Towards protein chromatography by design: Stochastic theory, single-molecule parameter control, and stimuli-responsive materials

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Abstract

The rapid rise of biological pharmaceuticals motivates a need for both predictive models and better materials for separations. Physical chemists now have the tools to deliver on an effort started almost 75 years ago to describe chromatographic separations through statistical methods. When combined with new support materials, a statistical model would enable the design and control of the iterative combination of many single-analyte events to produce an ensemble chromatogram. Because single-analyte events can now be measured and modeled directly using the latest experimental and computational methods, our perspective describes the development and implementation of a stochastic chromatographic theory based on these methods. Further, we comment on the use of stimuli-responsive materials for future applications. We believe that responsive materials when combined with state-of-the-art single-molecule experiments and theory could lead to cost-effective methods for predictive protein separation.

Introduction

Liquid chromatography is a multiscale problem where analytes separate in the mobile phase due to differential interactions with the stationary phase when pumped through a chromatographic column. The interactions depend on the nanoscale heterogeneity of the stationary phase as well as the chemical nature of the analytes.[1-5] Proteins are large macromolecules with complex secondary and tertiary structures, heterogeneous surface charge distributions, and hydrophobic/hydrophilic domains. Intrinsic heterogeneity in proteins makes empirical separation and purification of biologics time and energy-intensive. [6, 7]

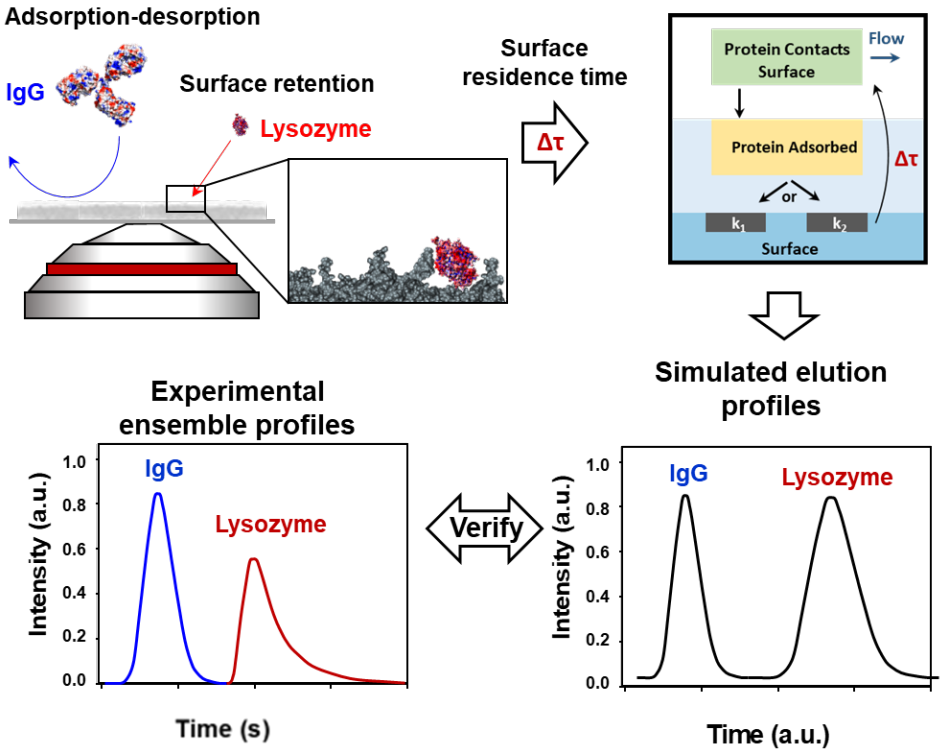

Figure 1. Connecting single-molecule parameters to stochastic theories for optimal separation. Schematic of single-molecule interactions of biomolecules with a chromatographic support at the nanoscale. Single-molecule experiments provide information, such as surface residence time (Δτ) for analyte-stationary phase interactions. One contributor to peak skewing/broadening is adsorption at rare surface sites, but simulations based on stochastic theory can be used to model the contribution from such heterogeneity to elution profiles. Different colored chromatograms compare simulated elutions with varying desorption rates for the rare surface sites.

Macroscale protein separation relies on empirical optimization of mobile and stationary phase parameters by qualitatively considering the physical and chemical properties.[8, 9] Because ensemble separation can be envisioned as the combined effect of all interactions between analytes and the stationary phase on the nanoscale,[10] quantitative optimization assisted by single-analyte knowledge is one means to improve separation efficiency. We have divided the protein separation problem into three facets (**Figure 1**): observing single-molecule interactions of biomolecules with the stationary phase, modeling properties such as peak asymmetry and broadening, and designing novel separations by combining experimental and theoretical single-molecule knowledge. Deviation from an ideal, Gaussian peak profile is due to mobile phase effects, column overloading, rare adsorption events, etc.[3, 4, 10-14] Here, we summarize how macroscale elutions can be understood and controlled through single-molecule understanding of the underlying solute-stationary phase interactions in this context and envision controlling various single-molecule properties to achieve better performance in ensemble separation.

In a previous perspective,[15] we outlined how single-molecule fluorescence experiments can provide insight into analyte-polymer interactions relevant to biologic separation. Here, we present the application of stochastic theories for nanoscale insight into local domain-specific biomolecule-polymer interactions and in modeling chromatographic peak broadening due to secondary site kinetics. We also summarize the need to use novel responsive materials in rational chromatographic designs and provide a design alternative for multiscale chromatographic separation.

Most of the widely used theoretical descriptions of chromatography are not designed to capture the time-dependent microscopic heterogeneity intrinsic in separation processes.[16, 17, 19] **Figure 2** illustrates the history and potential of three separate approaches to chromatographic theory that vary in complexity and physical descriptors. The Differential Equations model posited by Lapidus and Amundson[17] describes the chromatographic process as the motion of an analyte concentration front down a column. Peak shape in this model is described by the fluctuation of concentration in both time and space, together representing the diffusion of the analyte down the length of the column and on the surface of the stationary phase. van Deemter[16] proposed a simplification that condensed all calculations into three specific constants in contest with mobile phase velocity. Optimization in the van Deemter model now depends on one variable, the mobile phase velocity, at the cost of extensive *a priori* stationary phase characterization. Its simplicity established the van Deemter method as the standard descriptor for liquid chromatography. However, the van Deemter method is developed from a macroscopic perspective that cannot capture the heterogeneity of solute-stationary phase interactions at the nanoscale.

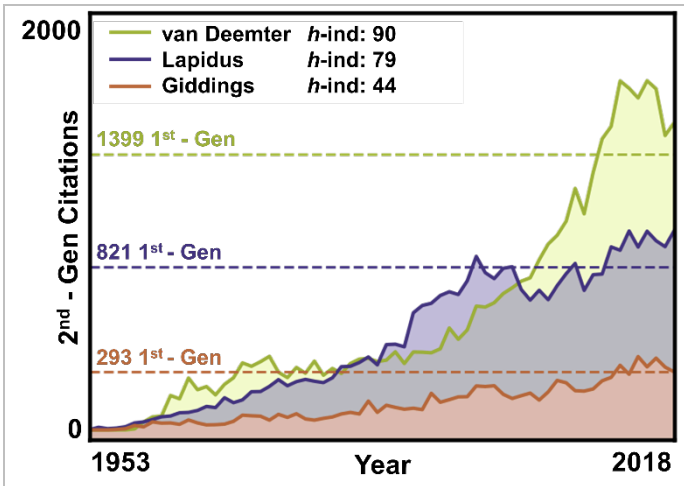

Figure 2. Theoretical advancements are primarily concerned with simplified descriptions of chromatography. Tracked citations for three prominent theoretical descriptions of chromatography ranging from simplified (van Deemter[16]), ensemble (Lapidus[17]), and single-molecule (Giddings[18]) methods. First-generation citations are marked with a dotted line. Second-generation citations, papers that cite articles that cited the original work, are depicted in the shaded populations. Citation data was gathered using Web of Science statistics tools provided by Clarivate Analytics.

Starting in the 1950s, the father of transition state theory, H. Eyring and his student J. Giddings, developed a statistical framework for separations that – in theory – was capable of linking microscopic analyte chemistry and physics to macroscale separations.[18, 20] Giddings and Eyring developed the stochastic model of chromatography by considering the overall separation process as a combination of a series of random adsorption and desorption processes. In the simplest picture of single-site adsorption, the analyte adsorption from mobile to stationary phase was characterized by a Poisson distribution, whereas desorption was assumed to follow first-order kinetics. The single-site adsorption model was further refined to account for more complex molecular interaction phenomena,[21, 22] and D. McQuarrie made further improvements[23] – the title of McQuarrie's Ph.D. thesis was "A Theory of Fused Salts and On the Stochastic Theory of Chromatography".[24] Pasti et al. expanded the stochastic description in the Lévy representation to connect the rare adsorption observables obtained from the single-molecule measurements to ensemble elution.[25] We recently adopted a Monte Carlo model, previously developed by Dondi and Cavazzini,[21] to relate chromatographic peak asymmetry to heterogeneous kinetics, as well as developed a new analytical metric to connect microscopic surface dynamics to the macroscopic chromatograms.[10]

Why use the stochastic theory now?

The state of experimental and computational technology in the mid-20th century led Giddings and Eyring to declare, "The general problem of chromatography is, in a practical sense, not solvable".[18] At that time, it was impossible to experimentally measure heterogeneities and deconvolute multiple contributions to ensemble observables and connect them to theory. Further, the statistical expressions for n- different adsorption sites on a heterogeneous stationary phase[23] could not be calculated because the necessary computational technology had not yet been

developed. Giddings and Eyring's statistical framework was ahead of its time, and despite the model's scientific rigor and promise, separations remain dominated by the same empirical descriptions used since the 1920s.

Measuring and modeling molecular interactions at unprecedented spatiotemporal resolutions is now a reality that has been made possible through the advancement of single-molecule methods and computational power.[26] The potential to link new single-analyte data and statistical theories hints at achieving predictive chromatography, a premise that is only achievable in Giddings' and Eyring's stochastic theory. For example, single-molecule and super-resolution methods have made it possible to experimentally observe the non-equilibrium interactions that drive separations. Such methods provide access to statistics about adsorption and desorption of individual molecules,[27-30] mobile/stationary phase exchange,[4, 31] and transport in nanoporous media unfettered by ensemble averaging and equilibrium assumptions.[32, 33] And cheap data storage and fast processors can model previously unsolvable nonlinear expressions and analyze large volumes of experimentally acquired data.[34]

Recently, the National Academies Press set out a vision[6] to transform separation science by adapting theory and data science approaches to model and predict separations. They identified a need to utilize computational methods to identify critical aspects of complex separation processes, including, but not limited to, kinetics and thermodynamics of molecular transport, transport in complex environments under molecular crowding conditions, and structural and dynamic properties of separation systems. The committee also identified the potential of conventional (such as temperature and pressure) and unconventional (such as electric, magnetic, and light) responsive materials as separation models. Thus, we assert that now is the time to revisit how a stochastic model of separations, informed by single-analyte experimental data and adapted

to exploit new responsive support materials, could allow us to achieve the goals set out by the National Academies study.

Relating single-molecule data to ensemble results

Ensemble chromatograms can be approximated as the collection of single-molecule elution histories. The van Deemter approach (**Figure 3A**) treats the column as a series of theoretical plates with uniform analyte concentration and predicts the maximum separation efficiency at minimum plate height. According to van Deemter,[16] plate height depends on three empirical constants: mobile phase velocity (μ): eddy diffusion (A), diffusion (B), and mass transfer resistance (C):

$$H = A + \frac{B}{\mu} + C\mu$$

Based on the van Deemter equation, the quality of a separation depends on empirical parameters such as column density and pressure. Separation optimization can be accomplished by tuning only one parameter, the mobile phase velocity (μ). However, the stated equation relies on physically unrealizable dynamics in the column, which hinders the translation of such a simplistic mathematical relation to more complex column descriptions.

Single-molecule models can describe the complexity in mathematically tractable terms but must extrapolate long-term behavior from microscale observations to relate to macroscale observables. Giddings and Eyring's stochastic theory provides a framework to assess the chromatographic peak shape as statistical populations differentiated by their interactions with the stationary phase surface. Giddings and Eyring describe the motion of each analyte molecule through a column as a random walk between the mobile and stationary phases[35] (**Figure 3B**). The distribution of the number of adsorption events is then a Poisson distribution which gives the probability of a single protein associating with an adsorption site r_m times:[18]

$$\psi_m = \frac{(k_a t_m)^{r_m}}{r_m!} exp[-k_a t_m]$$

Where r is the number of stochastic adsorption events for a given time, t, in the mobile phase, m; k_a - an adsorption rate constant; t_m - the total time in the mobile phase. This equation can be used to extract an elution profile from a single-molecule kinetic measurement, specifically observable rate constants. Utilizing the stochastic approach, previous single-molecule studies already demonstrated the influence of multiple adsorption sites with heterogeneous kinetics on elution profile, therefore, providing a conceptual link between peak broadening and adsorption on strong sites.[2, 4, 27] The stochastic theory can be expanded for multiple adsorption sites to calculate the adsorption probabilities and connect with observable rate constants, however, such calculations can be computationally demanding.[23] Increased data storage, faster processors, and advanced programming methods enable a linear increase in complexity of the stochastic theory, extending the original mathematics past the bounds of calculating one to two populations analytically. Therefore, the mosaic of populations that compose an elution, made apparent by single-molecule spectroscopic studies, can be used to predict elution accurately. A few proof-of-concept examples are highlighted next.

A study of reverse phase chromatography highlighted that the stochastic model of chromatography combined with single-molecule kinetic results can provide consistent macroscopic separation trends.[4] In another example, bulk-scale elution profiles of the salting-out of transferrin proteins from polymer nylon membranes matched stochastic simulations informed by single-molecule kinetic information. Single-molecule data confirmed a decrease in peak broadening as the salt concentration increased, consistent with macroscale observations.[2] These proofs-of-concept demonstrate that local chemistry and physics when measured precisely and without ensemble averaging, can be used to model elutions in ways that could be adapted for predictive simulations.

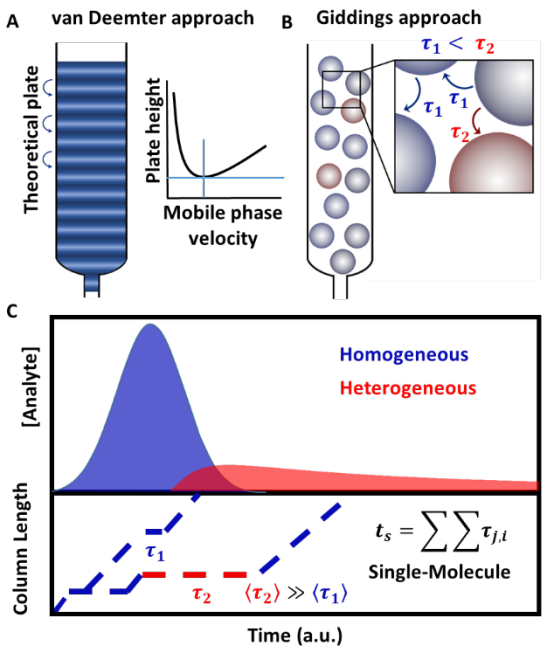

Figure 3. Macroscopic and microscopic description of chromatography. (A) The van Deemter description of chromatography predicts maximum separation at minimum plate height by empirical optimization. (B) Stochastic single-molecule description following Giddings incorporates adsorption heterogeneities by accounting for both homogeneous (time, τ_1) as well as rare heterogeneous interactions (time, τ_2). (C) (Top) The observed chromatogram with two underlying populations composed of homogeneous (blue) and heterogeneous (red) interactions. (Bottom) The underlying single-molecule contributions to the visualized subpopulations. Desorption times for homogeneous and heterogeneous interactions are τ_1 and τ_2, respectively. Time spent in the stationary phase (τ_1) is represented as a sum of i^{th} single-molecule event and j^{th} retention mode.

Theoretical and computational development

An essential step towards fully modeling elution is correlating calculations of the time an analyte molecule spends in the mobile phase and the structure of the chromatographic column. Many studies have provided detailed flow descriptions of single tracers through porous media to

describe the time an analyte spends in the mobile phase.[36] However, most theoretical models struggle to connect the physical quantities that define analyte motion to the structure of the column.[37] Capturing analyte behavior in the column, as well as in column connections, is key to achieving a complete theoretical model. Current theoretical models can account for multi-site adsorptions based on stochastic theory[18] and heterogeneous adsorption distributions using canonical Levy representations[25] in chromatographic simulations. The underlying assumption in these theories is that mobile phase effects do not contribute to chromatographic performance whereas in practice, mobile phase effects can introduce peak tailing and loss of analyte.[10] Combining new experimental methods to track analyte molecules moving through tortuous environments[1, 38] will underpin the development of mobile phase theory, bringing the separation sciences one step closer to predictive separations.

Another promising branch of theoretical development is chemometrics to connect surface chemistry to elution results. Ensemble metrics used to describe separation efficiency, such as the asymmetry factor, offer simple descriptors of chromatographic lineshape based on the same mathematics as the full width at half maximum.[10, 39] Linking the stochastic theory powered by the Monte Carlo simulations of Dondi[40] and Cavazzini[21] provides a new way to connect empirical chemometrics to microscopic dynamics and thus provides a path to the real-time adaptation of chromatographic conditions during elution. Further extensions to the theory should include ways to model interactions along patterned surfaces, mathematical expressions for mobile phase heterogeneity, and descriptions of nonlinearity originating from column connectors. By maintaining the statistical background of the theory, further advancements can be agnostic to the type of separation mode, offering broad applicability to a variety of separation industries.

Applicability of single-molecule and stochastic theory to industry

Predictive approaches that scale up single-molecule interactions to emulate ensemble separation with the support of statistical simulations could streamline the optimization of industrial scale separations. Ensemble elution profiles can be considered a statistical sum of numerous single-molecule events. In such a model, a single mechanism for adsorption onto the stationary phase leads to a Gaussian elution profile, whereas heterogeneous interactions lead to skewness or tailing in the elution profile (**Figure 3C**). The ability to link microscopic chemical and physical dynamics to macroscale separations *in silico* could thus reduce the need for iterative optimization during industrial scale-up.

Using stimuli-responsive materials as stationary phases with novel structure and functional properties

Development of novel stationary phase materials with stimuli-responsive behavior requires designing complex interfaces to control physical properties at the nanoscale. Predictive chromatographic performance of such materials can be achieved by manipulating the local chemistry and controlling the molecular scale solute-solvent interactions. In this section, we highlight recent progress[41-43] in how single-molecule experiments are helping to understand analyte motion in stimuli-responsive polymer brushes, 2D/3D porous structures, and 3D printed columns to aid in the design of predictive and efficient separations. We also point out future opportunities where single-molecule detail might be crucial for materials optimization.

Stimuli-responsive polymers:

Stimuli-responsive polymer brushes are an attractive material for separations because of their low cost, high flexibility, and tunable through a range of stimuli (e.g., pH, salt, temperature).[44, 45] Single-molecule methods have been implemented to understand how polymer brush chemical composition, height, and density influence analyte interactions with the polymer

support.[46-50] The influence of polymer conformational changes on analyte motion has been probed using single-molecule techniques, where it has been found that attractive Coulombic interactions and local geometric heterogeneities slow analyte diffusion.[51, 52] More recently, we reported that electrostatic-induced unfolding of immunoglobulin G (IgG) in an ion-exchange chromatography column slows IgG motion and elution, providing a crucial and direct link between single-molecule measurements and ensemble separations.[1]

On the macroscale, temperature-responsive separation is implemented by flowing the mobile phase at multiple temperatures, thereby heating the entire column and losing site-specific thermodynamic information.[53] Hybrid plasmonic nanoparticle/polymer materials are one option to understand and control local molecular interactions at various temperatures during the polymer phase transition. Plasmonic heterostructures coated with pNIPAM polymers offer local temperature control using plasmonic heating, and can be combined with analyte tracking in single-molecule experiments.[54, 55] There are a range of new redox-active polymer and other electro-driven membranes for chemical and biomolecular separations[56-58] that are ripe for study, especially because single-particle studies have shown that electrodes can reorganize at the nanoscale during electrochemical charging and discharging.[59]

***MOFs/COFs*:** Metal-organic frameworks (MOFs) and covalent organic frameworks (COFs) are emerging classes of materials that can be integrated into the solid stationary phase (**Figure 4A**).[55, 60, 61] MOFs consist of metal ions or clusters with extended conjugation of organic ligands to form 2D/3D porous structures. High porosity, mechanical stability, and tunable surface properties make MOFs suitable for separation and purification applications. Zeolitic imidazolate frameworks (ZIFs), a subclass of MOFs, have recently been explored to understand the protein capture behavior in MOFs. Zheng et al. showed that magnetic ZIF-8 nanocomposites (Fe_3O_4@ZIF8) can

selectively capture natural His-rich proteins, such as bovine hemoglobin.[60] Liu et al. demonstrated that hierarchical ZIF-8 structures can immobilize lysozyme via a physical adsorption mechanism.[62] COFs are extended 2D/3D organic structures with well-defined structural regularity, high surface area, tunable pore size, and high chemical and structural stability.[63] Various COF composites have been recently synthesized and shown to selectively capture small hydrophobic peptides while separating proteins through size-exclusion.[55] While the high physicochemical tunability of MOF/COFs is attractive for potential separation applications, a mechanistic understanding of the nanoscale molecular interactions is required for specificity biomolecular separation. Further investigations into other frameworks with responsive materials and novel chemical properties will enable better design of separation systems.

***3D printed materials*:** 3D printed columns with novel geometries assisted by stochastic single-molecule simulation and flow dynamics simulations can instigate a radical change in protein chromatography (**Figure 4B**).[64-66] Traditional liquid chromatography

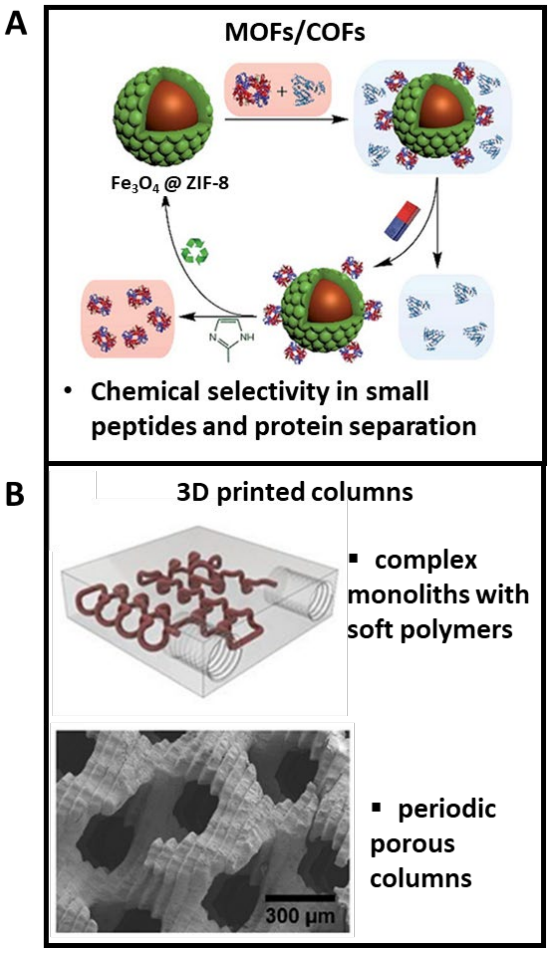

Figure 4. Responsive polymers with active functional groups and novel structures can be used as effective separation material. Some representative ideas include (A) MOFs/COFs. Adapted with permission from ref[60]. Copyright 2015 Royal Society of Chemistry. (B) 3D printed soft columns are presented. Adapted with permission from refs. [64, 65] Copyright 2019 Elsevier and Copyright 2018 American Chemical Society, respectively.

stationary phases consist of randomly packed spherical beads or monolithic networks where careful experiments and optimization is required to understand the separation behavior as *a priori* quantitative predictions are impossible. On the contrary, ordered and homogeneously packed beds have been shown to drastically increase separation efficiency in recent theoretical and experimental studies.[65, 67] However, manufacturing highly ordered columns with predefined structures is challenging with traditional techniques. Recent developments in additive manufacturing or 3D printing techniques enable layer-by-layer fabrication of highly ordered structures with precise morphologies. Future developments utilizing 3D printing and nanofabrication techniques to manufacture chromatographic supports with responsive materials will be the key to high-resolution separations with low cost and higher efficiency.[68]

On-chip multiscale separations

Multidimensional chromatography using responsive polymers as stationary phases could enable high-resolution separations of biologics. Microfluidic based devices offer portable, energy and time efficient platform for biologic separations specially in preparative procedures in protein and peptide analysis.[69-71] One tool employed in modern-day proteomics is 2D gel electrophoresis (2-DE) that relies on separation based on the isoelectric point of proteins and their molecular weights. In 2-DE, a mixture of proteins is sequentially separated using a gel strip and a gel slab kept between two electrodes. 2-DE is a multidimensional separation process that requires[72] (i) each separation step to be independent of the separation in other steps and (ii) the materials separated in one step must not get mixed in subsequent steps. These requirements are consequences of Giddings's definition of multidimensional (MD) chromatography.[73] However, designing an MD separation is challenging as the separation achieved in one dimension suffers degraded resolution in the 2nd

dimension due to diffusion. Introducing independent separation mechanisms of actively controlled polymers in subsequent separation steps could achieve high-resolution separations.

Figure 5A illustrates an on-chip separation system based on an LCxLC method, where LC denotes a separation in the liquid phase. In our proposed system, the two independent separation steps are size exclusion (^{1}D) and ion exchange (^{2}D) where a mixture of proteins can be sorted based on their size in ^{1}D by actively trapping proteins on a polymer hydrogel having a gradient of pore sizes. Smaller-sized proteins will enter the hydrogel pores and larger proteins will be excluded

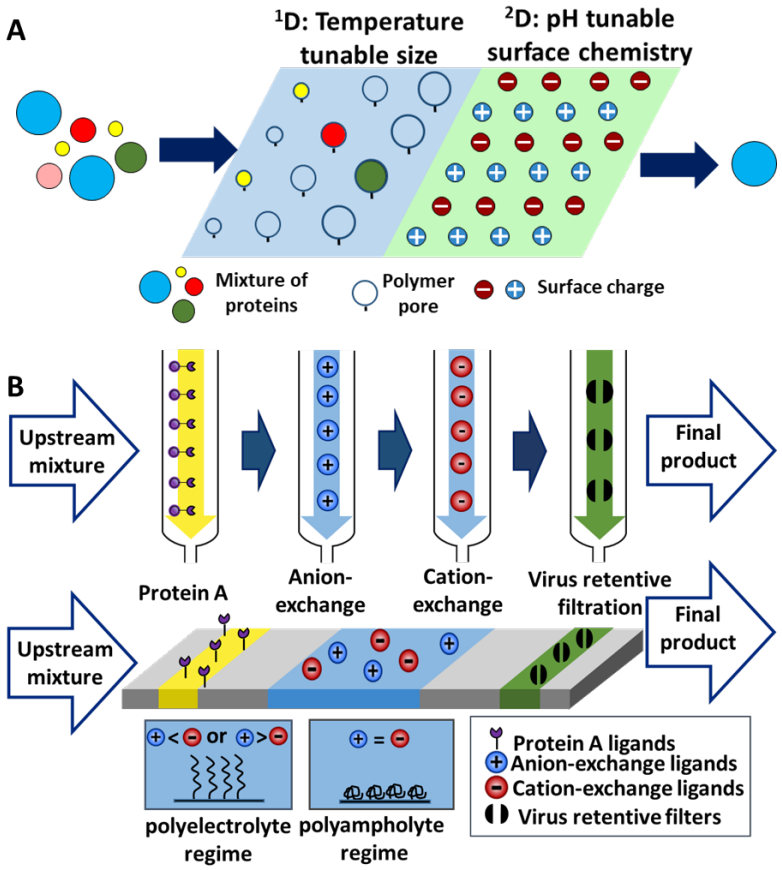

Figure 5. 2D chromatography on-a-chip, with a representative LCxLC method, where ^{1}D is size exclusion and ^{2}D is IEX. The type of polymers used here will work independently of each other and be selective. (B) A multiscale chromatographic process on a chip for protein separations. Schematic of a typical industrial antibody purification process (top), which begins with Protein A chromatography, followed by anion-exchange chromatography, cation-exchange chromatography, and virus retentive filtration. A proposed chip-based chromatography (bottom) can reduce the time and cost of the standard antibody column purification process.

and travel faster down the column. Proteins eluted from the size exclusion will be sorted further in ^{2}D where the surface chemistry will be modified with polyampholytic ion-exchangers. Stationary phase arrangements presented here will require an unprecedented level of accuracy in terms of sample preparation and probing the protein-surface interactions at the single-molecule level. Precise control of the separation mechanisms (pore size and surface chemistry) in each step will provide better resolution than conventional electrophoresis methods.

Advances in responsive polymer sample preparation and single-molecule studies of polymer–protein interactions with high spatiotemporal resolution will enable the design of a multidimensional separation on a chip. For instance, a polyampholyte-filled capillary may be tuned by varying solution conditions to function as a shape-selective substrate to capture an antibody of interest similar to affinity chromatography and an anion exchanger under similar solution conditions. Prior optimization for individual steps needs to be carried out separately to keep the degree of heterogeneous interactions to a minimum. Advanced sample preparation technologies, such as e-beam lithography to pattern polyampholyte hydrogels on the substrates and focused ion beam etching to shape the optically transparent substrates can be applied for the on-chip sample preparation. However, substrate-specific fabrication processes are needed to increase the thermal and radiation tolerance of the polymer-based microdevices to achieve high resolution in fabrication.[74] **Figure 5B** (top) demonstrates a simplified designed concept showing the sequence of purification in a standard antibody purification compared to the bottom panel in **Figure 5B** that demonstrates the on-chip implementation. In execution, we envision those multiple steps such as shape-based filtration and anion exchange can be conducted using the same polyampholyte material with different mobile phase conditions optimized using single-molecule experiments and stochastic theory. Even though microfluidic on-chip separations perform faster and require little

chemical consumption compared to alternative to traditional liquid chromatography on-chip separation still rely on empirical optimizations.[69, 71] The column-on-a-chip with single-molecule measurement capabilities will be significantly faster and more efficient, particularly in the early stages of an antibody-based biologic development before the completion of clinical trials and mass production. The microfluidic nature of the column-on-a-chip means that the entire multistep purification process can be tracked in real-time at the single-molecule level using fluorescently tagged proteins and advanced 3D single-molecule methods.

Real-time monitoring of all stages of the separation process will identify the bottlenecks and couple single-molecule observations to the previously discussed chromatographic theories, speeding chromatographic optimization through scientific observation of true physical phenomena. While the integrated single-molecule (experiment and theory) and responsive polymer approaches could lead to better performance in laboratory-based separations at small scales, additional challenges in scale up to industrial production need to be identified and addressed for practical applications, e.g., multicolumn continuous mode protein-A separation for monoclonal antibody separation.[75] The associated problems and their solutions are rather broad and system-specific, and so they are beyond the scope of this article.

Conclusions: separations by design

In conclusion, we discussed key steps to achieving high-resolution, predictive separations: the need to implement stochastic theories for a better understanding of the nanoscale heterogeneous interactions during protein separation and the use of responsive materials as separation systems for controlling molecular separation. Acquiring single-molecule knowledge of the separation system through state-of-the-art single-molecule experimental techniques and stochastic theoretical predictions of the desired molecular properties of an ideal system can fuel a

paradigm shift into futuristic separation systems. The predicted systems for ideal separation could be materialized through innovation in polymer science and chemical engineering for energy-efficient, high-resolution separations aligned with the central theme of the National Academies of Sciences report on the future of separation science.

Declaration of conflict of interests

The authors declare no competing financial interest.

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Christy F. Landes is the Kenneth S. Pitzer-Schlumberger Chair and Professor of Chemistry, Electrical & Computer Engineering, and Chemical & Biomolecular Engineering, and the Director of the Center for Adapting Flaws into Features (CAFF) at Rice University. She is also a fellow of the American Chemical Society, 2022. The Landes Group is comprised of chemists, applied physicists, and engineers who develop next-generation tools to image dynamics at soft interfaces at the limit of a single event. Her group devises new methods and models for controlling macroscale processes such as protein separations and photocatalysis using this super-resolved chemical knowledge. The group also uses advanced signal and image processing methods to improve accuracy and precision in low-signal measurements.

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(1) Misiura, A.; Shen, H.; Tauzin, L.; Dutta, C.; Bishop, L. D. C.; Carrejo, N. C.; Zepeda O, J.; Ramezani, S.; Moringo, N. A.; Marciel, A. B.; et al. Single-Molecule Dynamics Reflect IgG Conformational Changes Associated with Ion-Exchange Chromatography. *Anal. Chem.* **2021**.
(2) Moringo, N. A.; Bishop, L. D.; Shen, H.; Misiura, A.; Carrejo, N. C.; Baiyasi, R.; Wang, W.; Ye, F.; Robinson, J. T.; Landes, C. F. A Mechanistic Examination of Salting Out in Protein–Polymer Membrane Interactions. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116* (46), 22938-22945.
(3) Wirth, M. J.; Legg, M. A. Single-Molecule Probing of Adsorption and Diffusion on Silica Surfaces. *Annu. Rev. Phys. Chem.* **2007**, *58*, 489-510.
(4) Mabry, J. N.; Skaug, M. J.; Schwartz, D. K. Single-Molecule Insights into Retention at a Reversed-Phase Chromatographic Interface. *Anal. Chem.* **2014**, *86* (19), 9451-9458.
(5) Misiura, A.; Dutta, C.; Leung, W.; Zepeda O, J.; Terlier, T.; Landes, C. F. The competing influence of surface roughness, hydrophobicity, and electrostatics on protein dynamics on a self-assembled monolayer. *J. Chem. Phys.* **2022**, *156* (9), 094707.
(6) National Academies of Sciences, E.; Medicine. *A Research Agenda for Transforming Separation Science*; National Academies Press, 2019.
(7) Avorn, J. The $2.6 billion pill–methodologic and policy considerations. *N. Engl. J. Med.* **2015**, *372* (20), 1877-1879.
(8) Gritti, F.; Guiochon, G. The van Deemter Equation: Assumptions, Limits, and Adjustment to Modern High Performance Liquid Chromatography. *J. Chromatogr. A* **2013**, *1302*, 1-13.
(9) Pirrung, S. M.; van der Wielen, L. A.; van Beckhoven, R. F.; van de Sandt, E. J.; Eppink, M. H.; Ottens, M. Optimization of Biopharmaceutical Downstream Processes Supported by Mechanistic Models and Artificial Neural Networks. *Biotechnol. Prog.* **2017**, *33* (3), 696-707.
(10) Bishop, L. D.; Misiura, A.; Moringo, N. A.; Landes, C. F. Unraveling Peak Asymmetry in Chromatography Through Stochastic Theory Powered Monte Carlo Simulations. *J. Chromatogr. A* **2020**, 461323.
(11) Gritti, F.; Felinger, A.; Guiochon, G. Overloaded gradient elution chromatography on heterogeneous adsorbents in reversed-phase liquid chromatography. *J. Chromatogr. A* **2003**, *1017* (1-2), 45-61.
(12) Zhong, Z.; Lowry, M.; Wang, G.; Geng, L. Probing strong adsorption of solute onto C18-silica gel by fluorescence correlation imaging and single-molecule spectroscopy under RPLC conditions. *Anal. Chem.* **2005**, *77* (8), 2303-2310.
(13) Zhong, Z.; Geng, M. L. Microscopic origins of band broadening in chromatography. polarity distribution in C18 stationary phase probed by confocal ratiometric imaging of nile red. *Anal. Chem.* **2007**, *79* (17), 6709-6717.

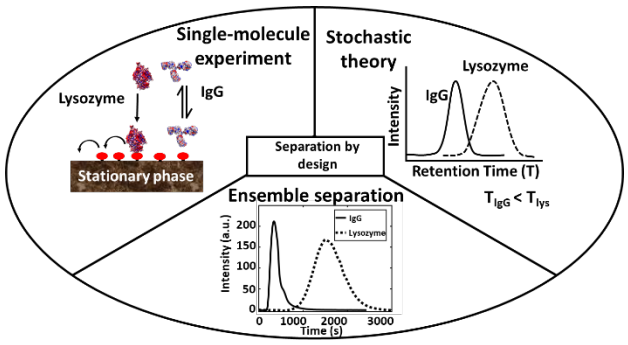
(14) Wick, C. D.; Siepmann, J. I.; Schure, M. R. Simulation studies on the effects of mobile-phase modification on partitioning in liquid chromatography. *Anal. Chem.* **2004**, *76* (10), 2886-2892.
(15) Calabrase, W.; Bishop, L. D.; Dutta, C.; Misiura, A.; Landes, C. F.; Kisley, L. Transforming Separation Science with Single-Molecule Methods. *Anal. Chem.* **2020**, *92* (20), 13622-13629.
(16) Van Deemter, J.; Zuiderweg, F.; Klinkenberg, A. v. Longitudinal Diffusion and Resistance to Mass Transfer as Causes of Nonideality in Chromatography. *Chem. Eng. Sci.* **1956**, *5* (6), 271-289.
(17) Lapidus, L.; Amundson, N. R. Mathematics of Adsorption in Beds. VI. The Effect of Longitudinal Diffusion in Ion Exchange and Chromatographic Columns. *J. Phys. Chem.* **1952**, *56* (8), 984-988.
(18) Giddings, J. C.; Eyring, H. A Molecular Dynamic Theory of Chromatography. *J. Phys. Chem.* **1955**, *59* (5), 416-421.
(19) Golshan-Shirazi, S.; Guiochon, G. Comparison of the Various Kinetic Models of Non-Linear Chromatography. *J. Chromatogr. A* **1992**, *603* (1-2), 1-11.
(20) Giddings, J. C. *Unified Separation Science*; Wiley New York, 1991.
(21) Cavazzini, A.; Dondi, F.; Jaulmes, A.; Vidal-Madjar, C.; Felinger, A. Monte Carlo Model of Nonlinear Chromatography: Correspondence Between the Microscopic Stochastic Model and the Macroscopic Thomas Kinetic Model. *Anal. Chem.* **2002**, *74* (24), 6269-6278.
(22) Pasti, L.; Dondi, F.; van Hulst, M.; Schoenmakers, P. J.; Martin, M.; Felinger, A. Experimental Validation of the Stochastic Theory of Size-Exclusion Chromatography: Retention on Single and Coupled Columns. *Chromatographia* **2003**, *57* (1), S171-S186.
(23) McQuarrie, D. A. On the Stochastic Theory of Chromatography. *J. Chem. Phys.* **1963**, *38* (2), 437-445.
(24) McQuarrie, D. A. A Theory of Fused Salts and On the Stochastic Theory of Chromatography. Oregon, Department of Chemistry, 1962.
(25) Pasti, L.; Cavazzini, A.; Felinger, A.; Martin, M.; Dondi, F. Single-Molecule Observation and Chromatography Unified by Lévy Process Representation. *Anal. Chem.* **2005**, *77* (8), 2524-2535.
(26) Shen, H.; Tauzin, L. J.; Baiyasi, R.; Wang, W.; Moringo, N.; Shuang, B.; Landes, C. F. Single Particle Tracking: From Theory to Biophysical Applications. *Chem. Rev.* **2017**, *117* (11), 7331-7376.
(27) Kisley, L.; Chen, J.; Mansur, A. P.; Shuang, B.; Kourentzi, K.; Poongavanam, M.-V.; Chen, W.-H.; Dhamane, S.; Willson, R. C.; Landes, C. F. Unified Superresolution Experiments and Stochastic Theory Provide Mechanistic Insight into Protein Ion-Exchange Adsorptive Separations. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (6), 2075-2080.
(28) Cooper, J. T.; Peterson, E. M.; Harris, J. M. Fluorescence Imaging of Single-Molecule Retention Trajectories in Reversed-Phase Chromatographic Particles. *Anal. Chem.* **2013**, *85* (19), 9363-9370.
(29) Wirth, M. J.; Swinton, D. J. Single-Molecule Probing of Mixed-Mode Adsorption at a Chromatographic Interface. *Anal. Chem.* **1998**, *70* (24), 5264-5271.
(30) Wirth, M. J.; Swinton, D. J. Single-Molecule Study of an Adsorbed Oligonucleotide Undergoing Both Lateral Diffusion and Strong Adsorption. *J. Phys. Chem. B* **2001**, *105* (7), 1472-1477.
(31) Wang, W.; Shen, H.; Moringo, N. A.; Carrejo, N. C.; Ye, F.; Robinson, J. T.; Landes, C. F. Super-Temporal-Resolved Microscopy Reveals Multistep Desorption Kinetics of α-Lactalbumin from Nylon. *Langmuir* **2018**, *34* (23), 6697-6702.

(32) Kisley, L.; Brunetti, R.; Tauzin, L. J.; Shuang, B.; Yi, X.; Kirkeminde, A. W.; Higgins, D. A.; Weiss, S.; Landes, C. F. Characterization of porous materials by fluorescence correlation spectroscopy super-resolution optical fluctuation imaging. *ACS nano* **2015**, *9* (9), 9158-9166.
(33) Dutta, C.; Bishop, L. D.; Zepeda O, J.; Chatterjee, S.; Flatebo, C.; Landes, C. F. Imaging Switchable Protein Interactions with an Active Porous Polymer Support. *J. Phys. Chem. B* **2020**, *124* (22), 4412-4420.
(34) Komsta, Ł.; Vander Heyden, Y.; Sherma, J. *Chemometrics in Chromatography*; CRC Press, 2018.
(35) Giddings, J. C. The random downstream migration of molecules in chromatography. *J. Chem. Ed.* **1958**, *35* (12), 588.
(36) Hlushkou, D.; Gritti, F.; Guiochon, G.; Seidel-Morgenstern, A.; Tallarek, U. Effect of Adsorption on Solute Dispersion: a Microscopic Stochastic Approach. *Anal. Chem.* **2014**, *86* (9), 4463-4470.
(37) Gritti, F.; Hochstrasser, J.; Svidrytski, A.; Hlushkou, D.; Tallarek, U. Morphology-Transport Relationships in Liquid Chromatography: Application to Method Development in Size Exclusion Chromatography. *J. Chromatogr. A* **2020**, 460991.
(38) Sarfati, R.; Calderon, C. P.; Schwartz, D. K. Enhanced Diffusive Transport in Fluctuating Porous Media. *ACS nano* **2021**, *15* (4), 7392-7398.
(39) Wahab, M. F.; Patel, D. C.; Armstrong, D. W. Total Peak Shape Analysis: Detection and Quantitation of Concurrent Fronting, Tailing, and Their Effect on Asymmetry Measurements. *J. Chromatogr. A* **2017**, *1509*, 163-170.
(40) Dondi, F.; Munari, P.; Remelli, M.; Cavazzini, A. Monte Carlo Model of Nonlinear Chromatography. *Anal. Chem.* **2000**, *72* (18), 4353-4362.
(41) Reznik, C.; Estillore, N.; Advincula, R. C.; Landes, C. F. Single molecule spectroscopy reveals heterogeneous transport mechanisms for molecular ions in a polyelectrolyte polymer brush. *J. Phys. Chem. B* **2009**, *113* (44), 14611-14618.
(42) Reznik, C.; Darugar, Q.; Wheat, A.; Fulghum, T.; Advincula, R. C.; Landes, C. F. Single ion diffusive transport within a poly (styrene sulfonate) polymer brush matrix probed by fluorescence correlation spectroscopy. *J. Phys. Chem. B* **2008**, *112* (35), 10890-10897.
(43) Tauzin, L. J.; Shuang, B.; Kisley, L.; Mansur, A. P.; Chen, J.; de Leon, A.; Advincula, R. C.; Landes, C. F. Charge-dependent transport switching of single molecular ions in a weak polyelectrolyte multilayer. *Langmuir* **2014**, *30* (28), 8391-8399.
(44) Stuart, M. A. C.; Huck, W. T.; Genzer, J.; Müller, M.; Ober, C.; Stamm, M.; Sukhorukov, G. B.; Szleifer, I.; Tsukruk, V. V.; Urban, M. Emerging Applications of Stimuli-Responsive Polymer Materials. *Nat. Mater.* **2010**, *9* (2), 101-113.
(45) Chen, W.-L.; Cordero, R.; Tran, H.; Ober, C. K. 50th Anniversary Perspective: Polymer Brushes: Novel Surfaces for Future Materials. *Macromolecules* **2017**, *50* (11), 4089-4113.
(46) García, T. A.; Gervasi, C. A.; Rodríguez Presa, M. a. J.; Otamendi, J. I.; Moya, S. E.; Azzaroni, O. Molecular transport in thin thermoresponsive poly (N-isopropylacrylamide) brushes with varying grafting density. *J. Phys. Chem. C* **2012**, *116* (26), 13944-13953.
(47) Wang, S.; Jing, B.; Zhu, Y. Single molecule diffusion on hard, soft and fluid surfaces. *RSC advances* **2012**, *2* (9), 3835-3843.
(48) Zhang, C.; Chu, X.; Zheng, Z.; Jia, P.; Zhao, J. Diffusion of ionic fluorescent probes atop polyelectrolyte brushes. *J. Phys. Chem. B* **2011**, *115* (51), 15167-15173.
(49) Elliott, L. C.; Barhoum, M.; Harris, J. M.; Bohn, P. W. Trajectory analysis of single molecules exhibiting non-Brownian motion. *Phys. Chem. Chem. Phys.* **2011**, *13* (10), 4326-4334.

(50) Elliott, L. C. C.; Barhoum, M.; Harris, J. M.; Bohn, P. W. Single Molecule Tracking Studies of Lower Critical Solution Temperature Transition Behavior in Poly(N-isopropylacrylamide). *Langmuir* **2011**, *27* (17), 11037-11043.
(51) Reznik, C.; Landes, C. F. Transport in supported polyelectrolyte brushes. *Acc. Chem. Res.* **2012**, *45* (11), 1927-1935.
(52) Daniels, C.; Tauzin, L.; Foster, E.; Advincula, R.; Landes, C. On the pH-responsive, charge-selective, polymer-brush-mediated transport probed by traditional and scanning fluorescence correlation spectroscopy. *J. Phys. Chem. B* **2013**, *117* (16), 4284-4290.
(53) Nagase, K.; Kanazawa, H. Temperature-Responsive Chromatography for Bioseparations: A Review. *Anal. Chim. Acta* **2020**.
(54) Contreras-Cáceres, R.; Sánchez-Iglesias, A.; Karg, M.; Pastoriza-Santos, I.; Pérez-Juste, J.; Pacifico, J.; Hellweg, T.; Fernández-Barbero, A.; Liz-Marzán, L. M. Encapsulation and Growth of Gold Nanoparticles in Thermoresponsive Microgels. *Adv. Mater.* **2008**, *20* (9), 1666-1670.
(55) Gao, C.; Lin, G.; Lei, Z.; Zheng, Q.; Lin, J.; Lin, Z. Facile Synthesis of Core–Shell Structured Magnetic Covalent Organic Framework Composite Nanospheres for Selective Enrichment of Peptides with Simultaneous Exclusion of Proteins. *J Mater. Chem. B* . **2017**, *5* (36), 7496-7503.
(56) Kim, K.; Baldaguez Medina, P.; Elbert, J.; Kayiwa, E.; Cusick, R. D.; Men, Y.; Su, X. Molecular tuning of redox-copolymers for selective electrochemical remediation. *Adv. Funct. Mater.* **2020**, *30* (52), 2004635.
(57) Su, X. Electrochemical interfaces for chemical and biomolecular separations. *Curr Opin Colloid Interface Sci.* **2020**, *46*, 77-93.
(58) Zhao, Y.; Mamrol, N.; Tarpeh, W. A.; Yang, X.; Gao, C.; Van der Bruggen, B. Advanced ion transfer materials in electro-driven membrane processes for sustainable ion-resource extraction and recovery. *Prog. Mater. Sci.* **2022**, 100958.
(59) Hoener, B. S.; Zhang, H.; Heiderscheit, T. S.; Kirchner, S. R.; De Silva Indrasekara, A. S.; Baiyasi, R.; Cai, Y.; Nordlander, P.; Link, S.; Landes, C. F. Spectral response of plasmonic gold nanoparticles to capacitive charging: morphology effects. *J. Phys. Chem. Lett.* **2017**, *8* (12), 2681-2688.
(60) Zheng, J.; Lin, Z.; Lin, G.; Yang, H.; Zhang, L. Preparation of Magnetic Metal–Organic Framework Nanocomposites for Highly Specific Separation of Histidine-Rich Proteins. *J Mater. Chem. B* . **2015**, *3* (10), 2185-2191.
(61) Anstine, D. M.; Sholl, D. S.; Siepmann, J. I.; Snurr, R. Q.; Aspuru-Guzik, A.; Colina, C. M. In silico design of microporous polymers for chemical separations and storage. *Curr. Opin. Chem. Eng.* **2022**, *36*, 100795.
(62) Liu, G.; Xu, Y.; Han, Y.; Wu, J.; Xu, J.; Meng, H.; Zhang, X. Immobilization of Lysozyme Proteins on a Hierarchical Zeolitic Imidazolate Framework (ZIF-8). *Dalton Trans.* **2017**, *46* (7), 2114-2121.
(63) Cote, A. P.; Benin, A. I.; Ockwig, N. W.; O'Keeffe, M.; Matzger, A. J.; Yaghi, O. M. Porous, Crystalline, Covalent Organic Frameworks. *Science* **2005**, *310* (5751), 1166-1170.
(64) Simon, U.; Dimartino, S. Direct 3D Printing of Monolithic Ion Exchange Adsorbers. *J. Chromatogr. A* **2019**, *1587*, 119-128.
(65) Gupta, V.; Beirne, S.; Nesterenko, P. N.; Paull, B. Investigating the Effect of Column Geometry on Separation Efficiency using 3D Printed Liquid Chromatographic Columns Containing Polymer Monolithic Phases. *Anal. Chem.* **2018**, *90* (2), 1186-1194.

(66) Salmean, C.; Dimartino, S. 3D-Printed Stationary Phases with Ordered Morphology: State of the Art and Future Development in Liquid Chromatography. *Chromatographia* **2019**, *82* (1), 443-463.
(67) Dolamore, F.; Fee, C.; Dimartino, S. Modelling Ordered Packed Beds of Spheres: The Importance of Bed Orientation and the Influence of Tortuosity on Dispersion. *J. Chromatogr. A* **2018**, *1532*, 150-160.
(68) Zhou, L. Y.; Fu, J.; He, Y. A Review of 3D Printing Technologies for Soft Polymer Materials. *Adv. Funct. Mater.* **2020**, *30* (28), 2000187.
(69) Rodríguez-Ruiz, I.; Babenko, V.; Martínez-Rodríguez, S.; Gavira, J. Protein separation under a microfluidic regime. *Analyst* **2018**, *143* (3), 606-619.
(70) Vicente, F. A.; Plazl, I.; Ventura, S. P.; Žnidaršič-Plazl, P. Separation and purification of biomacromolecules based on microfluidics. *Green Chem.* **2020**, *22* (14), 4391-4410.
(71) Kutter, J. P. Liquid phase chromatography on microchips. *J. Chromatogr. A* **2012**, *1221*, 72-82.
(72) Blumberg, L.; Klee, M. S. A Critical Look at the Definition of Multidimensional Separations. *J. Chromatogr. A* **2010**, *1217* (1), 99-103.
(73) Giddings, J. Concepts and Comparisons in Multidimensional Separation. *J. High Resolut. Chromatogr.* **1987**, *10* (5), 319-323.
(74) Carbonell, C.; Valles, D.; Wong, A. M.; Carlini, A. S.; Touve, M. A.; Korpanty, J.; Gianneschi, N. C.; Braunschweig, A. B. Polymer Brush Hypersurface Photolithography. *Nat. Commun.* **2020**, *11* (1), 1-8.
(75) Ramos-de-la-Peña, A. M.; González-Valdez, J.; Aguilar, O. Protein A Chromatography: Challenges and Progress in the Purification of Monoclonal Antibodies. *J. Sep. Sci.* **2019**, *42* (9), 1816-1827.

TOC Graphic:


Single-molecule experiment
Lysozyme
IgG
Stationary phase
Stochastic theory
Lysozyme
IgG
Intensity
Retention Time (T)
$T_{IgG} < T_{lys}$
Separation by design
Ensemble separation
IgG
Lysozyme
Intensity (a.u.)
200
150
100
50
0
0
1000
2000
3000
Time (s)