-free platform for kinetic studies of protein−small molecule interactions,; but more detailed studies will be needed to better understand the advantages and limitations of the method.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: skrylov@yorku.ca.

Notes

The authors declare no competing financial interest.

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