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Review

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Capillary Electrophoresis for Quantitative Studies of Biomolecular Interactions

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CONTENTS

The development of highly selective and sensitive analytical techniques has been a driving force for unprecedented advances in biotechnology, gene engineering, and drug discovery. Capillary electrophoresis (CE) is becoming a wider accepted analytical method in biology and medicine. CE offers short analysis time, high resolution, and minute consumption of samples and reagents, making it an attractive technique for mass bioassays and drug screening. Since the last *Analytical Chemistry* review in this field,[1] there have been published over 10 000 articles with CE as a topic. Within a variety of studies concerning CE, we have identified the intensively developing area of reversible biomolecular interactions which are defined as highly selective noncovalent binding of ligands with biomolecules. These affinity interactions control cell recognition, signal transduction, immune response, DNA replication, gene expression, and other cellular processes. The knowledge of quantitative parameters of binding reactions (equilibrium and/or rate constants) is essential for understanding the mechanisms of biological processes, which these reactions regulate. The present review covers a 3-year period between January 2012 and November 2014. We have attempted to select studies that demonstrate the newest and most impactive developments in the field of biomolecular affinity interactions.

KINETIC CAPILLARY ELECTROPHORESIS

In the case of 1:1 stoichiometry, two molecules, a target (T) and a ligand (L), take part in affinity interactions. In the review, we assign T to diagnostic and therapeutic targets and L to their affinity partners (e.g., diagnostic probes and affinity ligands), although such assignment is often arbitrary. Affinity interaction of L and T with the formation of an affinity complex (C) is described by the following equation:

$$\mathrm{L} + \mathrm{T} \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftarrows}} \mathrm{C} \tag{1}$$

where k_{on} and k_{off} are rate constants of complex formation and dissociation, respectively. Complex stability is typically described in terms of the equilibrium dissociation constant, $K_d = k_{off}/k_{on}$, or the equilibrium binding constant, $K_b = 1/K_d$. For a consistent review, we will list all equilibrium constants as K_d.

In the case of CE, the affinity partners L, T, and C are continuously separated in an electric field due to differences of their electrophoretic velocities, v_L, v_T, and v_C, respectively. The processes occurring within the capillary are described by a general system of partial differential equations:

$$\begin{cases} \dfrac{\partial L(t,x)}{\partial t} + v_{\mathrm{L}}\dfrac{\partial L(t,x)}{\partial x} = -k_{\mathrm{on}}L(t,x)T(t,x) + k_{\mathrm{off}}C(t,x) \\ \dfrac{\partial T(t,x)}{\partial t} + v_{\mathrm{T}}\dfrac{\partial T(t,x)}{\partial x} = -k_{\mathrm{on}}L(t,x)T(t,x) + k_{\mathrm{off}}C(t,x) \\ \dfrac{\partial C(t,x)}{\partial t} + v_{\mathrm{C}}\dfrac{\partial C(t,x)}{\partial x} = -k_{\mathrm{off}}C(t,x) + k_{\mathrm{on}}L(t,x)T(t,x) \end{cases} \tag{2}$$

where L, T, and C are concentration of L, T, and C, respectively, t is time, and x is the coordinate coaxial with the capillary length. The system of differential equations in eq 2 is common for all CE-based methods used for studying reaction 1. The apparent diversity of experimental CE approaches is a result of a variety of initial and boundary conditions: initial distribution of reaction components along the capillary and the ways in which they enter and leave the capillary during separation. The diversity of these conditions is what defines

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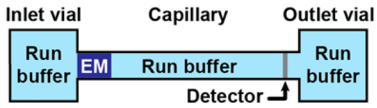
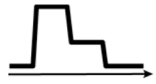
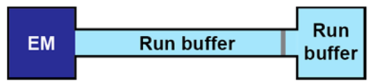
Table 1. Summary of KCE Methods

Name of KCE method[a]	Schematic of initial and boundary conditions[b]	Typical signal[c]	Proven determination of reaction constants	
			Parameter-based mode (parameters)	Pattern-based mode
NECEEM – non-equilibrium CE of equilibrium mixtures (Pre-equilibrated CZE)[d] (APCE – affinity-probe CE)[d]			NECEEM: K_d and k_{off} (areas and migration times) CZE, APCE: K_d (areas)	k_{on} and k_{off}
ppKCE – plug-plug kinetic CE (PF-ACE – partial-filling ACE)[d]			ppKCE: k_{on}, k_{off} (areas and migration times) PF-ACE: K_d (mobilities)	k_{on} and k_{off}
FA – frontal analysis (KCE/FA)			K_d (heights)	N/A[e]
cNECEEM – continuous NECEEM (FACCE – frontal analysis continuous CE)[d]			K_d (heights)	N/A[e]
ECEEM – equilibrium CE of equilibrium mixtures (ACE – affinity CE)[d]			K_d (mobilities) K_d, k_{on}, and k_{off} (peak heights and widths, migration times)[f]	k_{on} and k_{off}[f]
ACE – affinity CE, CACE – competitive ACE[g]			K_d (mobilities) K_d, k_{on}, and k_{off} (peak heights and widths, migration times)[f]	k_{on} and k_{off}[f]
SweepCE – sweeping CE			N/A[h]	k_{on} and k_{off}
HD – Hummel-Dreyer method			K_d (peak areas or heights)	N/A[e]
MASKE – macroscopic approach to studying kinetics at equilibrium			K_d, k_{on}, and k_{off} (peak heights and widths, migration times)	k_{on} and k_{off}
VP – vacancy peak method, VACE – vacancy ACE			K_d (VP: peak areas or heights, VACE: mobilities)	N/A[e]
sSweepCE – short SweepCE			N/A[h]	N/A[e]
sSweepCEEM – short SweepCE of equilibrium mixtures			N/A[h]	N/A[e]

[a]Short descriptions of the main methods can be found in refs 2, 10, and 11. [b]Abbreviations used: L, detectable ligand; T, target; EM, equilibrium mixture of L and T; EM^0, equilibrium mixture of L^0 (undetectable ligand) and T. In the presence of a typical electroosmotic flow directed from left to right, the signal on the detector does not depend on the outlet-vial contents. For simplicity of the experimental procedure, the outlet vial can be filled with the run buffer. [c]Assuming that L is detected and T is not, the detected signal is proportional to ([L] + [C]). [d]Outdated method name. [e]Analytical solution (mathematical expression describing the dependence of signal on time) is not available yet. [f]MASKE mathematical approach is used under an assumption of [L] ≪ [T], see ref 12. [g]In the competitive ACE method, T is the mixture of neutral and charged targets. [h]Analytical solution (equilibrium and/or rate constants as functions of parameters from the electropherogram) is not available yet.

different experimental approaches for studying reaction 1 as shown in Table 1.

When L and T are mixed and incubated, dynamic equilibrium between L, T, and C is eventually established. Strictly speaking, reaction 1 cannot be maintained in equilibrium during CE separation, but some initial and boundary conditions shown in Table 1 allow for the assumption of equilibrium with sufficient accuracy. Such "pseudo-equilibrium" methods were introduced first due to their relative simplicity. They have been typically called affinity CE methods and used to determine K_d only. While determining K_d by pseudoequilibrium affinity CE methods, the following parameters from an electropherogram are used: peak areas, peak heights, migration times, and others. However, peak-shape distortions (band broadening and tailing), associated with the nonequilibrium nature of reaction 1 inside the capillary, are

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often observed and affect the accuracy of K_d determination. Moreover, it has always been appreciated that the shapes of the peaks depend on k_{on} and k_{off}, and the values of k_{on} and k_{off} can, thus, be determined from analyzing the shapes. Deconvolution of kinetic parameters from electropherograms was (and is), however, a difficult to solve problem.

There are two general approaches for finding K_d, k_{on}, and k_{off} from the analysis of electropherograms: an approach that requires an analytical solution of the system of equations in eq 2 and a brute-force approach that involves in silico solution of this system. The analytical approach can, in turn, be split into two categories, namely, parameter-based methods and pattern-based methods. The parameter-based methods require analytical solutions that explicitly link K_d, k_{on}, and k_{off} with parameters from the electropherogram (e.g., peak areas, peak heights, peak widths, and migration times). The pattern-based approach requires an analytical solution of system of equations in eq 2 in a form of time-dependence of signal, S, which is proportional to a sum of concentrations: e.g., $S(t) \sim (L(t) + C(t))$. This solution is fitted into the experimental time-dependence of signal while varying k_{on} and k_{off}, and the best fit reveals the values of k_{on} and k_{off}. Both types of analytical solutions can only be obtained by the linearization of eq 2 under some simplifying assumption. The analytical solutions are difficult to find, they typically require efforts of professional mathematicians. The brute-force approach requires much more complicated and time-consuming computation; therefore, it is rarely used.

Despite the difficulties, some practical mathematical approaches for extracting kinetic information from electropherograms, obtained under different initial and boundary conditions, have been developed over the past decade. Methods, utilizing this advanced data treatment to obtain not only K_d but also k_{on} and k_{off} from electrophoretic signal were named methods of kinetic CE (KCE). It is important to emphasize that KCE methods are able to determine the unknown concentration of T in a calibration-free approach,[2] and, in some cases, stoichiometry of affinity interaction.[3,4]

If KCE is defined as the CE separation of T, L, and C, which are allowed to interact during separation, with a purpose of studying the mechanism of reaction 1 or for finding the unknown concentration of T, then the concept of KCE embraces all CE-based affinity methods. Therefore, for simplicity we use the term of KCE for all CE-based affinity methods (see Table 1). It should be noted that, in this work, the term of affinity capillary electrophoresis (ACE) refers only to a single KCE method for which it was originally suggested.[5]

For uniformity, we assume that L is a detectable molecule so that both L and C can be detected. It is essential for all KCE methods that mobility of C must be significantly different from that of L. Obviously, this condition is easier to satisfy when the molecular size of L is smaller than that of T. Different techniques may be applied to observe L and C. Some of them are based on label-free detection (ultraviolet (UV), mass spectrometry (MS)) while others use labeling of L, for example, with a fluorescent tag.

Other processes involving T and L, such as diffusion, adsorption to capillary walls, and binding with a different from 1:1 stoichiometry, may need be taken into consideration. In such a case, additional terms must be added to the system of differential equations in eq 2. The analytical solution of this complex system of equations is impossible, and the brute-force approach has to be used. Moreover, even for KCE methods which can be adequately described by eq 2, only the brute-force approach is a universal way of finding k_{on} and k_{off}. As we mentioned above, the partial differential equations can be linearized to solve eq 2 analytically only for a few KCE methods, for which some simplifying assumptions may be found. It is even more difficult to find a solution that would facilitate the parameter-based approach for finding K_d, k_{on}, and k_{off}.

The lack of simple solutions of eq 2 and the requirement of using extensive numerical computation were, and to some extent are, key factors preventing a widespread application of KCE methods for finding k_{on} and k_{off} of biomolecular interactions. We anticipate that advances in computing and availability of user-friendly software for KCE simulation will overcome these challenges in the near future. We would also like to see more KCE methods to be served with parameter-based approaches for extraction of k_{on} and k_{off} values from KCE electropherograms.

APPLICATIONS

Aptamers. Aptamers are oligonucleotide or peptide molecules that can bind to their targets with high affinity and specificity due to the complementarity of their three-dimensional structures. Nucleotide-based aptamers are short single stranded DNA (ssDNA), RNA, or nucleic acid analogues. In the 1980s, the studies on human immunodeficiency virus and adenovirus shed light on the regulative role of short nucleic acids. Fundamental research on aptamers has arisen since the in vitro selection process called systematic evolution of ligands by exponential enrichment (SELEX) was introduced by both Szostak's and Gold's groups in 1990. Aptamers have been investigated in numerous studies concerning their application as diagnostic and therapeutic tools and biosensing probes.[6−9]

Since the protein-unbound oligonucleotides are readily separated by zone electrophoresis from proteins and oligonucleotide−protein complexes, KCE methods are successfully applied for studying binding of oligonucleotides to proteins. Hence, KCE can serve as a generic tool for studies of oligonucleotide aptamers that mimic antibodies in their function of high-affinity binding to protein targets. Two main areas should be distinguished: (i) KCE for quantitative characterization of aptamer-target binding and (ii) KCE-based aptamer selection.

KCE for Quantitative Characterization of Aptamer-Target Binding. In general, aptamers are more stable than antibodies especially with regards to temperature and pH. At the same time, various experimental conditions influence biomolecular conformations, thus, affecting aptamer-target binding. Girardot et al. employed microchip electrophoresis in a frontal analysis continuous mode to provide new insights into the interaction between a nucleotide-based aptamer and its target (lysozyme).[13] Their study has evidenced the influence of different factors (nature and ionic strength of background electrolyte, nature and concentration of an added divalent cation, thermal treatment of the aptamer) on the aptamer−lysozyme interaction. These results, in parallel with studying both nanoparticle-aptamer conjugates[14,15] and immobilized aptamers,[16] may help to develop new miniaturized devices for molecular diagnostics.

Oligonucleotide aptamers were reported to inhibit the dealkylation function of the AlkB protein from *Escherichia coli* (*E. coli*), which belongs to oxoglutarate-dependent oxygenases.[17] This subfamily of proteins is known to enable the

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repair of methylation in DNA and RNA; thus, such oxygenases may restore normal cell functioning. In contrast, many anticancer drugs cause damage of tumor cells through methylation of their nucleic acids. Consequently, conservation of such methylation damages via inactivation of oxoglutarate-dependent oxygenases in tumor tissues will increase the probability of death of cancerous cells. It was shown that K_d values for the aptamer−enzyme complex were in the nanomolar range indicating high affinity of the studied DNA aptamers toward AlkB.[17] The selected aptamers were found to inhibit the demethylation function of AlkB. The inhibition assay reveled correlation between the values of the inhibition constants and K_d. The following kinetic analysis indicated that the aptamers interacted with the protein aside from the AlkB active site. We anticipate the further selection of DNA aptamers for human homologues of AlkB-protein and their consequent evaluation as chemotherapy enhancers.

Peptide nucleic acid (PNA) is an analogue of DNA with an electro-neutral polyamide backbone. In PNA, the natural nucleobases are linked via peptide bonds. There is no electrostatic repulsion between PNA and DNA strands, and this synthetic oligonucleotide-analogue readily hybridizes to the folded DNA through strand invasion. Therefore, PNAs may be utilized as affinity probes for the secondary-structured DNA. A KCE approach was adopted to characterize binding between PNA and hairpin-structured 60-base DNA strands (HP2w and HP3w).[18] PNA and polyethylene glycol formed a diblock copolymer (PEG-*b*-PNA), which was used as an affinity probe. A base sequence of the PNA-part of the probe was complementary to a hairpin-structured segment of the ssDNA. For the PEG-*b*-PNA probe bound to HP3w, the K_d value was found to be 280 times lower than micromolar K_d for the DNA-based probe (PEG-*b*-DNA) bound to HP2w. Similar allele-specific PNA probes modified by PEG were used to estimate allele frequency of single-nucleotide polymorphisms in DNAs obtained after the pooling procedure.[19−21]

Aptamers that bind small molecules can serve as universal biosensing tools. Measuring the values of K_d and k_{on} for interaction between an aptamer and a small molecule helps to determine the effectiveness of the aptamer-based sensors. The K_d values are often measured in a series of experiments by varying either aptamer or target concentrations. A new analytical tool, laser-tweezers, was offered to determine K_d for aptamer interaction with adenosine triphosphate (ATP) at the single-molecule level from only one ATP concentration (100 μM).[22] As a reference, the NECEEM method (nonequilibrium capillary electrophoresis of equilibrium mixtures) was successfully used to validate the laser tweezers technique: $K_d = 2.0 \pm 0.2$ μM measured by applying a Hess-like cycle was in good agreement with the $K_d = 2.4 \pm 0.4$ μM obtained from KCE data using Langmuir-isotherm analysis.

KCE is suitable for evaluation of aptamer binding with complex targets. Recently *Lactobacillus acidophilus* and *Escherichia coli* as well as their protoplasts were treated as targets for a random ssDNA aptamer.[23] By using the ACE and NECEEM methods, it was found that protoplasts without a cell wall had apparently strong interaction with ssDNA ($K_d \sim 10^{-9}$ M) but bacteria did not bind aptamers. However, alcohol treatment of *E. coli* led to ssDNA binding ($K_d \sim 10^{-8}$ M). Therefore, bacteria pretreatment may be considered as a useful procedure in whole-cell aptamers selection.

KCE-Based Aptamer Selection. Non-SELEX and CE-SELEX are popular KCE approaches for aptamer selection from random oligonucleotide libraries. We should clarify these terms because sometimes they are mixed. In 2004, Mendonsa and Bowser[24] successfully used KCE as an alternative partitioning method to affinity chromatography in SELEX and named this new approach CE-SELEX. In 2006, Berezovski et al.[25] reported another version of KCE-based aptamer selection, which was called non-SELEX for the fact that it did not involve PCR amplification and strand separation between the rounds of KCE-based partitioning.

In recent years, non-SELEX and CE-SELEX, mainly by using NECEEM for both partitioning and determination of binding parameters, have been frequently used as a fast and efficient way to develop aptamers against different proteins, for example, bovine catalase ($K_d \sim 10^{-7}$ to 10^{-5} M),[26] human leptin protein ($K_d \sim 10^{-6}$ M),[27] human thrombin ($K_d \sim 10^{-10}$ to 10^{-8} M),[28,29] Ara h1 protein ($K_d \sim 10^{-7}$ M),[30] recombinant human vascular endothelial growth factor 165 ($K_d \sim 10^{-8}$ M).[31]

The boronate affinity monolithic capillary was used as a platform for target immobilization and rapid selection of high-specificity glycoprotein-binding DNA aptamers. A new SELEX method based on a monolithic capillary allowed for efficient selection of the aptamers in six rounds and the K_d values were in a range of 10^{-8} M.[32] This SELEX approach needed fewer rounds of selection than conventional SELEX but more than typical CE-SELEX or non-SELEX with NECEEM-based partitioning.

NECEEM provides a means to predict partitioning efficiency during the non-SELEX procedure. Recently, Yu and Yu[33] have analyzed selective enrichment of NECEEM-based non-SELEX and evaluated the efficiency of partitioning target-bound ligands from free ligands. They predicted the levels of enrichment of non-SELEX selection under different conditions such as protein concentrations and efficiencies of partitioning.

Ashley and Li[27] applied both the fluorescence-intensity method with the protein immobilized onto a 96-well microplate and the NECEEM method to evaluate aptamers' binding affinity against human leptin protein. NECEEM yielded K_d values in the high nanomolar range, whereas the fluorescence intensity method gave K_d values in the low micromolar range. It was concluded that the immobilization of smaller targets onto the plate surface can affect the ability of the target to bind DNA, giving rise to higher K_d.[27] A similar algorithm of aptamer selection utilizing CE-SELEX and subsequent K_d validation using a fluorescence-polarization technique was adopted by Jing and Bowser.[31] At first, high-affinity aptamers ($K_d \sim 10^{-8}$ M) for recombinant human vascular endothelial growth factor 165 were identified. Then, affinities of the abundant and randomly chosen aptamers were characterized using two orthogonal methods, NECEEM and fluorescence polarization. Similar to the findings of Ashley and Li,[27] the use of the microplate-based fluorescence-polarization method resulted in higher K_d values in comparison to those obtained with the NECEEM method.[31]

Immune system disorder can lead to anaphylaxis caused by natural food ingredients. NECEEM-based SELEX was used to select DNA aptamers against one of the most important peanut allergens, Ara h1.[30] The K_d value of a best performing aptamer was determined independently by three different approaches, which were surface plasmon resonance, fluorescence anisotropy, and NECEEM, 353, 419, and 450 nM, respectively. The selected aptamer due to its high affinity and specificity for the target protein was successfully used for bioassay development to detect Ara h1 protein in both buffer and food matrix samples.[30] Lipopolysaccharide (LPS), being an endotoxin, can

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trigger a lethal septic shock; therefore, fast and precise recognition of LPS from the environmental sources is very important. Kim et al. utilized non-SELEX with NECEEM partitioning to identify 10 different ssDNA aptamers displaying specific binding to LPS with the K_d values in the nanomolar range.[34] The aptamer with the highest affinity toward LPS was chosen to create an impedance biosensor on a gold surface. Before immobilization onto the gold electrode, surface-plasmon-resonance experiments were carried out and confirmed the LPS recognition ability of the immobilized aptamer. The developed electrochemical aptasensor demonstrated exceptional sensitivity and specificity in a linear detection range of 0.01 to 1 ng/mL of LPS. Moreover, the aptasensor showed considerably reduced detection time in comparison to the traditional Limulus amoebocyte lysate assay.

In summary, KCE provides a highly efficient and versatile platform for the development and evaluation of aptamers. However, the research community still needs to resolve a number of issues before aptamers can find wider applications. For example, in vivo, the small size of aptamers makes them prone to renal filtration; moreover, nonmodified aptamers are highly susceptible to degradation by nucleases. Further research onto aptamers and aptasensor systems need to be pursued to extend the frontiers of their bioapplications.

Small Molecules. In the context of this review, a small molecule is an organic compound with molecular weight of less than 1 kDa that may participate in biological processes. These compounds can act among others as drugs, signaling molecules, and pesticides. Small molecules which selectively bind to therapeutic and diagnostic targets may be used as drug leads and diagnostic probes.

Heparin, a highly sulfated polydispersed glycosaminoglycan, is the most widespread clinical anticoagulant; it binds antithrombin III (AT-III), a member of serine proteinases superfamily, accelerating its antagonist effect on blood coagulation. KCE experiments by using the ACE method were carried out for a number of heparin samples derived from various sources (porcine, bovine, and ovine mucosa) and K_d values ranging from 14.2 to 56.1 nM were determined.[35] The good correlation of K_d values with the in vitro anticoagulation activity confirms that the affinity for the AT-III target is an important feature of heparin samples and can be used for fast screening of heparin drug quality. The interaction of heparin with selected peptide fragments of apoB-100, apoE, and low- and high-density lipoproteins (LDL and HDL) were studied by capillary electrochromatography and quartz crystal microbalance (QCM) techniques.[36] It was shown that heparin interactions are stronger with apoB-100 peptide than with apoE peptide fragment, and the sulfate groups in heparin play an especially important role in interactions with apoB-100 peptide fragments, LDL, and apoE-containing HDL. Affinity interaction between the AT-III-protein and a set of ligands, including the low molecular weight heparins (LMWHs) and the synthetic pentasaccharide drug fondaparinux, was investigated by the ACE method.[37] Most of the studied ligands of AT-III were polydisperse materials containing saccharide chains of different length and structures. However, a unique bis-sulfated *N*-sulfoglucosamine residue is known to be the main structure responsible for specific interaction with AT-III. Thus, the percentage of sulfoglucosamine residues was established for each studied ligand using 2D-NMR and utilized to interpret the KCE results.[37] The described approach can be helpful for the optimization of heparin depolymerization methods or for quality assurance in the production of LMWHs for clinical use.

Human serum albumin (HSA), being the most abundant protein in human blood plasma, is the main target during drug studies. The interactions between some nonsteroidal anti-inflammatory drugs (naproxen, ibuprofen, and flurbiprofen) and HSA or bovine serum albumin (BSA) have been examined by means of two complementary techniques, isothermal titration calorimetry (ITC) and by the frontal analysis method of KCE (KCE/FA).[38] It was concluded that ITC can measure with high precision the strongest drug−albumin interactions but the interactions with more than 1:1 stoichiometry can be better determined by means of KCE/FA. Dexamethasone (DXM) is a potent glucocorticosteroid with an anti-inflammatory and immunosuppressive activity. KCE/FA was used to examine the interaction of DXM with HSA and BSA under physiological conditions (phosphate buffer, pH 7.4).[39] The number of binding sites, K_d values, and other binding parameters were calculated both from KCE/FA and from the equilibrium dialysis experiment; the latter was considered to be a reference method. KCE/FA yielded approximately 3 times lower K_d values ($\sim 10^{-4}$ M) than equilibrium dialysis. The results for DXM might be explained by inherent differences of the two experimental procedures and/or by nature of interacting samples.

The competitive binding of drugs (retinol and retinoic acid) were investigated by the ACE method and the study indicated that retinoic acid was able to replace retinol from HSA and vice versa in the case of BSA.[40] The K_d values of retinol were found to be 7.8 μM (0.19 μM) and of retinoic acid were 3.0 μM (0.44 μM) with HSA (BSA), respectively. The interaction between the cardiovascular drug isoprenaline hydrochloride and BSA was determined by two KCE methods: NECEEM and ACE.[41] The obtained binding parameters may help in gaining some insights into the possible drug/protein interactions and in the early evaluation of the pharmacokinetic profile of the drug during cardiovascular drug screening.

Interactions of drug candidates with biopolymers of the synovial fluid affect drug targeting to the articular cartilage as well as clearance from the synovial space upon intra-articular administration. Hyaluronic acid (HA) and HSA are two main components existing in the synovial fluid. The affinity of seven cationic amino acids and dipeptide β-naphthylamide derivatives toward HA and HSA was investigated in order to shed light on the influence of chemical properties of the drug-candidates on the K_d values.[42] Using KCE/FA it was demonstrated that for the ligand−HA interaction the K_d values changed from 7.5 to 23 mM whereas for the ligand−HSA interaction the K_d values varied in the range of 10^{-3}−10^{-6} M. The interactions of drug-candidates with HA were highly dependent on the number of amino-groups in the ligand. This trend was not observed for the interactions with HSA. The obtained affinity data might provide useful information in the design of cartilage adhesive prodrugs.[42] Three methods (QCM, partial-filling affinity CE (PF-ACE), and molecular dynamics) were used to clarify the temperature effect on the interaction of a chondroitin-6-sulfate with low-density lipoprotein and with a peptide fragment of apolipoprotein B-100.[43] All three techniques demonstrated a nonlinear dependence of K_d on temperature at temperatures above 37 °C for binding of chondroitin-6-sulfate to LDL and the peptide fragment.

ACE was used to study quantitatively the noncovalent interactions between β-lactoglobulin (β-LG), a milk whey

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protein, and two lantibiotics, nisin (a dairy biopreservative lantibiotic) and duramycin (a bovine mastitis treatment lantibiotic).[44] Protein adsorption to the capillary walls was found to be insignificant; therefore, capillary coating was not necessary. The interactions were studied at optimum pH levels that are close to the physiological pH 6.5 of milk. Nisin was maintained at pH 6, while duramycin at pH 6.5. The K_d values were determined to be 3.2 nM for nisin and 4.5 nM for duramycin. These results demonstrated that KCE is a suitable approach for characterizing the interaction between lantibiotics and β-LG.

Ethidium bromide (EtBr) is a DNA intercalating molecule commonly used as a molecular probe for DNA visualization. Under specific conditions it may lead to mutations, carcinogenesis, and other damages. The PF-ACE method was applied to investigate noncovalent interactions between double-stranded DNA (dsDNA) oligonucleotide (Dickerson dodecamer) and EtBr or DNA ligands of a new type, which are based on oligophenylene derivatives.[45] Two background electrolytes (BGEs) were used in the experiments: Tris-borate, pH 8.0, ionic strength of 14.3 mM (BGE1), and sodium phosphate, pH 7.5, ionic strength of 133 mM (BGE2). A partial adsorption of hydrophobic positively charged oligophenylene derivatives to hydroxypropylcellulose-coated capillary was observed and taken into account during data treatment. The K_d values of the DNA−EtBr complex (~65 μM in the BGE1 and 238 μM in the BGE2) were found to be significantly lower than K_d of DNA complexes with oligophenylene derivatives.

Cyclodextrins. Extensive current applications[46] and future perspectives of native and synthetic cyclodextrins (CDs) are based on their several unique properties including the ability to form reversible host−guest complexes and discriminate between achiral and chiral compounds. In addition, CDs are biocompatible which favors their use as vehicles in vivo.

Interactions between nine drugs and six CDs or their polymers were studied by ACE and NMR at pH 2.5.[47] After preliminary experiments, four drugs and both β-CD and poly(β-CD) were selected for the quantitative study of the interactions at pH 2.5 and 7.0. By comparing the results obtained with the β-CD and poly(β-CD), it was found that the K_d values were up to 5 times lower for polymers than for monomers. The 2D-NMR results indicated that the structure of the polymeric network favored the inclusion of the guest in the hydrophobic cavity of the CD units. Moreover, the polymers of CD had shown very high enantioselective abilities at both pH 2.5 and 7.0. The relationship between K_d and enantioseparation ability was investigated for sulfethylthio-β-CD (SET-β-CD) bearing a single negative charge and sulfooxymethylhexylthio-β-CD (SMHT-β-CD) carrying three negative charges.[48] SMHT-β-CD provided a significantly greater enantioseparation of enantiomerically related drugs than SET-β-CD. This was caused by differences in both K_d values of enantiomer-chiral selector and the mobilities of the complexed enantiomers.

Sugammadex, a new modified γ-cyclodextrin, reverses the neuromuscular blockage induced by rocuronium by forming a strong complex with this muscle relaxant. To evaluate possible interactions with potentially coadministered drugs, interactions between sugammadex and penicillins were investigated by the ACE method.[49] The obtained K_d values were in the range from 2.6 to 11 mM for amoxicillin, ampicillin, oxacillin, and dicloxacillin. No complex formation with sugammadex could be detected for both penicillin G and piperacillin. Differences in the chemical structures of penicillins are suggested to be responsible for varying binding strength of different penicillins to sugammadex. The mobility study demonstrated that interactions between sugammadex and coadministered penicillins could reduce the pharmacological effects of both.

The highly charged CDs pose a challenge in KCE experiments carried out in CD-containing BGE due to the necessity to correct for changes in BGE ionic strength, temperature, and viscosity. Being a function of CD concentration, the viscosity affects both electrophoresis and formation of analyte−CD complex. Advanced correction was applied for precise determination of the K_d values of neutral (*R*,*R*)-(+)- and (*S*,*S*)-(−)-hydrobenzoin and R- and S-bromomethylpropanol complexing with charged β-CD derivative.[50]

The sulfobutylether-β-cyclodextrin (SBE-β-CD) is a modified CD carrying approximately seven negatively charged side chains, which can potentially interact electrostatically with the guest molecule. Bile salts are also negatively charged at physiological pH, and the concomitant repulsion from SBE-β-CD could potentially reduce their affinity for this CD. Correction for BGE ionic strength, temperature, and viscosity was used to study the interaction between bile salts and the SBE-β-CD in ACE.[51] To obtain the necessary correction factors the experiments were carried out at constant power (0.55 W) and ionic strength (that lead to an average capillary temperature of 25 °C) as well as at constant voltage (10 kV) but varying ionic strength.

Pesticides are chemicals that are mainly used in agriculture to control pests and plant diseases. Many of them are resistant to degradation under natural conditions and exhibit pronounced ability to accumulate in living organisms. As a result, elimination of these chemicals from the ground is an essential and challenging task. Recently, cyclodextrins have been studied as potential complexation agents to extract pesticides from the soil.[52] K_d values for interaction between seven common pesticides and different types of CDs were measured by ACE, including its competitive version. A promising correlation between the pesticide-CD affinity and the percentage of pesticide retrieval from contaminated soil was found.

Proteins. A proteomic research is an important and fast growing field of application of capillary electrophoresis today. Proteins, peptides, and polypeptides are key participants in all biological regulatory pathways. Via interaction with target molecules they perform many different and varied biological functions, including enzymatic regulation of metabolic reactions, immune response, DNA replication, ionic and molecular transport. KCE methods are a powerful tool for binding studies of proteins and peptides toward their targets, and such studies are pivotal to the progress of the whole set of protein-related sciences. It should be noted, however, that separation of native proteins from each other by CE may be a difficult task for some protein. SDS-gel CE can easily separate proteins based on differences in their molecular sizes but in a denaturing mode incompatible with dynamic affinity interactions described by reaction 1.

A KCE-based platform to identify inhibitors of protein−protein interactions (Figure 1) was developed by Rauch et al. for the complex of heat shock protein 70 (Hsp70) and Bcl2-associated anthanogene 3 (Bag3).[53] The Bag3−Hsp70 pair is known to stabilize a number of key oncogenes that makes this complex a promising anticancer target. The NECEEM method yielded K_d = 23 nM for the Hsp70−Bag3 complex. A library of over 3000 small molecules was then screened (Figure 1), and the results were compared with previously obtained data from a

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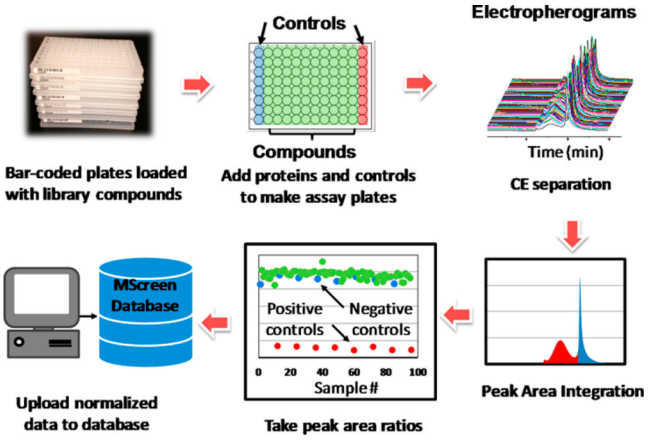

Figure 1. Step-by-step procedure for KCE-based screening inhibitors of protein−protein binding. Both library compounds and controls were loaded in 96-well plates. Then, Hsp70 labeled by Alexa-Fluor-488 was added. KCE electropherograms revealed "bound-to-free" ratios for the studied protein pairs. The obtained ratios were compared with control data. Variations in more than 3 standard deviations from control were considered hits. Reprinted from ref 53. Copyright 2013 American Chemical Society.

protein interaction assay based on flow-cytometry. The NECEEM-based screening resulted in a lower hit rate and a greater chance for the discovered compounds to be reconfirmed during the following testing steps. The NECEEM-based screening has thus revealed its greater specificity. These findings were rationalized by differences in protein modifications required to carry out KCE and flow cytometry assays. KCE was found to be a reliable reference for testing hits identified by higher-throughput technique. Increases in throughput are required to make the KCE-based platform suitable for primary screening, but the throughput of KCE methods is sufficient for secondary screening.

Peculiarities of NECEEM-based immunoassay were analyzed by Giovannoli et al.[54] As a test system they used a fluorescein-labeled HSA and a polyclonal anti-HSA. A K_d value of 75 nM was measured for the interaction between the antiserum and the labeled protein. It was determined that a high level of protein loading with the fluorescent probe must not always be considered as a positive feature because it may generally lead to multiple peaks in electropherograms.

Bohr discovered cooperative binding of oxygen to hemoglobin over a hundred years ago. Since then, many vital proteins have been shown to bind multiple ligands in a cooperative manner. Now, antibodies are under intensive studies. Wang et al. selected monoclonal anti-FLAG M2 antibody (M2) and the FLAG peptides to investigate the nature of selective affinity of M2 toward multiple FLAG peptides.[55] FLAG peptides were labeled with a fluorescent tag and used in NECEEM experiments which allowed separation and quantification of 1:1 and 1:2 M2−FLAG complexes. The binding process was described by two successive steps (complexation of M2−FLAG and then FLAG−M2−FLAG) with the corresponding functional and intrinsic equilibrium constants. These two constants were found to substantially differ (10^3−10^4-fold) from each other, the functional one being nanomolar. The work demonstrated negative cooperativity of anti-FLAG M2 antibody upon the binding of FLAG peptides.

The production of protein-based drugs strongly requires the knowledge of purity and affinity of the source protein. Protein−receptor binding was studied to establish the composition and affinity of the biomanufactured "nanobody" EGa1, which is the binding fragment of a heavy-chain-only antibody.[56] EGa1 is known to be a ligand that blocks responses of the epidermal growth factor receptor (EGFR), which is overexpressed on the surface of tumor cells. ACE analysis of the EGa1−EGFR interaction yielded nanomolar K_d.[56] Using a cellular binding assay, a comparable K_d value was obtained upon the EGa1-binding to EGFR on human carcinoma cells. In addition, the CE instrument was coupled with electrospray-ionization mass spectrometer (CE−ESI-MS) to further study the composition of EGa1. These experiments demonstrated that the "nanobody" EGa1 was heterogeneous, representing a mixture of highly related proteins possessing very similar affinity toward EGFR.

Creating drugs with weak intrinsic immunogenicity is a significant task of the pharmaceutical industry. The dendrons of L-lysine are synthetic nonimmunogenic carriers that can be used for the production of synthetic vaccines. The number of binding sites and the corresponding successive equilibrium constants between dendrigraft poly-L-lysine of generation 3 (DGL-G3) and HSA under physiological conditions were determined.[57] The KCE method (frontal analysis continuous capillary electrophoresis, FACCE) demonstrated that HSA has two binding sites with DGL-G3 with the following successive constants K_{d1} = 32 μM and K_{d2} = 33 μM. For example, these binding constants should lead to only 5% free DGL-G3 in the plasma with 40:1 HSA/DGL-G3 mass ratio. It was also shown that the interactions between DGL-G3 and HSA corresponded to a model of cooperative sites. During the subsequent study, the interaction between HSA and different generations of DGL was assayed in a physiological run buffer.[58] The K_d values and stoichiometry of the interaction were estimated by using FACCE in a polycationic modified capillary. When the K_d value decreased, then for the corresponding HSA−DGL pair the (1:n) binding stoichiometry also decreased, as shown in Figure 2. The result was in good agreement with the rise of ligand

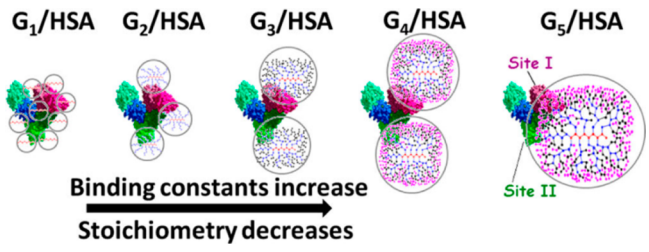

Figure 2. Interaction between five first generations (G_1−G_5) of dendrigraft poly-L-lysine and HSA. Reprinted from ref 58. Copyright 2014 American Chemical Society.

(DGL) size. The size growth led to a greater negative charge of DGL and consequently to stronger electrostatic repulsion of ligand from ligand and ligand from protein (HSA is known to have two negatively charged interaction sites). Finally, two ligand topologies (linear and dendrigraft) were investigated to compare affinity and stoichiometry of dendrimer−HSA interaction. The linear poly(L-lysine) led to much lower stoichiometry compared to dendrigraft poly(L-lysine) of similar molar mass because of much higher flexibility and contour length.

Conformational changes represent an important way of regulating biological functions of proteins within the complex environments typical for living organisms. The specific

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protein−protein binding is known to result in modulation of gating properties, surface expression, and subunit assembly of Kv-channels. ACE was employed to quantitatively evaluate the interactions between Kv-channel-interacting proteins (KChIPs) and the N-terminus of Kv4 pore-forming α-subunits (KvN) as well as between KChIP4a/related mutants and Ca^{2+}.[59] The K_d values for KChIP4a/KvN and KChIP1/KvN complexes and for KChIP4a with Ca^{2+} were found to be 0.12, 0.19, and 0.014 μM, respectively. In the presence of Ca^{2+} (10 μM), K_d of KChIP4a/KvN decreased down to 0.015 μM. The conformational change of KChIP4a induced by Ca^{2+} affects protein binding with the Kv-channel. Ca^{2+} binding activates another protein, human tissue trans-glutaminase (TG2), in the form of an extended "open" conformation, while the binding of guanidine di- or triphosphate deactivates this enzyme in a compact "closed" conformation. The large-scale Ca^{2+}-induced conformational changes associated with the regulation of TG2-activity were demonstrated using a KCE method termed equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM).[60] The measurement yielded the following descriptive parameters K_d = 38 μM, k_{open} = 14 × 10^{-2} min^{-1}, and k_{close} = 5.0 × 10^{-2} min^{-1} for calcium binding. These results establish KCE as a real-time approach for studying protein dynamics and function in general.

NECEEM was used to find that the specificity of methyl-CpG binding domain 2b (MBD2b) protein to methylated DNA decreases as more MBD2b monomers bind to the same segment of DNA.[61] MBD-family proteins can precisely bind methylated DNA sequences and mediate gene transcription, thus, MBD2b was used as a model MBD-family protein with the maximum affinity to synthesized dsDNA of variable length (20−80 bp) and of varying methylation density. It was demonstrated that several MBD2b proteins can bind to one DNA molecule with a DNA length-dependent stoichiometry. This study gives an additional opportunity to make improvements to MBD protein-based assays for monitoring DNA methylation.

Others. The affinity of boronic acids to cis-diol-containing biomolecules such as saccharides, nucleosides, and glycoproteins is utilized for sensing, separation, drug delivery, and creation of functional materials. The ACE method was utilized to examine the interactions between 14 boronic acids and 5 typical monosaccharides.[62] In particular, effects of pH and temperature on the binding strength were studied. As compared with the existing techniques, such as ^{11}B NMR and Alizarin Red S assays, the ACE method demonstrated several important advantages, which included the possibility of simultaneously studying multiple interactions, low requirement for the purity of the interacting substances, wide applicability, and high accuracy and precision.[62] A probe which is receptive to sugars should also be efficient for the recognition of peptidoglycan layers that exist in some bacteria. A new squarylium cyanine dye conjugated with boronic acid (SQ-BA) was created for fluorescently tagging Gram-positive bacteria to facilitate CE−LIF with longer excitation and emission wavelengths of 630 and 660 nm, respectively.[63] For fructose-SQ-BA, the K_d value of 1.58 mM was found; it is lower than typical K_d values for boronic acid receptors (10−100 mM).

Heavy metals are expanding environmental pollutants with growing abundance due to industrial growth. Therefore, understanding the exact mechanism of their toxicity and the development of sensitive, accurate, quantitative methods of their detection is of great importance. The toxicity of heavy metal ions is linked to their binding properties with biomolecules. In this context, the interaction between Pb^{2+} and the most widespread bovine serum proteins (albumin, transferrin, and IgG) was studied with ACE and differential pulse stripping voltammetry.[64] Both techniques demonstrated similar K_d values for every proteins−Pb^{2+} complex, which indicated the absence of Pb specificity toward definite serum protein.

The K_d calculation for ion−protein interactions is challenging because the binding stoichiometry is usually unknown. Therefore, the protein mobility shift in the presence of the ions was used instead of K_d to assess and rank the influence of various ions on ovalbumin, β-lactoglobulin, and BSA.[65,66] For more than 75% of the studied protein−ligand pairs, significant interactions were observed with a very small confidence interval due to the excellent precision of migration time measurements by the ACE method. To solve the stoichiometry problem, in silico research was coupled with the experimental study of the noncovalent complex between the hexaarylbenzene-based receptor and the potassium cation K^+.[67] Employing quantum mechanical calculations, the most probable structure of the complex species was predicted, whereas the complex strength was evaluated by ACE. As a result, for potassium binding in methanol, the K_d was estimated to be 633 μM.

Desferrioxamine (DFO), an iron chelator, is currently used to treat iron overload diseases in most animal and clinical studies. However, under certain circumstances, DFO is able to promote iron donor hemin-induced protein oxidation. ACE was applied to investigate the interaction between DFO and hemin.[68] The high binding affinity between hemin and DFO (K_d = 28.9 μM) seemed to be the key factor in promoting hemin-catalyzed formation of cytotoxic radicals, such as superoxide anion ($O_2^{\bullet-}$), which was related to the potential toxicity of this drug in clinical use.

To increase the solubility of drugs in water and prevent their crystallization in supersaturatable drug-delivery systems, special techniques should be applied. Polymers are frequently utilized as substances that enhance a water solubility of drugs and, consequently, increase bioavailability of orally administered drugs. However, polymer−matrix interactions with drugs are complicated and their strengths are typically not known. To address this issue, equilibrium constants of complexation between an aryl propionic acid derivative (APAD) and polyvinylpyrrolidone (PVP K30) or vinylpyrrolidonevinyl acetate copolymer (Kollidon VA64) were investigated by ACE at different pH for 17 APADs.[69] The K_d values of the APAD−polymer complex were found to decrease with increasing APAD lipophilicity and the K_d values were lower at pH 4 than at pH 9. It was shown that the hydrophobic effect prevailed over the hydrogen bonding. The comparison of two studied polymers, Kollidon and PVP, demonstrated higher affinity of the former toward the APAD compounds.

INSTRUMENTATION AND METHODOLOGY

The use of KCE methods requires advanced CE instrumentation and methodologies, and here we present an overview of the recent progress in these relevant aspects.

Sample Preparation. A large-volume-sample stacking with an electroosmotic flow (EOF) pump (LVSEP) and PF-ACE methods were applied to study affinity of glycoprotein oligosaccharides to some plant lectins.[70] LVSEP was used for the sample preconcentration. In this procedure, capillary walls

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were coated by polydimethylsiloxane and hydroxypropylcellulose was added to BGE. The technique exploited EOF dependence on the ionic strength of BGE being inside the capillary and allowed for sample enrichment at the capillary inlet. The concentration of analyte (oligosaccharides derivatized with aminopyrene-trisulfonic acid) in the LVSEP-formed plug was determined to be ~900 times higher than that in the plug created by an ordinary injection. Affinity interactions of the preconcentrated samples were then examined by the PF-ACE method. The study demonstrated that the hyphenated LVSEP/PF-ACE method is promising for analysis of minor components in the glycan solutions.

To simplify immobilization of ligands on the inner capillary wall while maintaining their activity, Fukushima et al. developed a sample-preparation method based on the protein encapsulation by the alginate hydrogel formed inside the capillary.[71] Avidin was chosen as a model affinity ligand for its ability to strongly bind biotin. The amounts of injected and immobilized avidin were estimated by KCE/FA. The hydrogel formed inside the capillary was stable at different pH values; therefore, the pH switching did not influence the elution of the hydrogel resulting in the fact that biotinylated analytes were concentrated and eluted by a simple change of pH. The study has demonstrated that various proteins could be immobilized quantitatively by the created alginate hydrogel. Another example of ligand immobilization was proposed by Gao et al. to study the affinity binding between aptamers and thrombin inside a microchip channel.[72] The aptamers were immobilized on PEG monolith, which is biocompatible and resistant to protein adsorption. In situ fluorescent observation of the binding process offers another path for investigating the ligand−target interaction, which has potential applications in drug discovery.

An online screening method for CC chemokine receptor 4 (CCR4) ligands had been developed with CE.[73] Cells expressing CCR4 were cultured and immobilized on the inner wall of the capillary as the stationary phase. Therefore, it was possible to almost totally preserve the native conformation of the target receptors. A known antagonist of CCR4, one of the lactam analogues, was used to evaluate the bioactivity of the cell layer and stability of this method. The binding activities of the CCR4 on immobilized cells did not change. Similar cell-immobilization approaches may be efficient for drug screening.

Capillary Coatings. At the physiological pH, which is typically used for affinity interactions, an internal surface of the fused silica capillary carries a negative charge. The electrostatic interaction results in adsorption of positively charged biomolecules onto the capillary wall. The high propensity of proteins to be adsorbed can easily prevent the electrophoretic migration (and, thus, detection) of low-abundance biomarkers. The best strategy is a modification of the inner capillary surface with either dynamic or static coating to prevent protein adsorption.[74−78]

A simple and universal technique for screening various capillary coatings for protein analysis in KCE was proposed.[75] In this method, first, a short plug of the protein is injected into the capillary. Then, the protein-plug is slowly pushed through the capillary by applying a low pressure to guarantee significant Taylor dispersion. The measurements are done on a non-modified commercial setup in a pseudo-two-detector approach. Instead of using two detectors along the capillary, the distance between the detector and the capillary inlet is changed. Thus, the doubled number of measurements results in mimicking a response from two detectors. Two signal characteristics are analyzed, namely, peak area and shape. Their changes between observations with different distances to the detection point are used to evaluate the extent of protein adsorption and to classify antiadhesive properties of different wall chemistries. The proposed method may be readily applied to optimize the KCE study of protein−ligand interaction. Suppressing protein adhesion is absolutely essential for the pattern-based approach of kinetic data extraction from electropherograms.

A semipermanent capillary coating comprised of dioctadecyldimethylammonium bromide (DODAB) and polyoxyethylene-8 (POE-8) stearate was demonstrated to greatly reduce protein adsorption at physiological pH.[77] The coating exhibited the following properties: (i) it did not inhibit protein−DNA complex formation, (ii) it prevented the adsorption of the analytes, and (iii) it supported an electroosmotic flow required for many applications of KCE. The use of the DODAB/POE-8 stearate coating may help diversify the number of protein−ligand studies including KCE-based aptamer selection. In the next study, a number of capillary coatings (LPA, PVA, CElixer, UltraTrol LN) were screened to reduce adsorption of the His-tagged DNA mismatch repair protein MutS to the capillary wall.[76] UltraTrol LN was found to be the most effective coating which was also confirmed with the prevention of adsorption of His-tagged fat mass and obesity-associated protein. Under typical conditions, the coating reduced protein adsorption to a level at which accurate KCE analysis of protein−DNA interactions was possible.

The use of a polyelectrolyte multilayer (PEML) coating allowed for improving the performance of the KCE/FA method.[78] The coating was created by alternatively flushing the capillary with positively charged polyelectrolyte Polybrene and negatively charged polyelectrolyte dextran sulfate. HSA and six drugs were chosen as an experimental model to confirm the advantage of the capillary-surface modification. The PEML coating was shown to efficiently decrease the protein adsorption on the capillary wall.

KCE-MS. Being a highly sensitive and informative method of detection, MS has an important limitation, it requires volatile low-salt buffers for high-sensitivity measurements. On the other hand, the buffer must be suitable for undisturbed biomolecular interactions. A search for suitable buffers has been recently conducted. Three protein−ligand pairs were studied in the near-physiological buffer (Tris-acetate) as well as in the three volatile ammonium buffers, namely, acetate, bicarbonate, and formate, which are typically used in MS.[79] The stability of the protein−ligand complexes was not significantly affected by the volatile buffers. In addition to maintaining complex stability, such buffers used as the BGE supported the separation of free ligands from the protein−ligand complexes by KCE. The limit of detection was improved by about 2 orders of magnitude when Tris-acetate was substituted with volatile buffers during MS-identification of small molecules. This research demonstrated that KCE with volatile BGE can be readily coupled with MS granting label-free studies of kinetic processes in protein−ligand affinity complexes.

Studying affinity interactions between drug leads and proteins from blood plasma is an essential step during creation of new medications because their pharmacodynamics and subsequently pharmacokinetics is defined by drug−protein binding parameters. Vuignier et al. hyphenated KCE/FA to ESI-MS to improve the sensitivity of the analysis to the level that allows the direct study of binding properties of drugs toward plasma proteins.[80] To efficiently transform the KCE/

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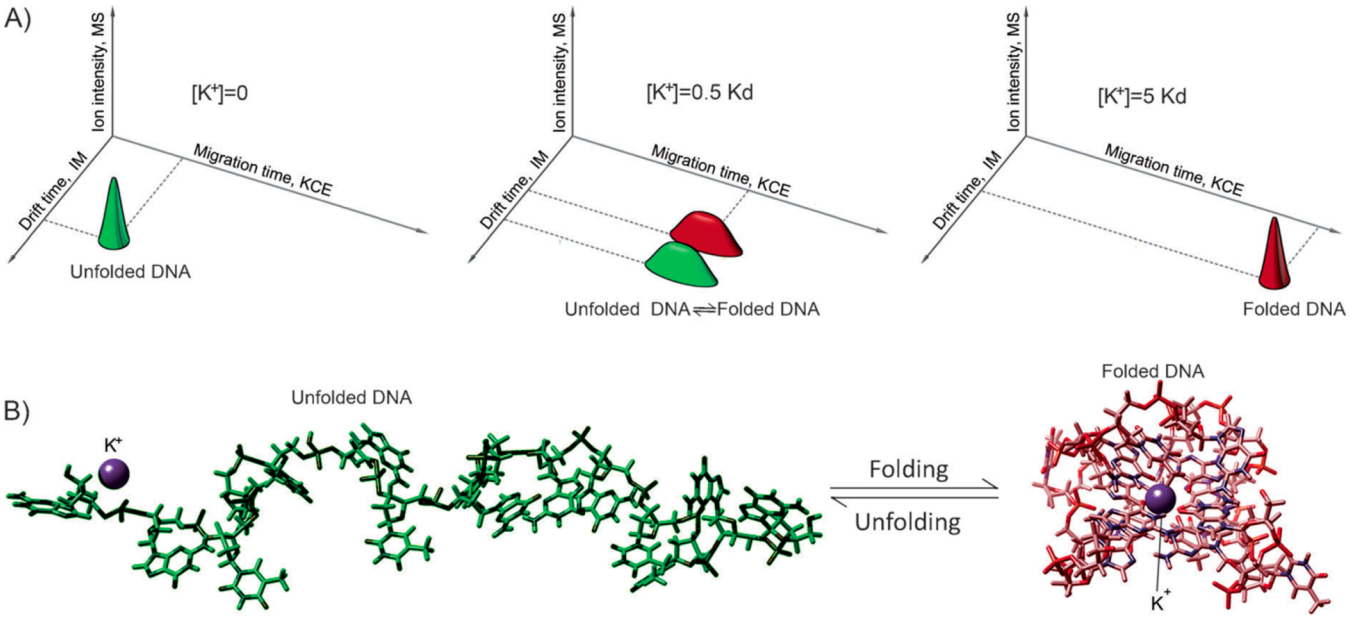

Figure 3. Unfolded (green) and folded (red) forms of G-quadruplex DNA. (A) Two-dimensional separation of conformers, in solution (KCE-axes) and then in a gas phase (IM-axes), results in a series of schematic 3-dimensional plots. (B) Potassium ion mediates DNA folding in a compact G-quadruplex structure. Reprinted with permission from ref 82. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

FA-UV method into KCE/FA-MS, the authors considered and optimized different factors, such as the buffer composition, the rinsing step, and the ESI and MS parameters. The optimized KCE/FA-MS technique was employed to examine interactions between six drugs and α_1-acid glycoprotein as well as between BSA and three drugs, including basic, neutral, and acidic compounds. KCE/FA-UV and equilibrium dialysis experiments successfully qualified the new KCE/FA-MS method, which provided for ligand−protein complexes with the K_d values ranging from ∼10 to 60 μM.

Mironov et al. coupled ECEEM with label-free ESI-MS to measure affinity constants for interaction between β-cyclodextrin and eight small-molecule drugs.[81] ECEEM-ESI-MS was also compared with two other approaches: direct infusion mass spectrometry and ECEEM with UV detection. Both ECEEM-UV and ECEEM-ESI-MS were found to provide more reliable K_d values than direct infusion of the equilibrium mixture into a mass spectrometer without the separation step. A conclusion was made that direct infusion MS should not be used for studies of affinity interactions. In the ECEEM-ESI-MS studies of drug−CD interactions, the signals from certain small molecules overlapped with each other at the ECEEM step. However, the subsequent ESI-MS quantitation resolved all reacting drug molecules from the mixture and allowed the determination of K_d for all pairs. These results indicate that ECEEM-ESI-MS can be utilized for separation of binding partners and their rapid screening through the m/z ratio. The new approach may lead to the higher-throughput examination of multiple drug-leads.

Some sequences of DNA/RNA may fold into a 3-dimensional structure called G-quadruplex (GQ). GQs are known to participate in several important biological processes including the ones associated with cancerogenesis. In the consequent research, Mironov et al. applied ECEEM-ESI-MS to examine conformational dynamics of DNA GQ as a function of the presence or absence of the potassium ion.[82] Peak shift and widening in ECEEM electropherograms were thoroughly interpreted allowing for estimation of k_{on}, k_{off}, and K_d for DNA−metal affinity interactions and facilitating qualitative study of DNA-GQ folding/unfolding (Figure 3). Ion mobility (IM) spectroscopy was used to confirm DNA folding. Finally, to reveal possible effectors of GQ-folding/unfolding a number of DNA binding dyes and an anticancer drug, cisplatin, were examined using the presented KCE-MS approach.

To simultaneously benefit from low sample consumption and low detection limits, KCE was hyphenated with inductively coupled plasma mass spectrometry (KCE-ICP-MS), and such an approach was applied to study the stability constants of organometallic−protein complexes.[83] Following the optimization of experimental conditions, the interaction between four organotin compounds (trimethyltin, tripropyltin, tributyltin, triphenyltin) and HSA was investigated. Comparative sets of ACE and NECEEM experiments were carried out with ICP-MS used for detection. The equilibrium constants for complexes of organotin compounds with HSA under an assumption of 1:1 molar ratio were determined. Both KCE methods were found to be applicable for quantitative studies of such interacting systems. Stern et al. applied KCE-ICP-MS utilizing two competing ligands, humic acid and EDTA, to examine the trace concentrations of Th, Hf, and Zr in natural waters.[84] It was shown that tetravalent metals can strongly complex humic substances at pH values relevant to natural systems (pH from 3.5 to 7). The results confirmed that KCE-ICP-MS is suitable to study organometallic complexation.

Computational and Mathematical Approaches. In electrophoresis, the velocity of EOF is usually measured by adding small neutral substances to BGE. In an electric field, neutral molecules are expected to have the velocity of the BGE flow and, thus, can be used as EOF markers. However, in reality, neutral marker molecules can have affinity to BGE ions and form complexes with nonzero effective charges. The velocity of such complexes will not be representative of the EOF velocity. To address this issue, the electrophoretic mobilities of different EOF markers were investigated by

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combination of CE measurements and molecular dynamics simulations.[85] It was proven that neutral molecules possess solute- and BGE-specific mobilities. In a similar approach, molecular dynamics and two experimental techniques (QCM and PF-ACE) demonstrated a nonlinear pattern of the chondroitin-6-sulfate affinity toward LDL at temperatures above 37 °C.[43]

A CE-based technique was utilized to efficiently perform a catalytic and inhibition study of cytochrome-P450-mediated reactions.[86] In this technique, an advanced injection procedure exploiting transverse diffusion of laminar flow profiles (TDLFP)[87] was applied. In essence, a series of short plugs of the enzyme and its substrates were entered into the capillary followed by their rapid mixing. Mathematical modeling supported the hypothesis of a uniform distribution of reactants in the final reaction mixture. The study yielded kinetic and inhibition characteristics of reactions of cytochrome P450 (2C9's isoform), diclofenac, and sulfaphenazole used as the enzyme, substrate, and inhibitor, respectively. The method was partially validated and suggested for application in online screening of drug metabolism mediated by cytochrome P450 enzymes.

To represent electropherograms under test conditions, one-dimensional dynamic simulation software called "Simul 5 Complex" was developed.[88] The brute-force numerical calculations done with this software were successfully verified[88−90] by reproducing the published results obtained by several methods (ACE, HD, VACE, VP, KCE/FA, FACCE) used for the determination of equilibrium constants of reversible binding. In parallel to its experimental evaluation, Simul 5 Complex was used to test the applicability and limitations of ACE and VACE methods.[91]

An original parameter-based method for finding k_{on} and k_{off} was proposed for the macroscopic approach to studying kinetics at equilibrium (MASKE[11]).[92] The authors have found an analytical solution for the system of partial differential equations using three assumptions, namely, (i) affinity reaction matches to eq 1, (ii) KCE signal consists of two separated peaks, and (iii) the concentration of T does not change during the experiment. This mathematical approach named a "two-peak approximation" allowed the determination of k_{on} and k_{off} with relative errors of less than 10%. Though the method was originally developed to satisfy conditions of the MASKE approach assuming that the concentration of T remains constant, Kanoatov et al. have recently demonstrated a validity of the "two-peak approximation" mathematics for the ACE and ECEEM experimental data.[12] Figure 4 illustrates similarities and differences between MASKE and classical ACE. In the described study, the authors obtained an approximate analytical solution of eq 2 for ACE and ECEEM conditions using the MASKE mathematics. In silico study confirmed that MASKE mathematics gives acceptable accuracy for finding k_{on} and k_{off} if the concentration of T exceeds the concentration of L by at least a factor of 5. Importantly, such a requirement is usually fulfilled in ACE and ECEEM experiments. In general, the method can be applied to new and historical ACE and ECEEM electropherograms provided that the condition of $[L] \ll [T]$ is satisfied. Kanoatov et al.[12] validated the proposed method by analyzing published ACE data with known rate constants. Finally, the new technique was applied to extract unknown k_{on} and k_{off} from recently published KCE data. Thereby, the power of the MASKE-based mathematical method for finding rate constants in ACE and ECEEM experiments was successfully

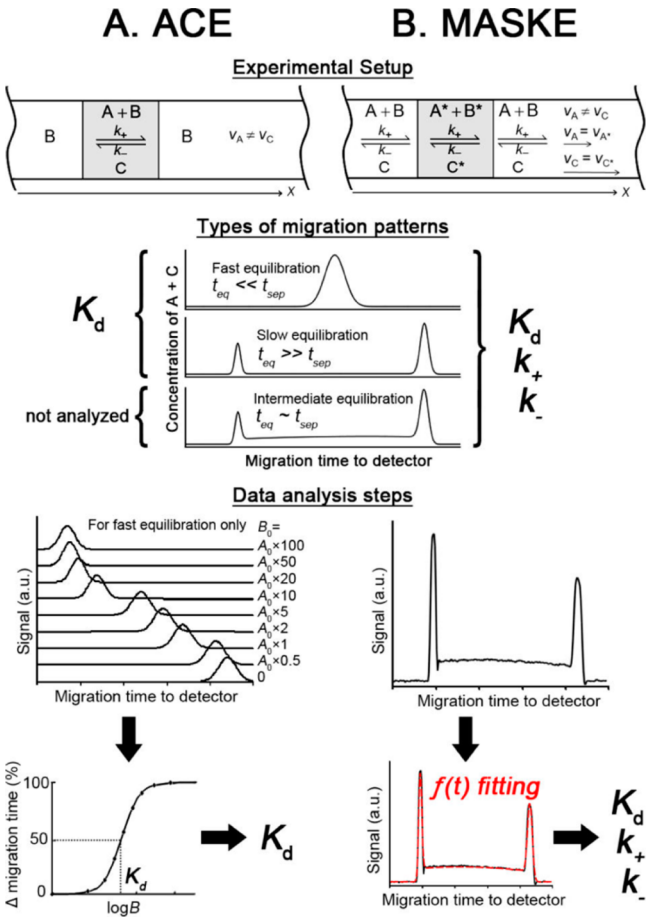

Figure 4. Schematic representation of the ACE (column A) and MASKE (column B) methods. Rows demonstrate (from top to bottom): (i) initial experimental conditions, (ii) typical patterns of electropherogram and quantitative parameters extracted from the KCE signal, and (iii) differences in data treatment. Reprinted from ref 12. Copyright 2014 American Chemical Society.

demonstrated. We anticipate wide application of the proposed approach for both prospective and retrospective data treatment.

Validation. For a long time, researchers in universities, as well as in clinical and industrial laboratories, mainly aimed to obtain quantitative data, which would be widely accepted as correct, but they did not accompany their efforts by quality control testing. During the last 2 decades, validation of bioanalytical methods has been developed and formulated as rules for the pharmaceutical industry that has stimulated the introduction of the validation process in the field of science and research. These validation procedures were compiled into guidances[93,94] and have not yet been written into general law. Nevertheless, while using modern bioanalytical methods, scientists should also pay particular attention to the validation of the applied methods. Although the guidance is mostly intended for chromatography methods and ligand-binding assays (LBA), these rules provide also a good basis for validation of KCE methods.

Overall, the validation process consists of at least two distinct steps: instrumentation (hardware and software) qualification and analytical method validation. By definition, method validation is the process of establishing the performance characteristics and limitations of a method and the identification of the influences which may change these

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characteristics and to what extent. The typical characteristics are selectivity, accuracy, precision, sensitivity, reproducibility, and stability. This list can be expanded or reduced depending on the specific task. The validation parameters usually are determined and presented together with other experimental results or in separate chapters.[86,95,96]

Selectivity (*specificity*) is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be contained in the sample. Specificity refers to methods, which produce a response for a single analyte, whereas selectivity refers to methods that produce responses for a number of chemical entities, which may or may not be distinguished. In KCE the selectivity undergoes both matrix (background electrolyte) effects and interference from substances physicochemically similar to the binding partners, for example, drug metabolites, isomeric compounds, and degradation products. In such situations, overlapping peaks may be observed and sophisticated detection procedures should be utilized for the selection of useful signals. They include, for instance, CE−LIF with fluorescently labeled ligands,[11] CE−UV equipped by a wide-range photodiode array detector,[95] KCE-ESI-MS,[81,82] and KCE-ICP-MS.[97] Advanced data treatment (chemometrics) gives additional benefits to the handling of CE-data where ideal baseline separation of electrophoretic peaks cannot be achieved.[95] It should be noted these highly selective methods work properly if the contaminants possess an affinity to molecules under study significantly weaker than interaction of the investigated molecules with respect to each other. A general recommendation is to use for the analysis the most pure available substances. In some cases, pretreatment and/or preseparation procedures of interacting components may assist in research. For example, an irregular fraction of DNA was decreased several orders of magnitude when DNA counterions were removed by dialysis against deionized water in the presence of a strong electric field.[98,99] The selectivity can be assessed by determining how K_d changes with changing concentrations of known impurities; lesser changes in K_d indicate better selectivity. The common "gold" scheme to test the selectivity is to compare the measured values with independent results obtained by the different methods in various laboratories.

Accuracy is the measure of exactness of an analytical method or the closeness of agreement between an accepted reference value and the value found in a sample. Like for the selectivity, a crucial test for accuracy of binding experiments is to compare the acquired constant with one obtained by other well-established methods. For example, the multimethod KCE toolbox has been proposed to combine different types of KCE technique,[2] whereas QCM and a microscale thermophoresis were used to validate the results of partial-filling ACE.[100]

Precision measures agreement among test results when the method is applied repeatedly to multiple samplings of a homogeneous sample, that is the distribution of the results near their average value. *Sensitivity* (in LBA coincides with the lower limit of quantitation, LLOQ) is defined as the lowest analyte concentration that can be measured with acceptable accuracy and precision. The analyte response at the LLOQ should be at least 5 (typically 10) times higher than the response to the blank injection. The precision is affected by both a systematic error (bias) and a random error (variability), which sum up to the total error. Precision can be further divided into *repeatability* (intra-assay precision), *intermediate precision* (within laboratory), and *reproducibility* (among different laboratories).

Typically, to estimate K_d by KCE methods with simple mathematical approaches for finding K_d, a series of experiments, where the concentration of one component is varied while keeping that of the other component constant, should be performed. Then, K_d is calculated from the dependence of analyte velocity or from the concentration ratio of unbound and bound analytes. Although multiple measurements, in general, have a positive effect on the precision of the method, thus, decreasing its random error, for a series of nonidentical samples with different concentrations, additional systematic errors can appear and reduce the overall precision of the determined constants. KCE methods based on advanced mathematical approaches are able to process a full electrophoretic signal and extract information from band shapes, not only from peak amplitudes or analyte velocities. As a result, such KCE approaches possess intrinsically high accuracy and precision. For example, the NECEEM method calculates equilibrium and kinetic parameters of binding complex from a single electropherogram,[101] and allows potential achievement of a greater precision for the same number of measurements compared with ordinary KCE approaches. However, advanced KCE methods employ certain simplifications and approximations to obtain an analytical solution of the system of eqs 2. Using a simplified mathematical model can affect the accuracy of the calculated data. Therefore, the KCE-mathematical approaches should be validated by a comparison of the results of simplified calculations with data received in the in silico brute-force approach.[12] Another limitation of pattern-based approaches for data processing is its susceptibility to the influence of contamination and peak-shape irregularities. If the electropherogram is not "clean", the pattern-based approach for data processing can lead to significant systematic errors.

Analytical method validation is the determination of the degree of reliability of the data. In this connection, we should note that a researcher must use all available techniques to achieve accurate and precise results, although this requirement is not explicitly included in the method validation but is a necessary condition for obtaining reliable data.

Precise evaluation of quantitative parameters of reversible binding is not trivial because temperature, viscosity, and ionic strength of the BGEs influence the mobilities of analytes. The temperature inside the capillary tube has a tendency to increase due to Joule heating despite the active cooling systems used in most instruments, because their capability is limited as well as they leave "hot spots" in the detection region and at the capillary ends. It is necessary to determine the real internal temperature in order to overcome large systematic errors in quantitative KCE. A probeless temperature determination approach[102] that requires only measurements of current against voltage for several voltages and data treatment using an iterative algorithm was developed for commercial CE instruments equipped with both liquid[103] and forced-air[104] systems for cooling the capillary. The influence of a nonthermostated capillary inlet on accuracy of ACE data was examined, and it was demonstrated that uranyl mobility values undergo a significant bias at temperatures higher than 35 °C.[105]

The addition of a ligand or a target to the BGE can change the viscosity of the solution and, consequently, electrophoretic mobilities of ionic particles. The changes in the buffer viscosity can be observed by a slight change in the migration time of the EOF marker. If charged substances are studied in KCE, the

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attention must be paid to increasing ionic strength, which influences the ionic mobilities significantly.[106−108] A procedure based on BGE temperature control, viscosity ratio determination, and electrophoretical measurements performed both at constant and at variable ionic strengths was designed for precise data correction in KCE with neutral analytes and charged complexation agents.[50,51,109] The effect of various operational parameters, namely, electric field, pH, buffer concentration, and nature of buffer co-ion on the enantioseparation of the model compounds, was investigated based on the difference in migration time of the analyte in the absence and presence of negatively charged β-CD derivatives.[48] Nonpeptide substrates of human sirtuin were synthesized and evaluated for a CE-based enzyme assay. The assay was subsequently validated with respect to dynamic range of concentrations, linearity, limit of detection, limit of quantification, repeatability, interday precision, and recovery.[110]

Poor accuracy and repeatability of binding data in KCE may be caused by adsorption of macromolecules (e.g., proteins) onto the inner surface of the capillary. Researchers may choose appropriate rinsing procedures,[65] permanent and dynamic capillary coatings,[75] or semipermanent capillary coatings[76,77] to reduce protein adsorption and improve accuracy and repeatability.

KCE techniques make it possible to collect additional information about binding processes due to extracting k_{on} and k_{off} values from the shape of the reaction zone. Therefore, KCE have high requirements to the shape of the injected plugs. Pressure-driven sample injection in CE results in asymmetric peaks because of difference in shapes between the front and the back edges of the sample plug. Recently, a simple procedure has been proposed to reduce such a shape difference in the injected sample through the creation of identical parabolic profiles at both boundaries of the plug.[111] Advantageously, the propagation-based correction procedure also facilitates moving the sample into the efficiently cooled part of the capillary for CE instruments with active cooling.[105]

Stability tests confirm that the analyte (and internal standard) does not break down under typical laboratory conditions, or if degradation occurs, its extent is known and can be avoided by appropriate sample handling. Adsorption to the container walls or tubing is one of the factors affecting the stability of samples. There was found an essential DNA adsorption to the surface of the sample vials, in which DNA−protein mixtures were prepared before a KCE study.[112] The problem can be considerably lessened by the treatment of the vial surface with passivating agents, such as masking DNA or BSA.

CONCLUDING REMARKS

KCE methods have been validated by other techniques during their intensive development over the past decade. Nowadays, more and more research group use KCE as a standard reliable approach for studying biomolecular interactions. Among others, NECEEM stands as a solid and most advanced tool for both selection and quantitative characterization of oligonucleotide aptamers, which are highly promising for applications in diagnostics and therapy. KCE-based discovery of small-molecule protein binders will result in the development of new drugs and more selective and versatile molecular probes. Subsequent KCE research for drug−cyclodextrin binding complexes is the next logical step in the creation of efficient drug-formulation. It is also time to start the development of kinetic chromatography. It could expand the arsenal of available separation modes while potentially adopting data processing approaches similar to those developed for KCE.[113]

In this review, we have tried to clarify the nomenclature for KCE methods and invite a wider research community to using this approach. In our view, further progress of the KCE platform should involve (i) methodological development of the KCE approach, (ii) detailed study of advanced computational models and building new mathematics with user-friendly solutions, thus, to fill the gaps in Table 1, (iii) enhancement of technical tools for the KCE methods, (iv) miniaturization of KCE technical basis, (v) application of KCE methods to new ligand−target systems, (vi) validation of KCE methods, and (vii) hyphenation of KCE with other instrumental platforms to realize multidimensional analysis and accelerate analytical capabilities of KCE-based methods. These efforts should make KCE more powerful and accessible to a larger research community.

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Notes

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Dr. Alexander S. Stasheuski studied biophysics at the Belarusian State University (Minsk, Belarus). He received his M.Sc. degree in Physics under the supervision of Dr. Victor A. Galievsky (2009) and Ph.D. degree in Physics and Mathematics under the supervision of Prof. Boris M. Dzhagarov (2013). Both academic degrees were obtained in the B.I. Stepanov Institute of Physics of the National Academy of Sciences of Belarus, where he was then employed for 2 years as a Researcher in the Laboratory of Molecular Photonics. In 2014, he was awarded the Banting Postdoctoral Fellowship and joined Prof. Krylov's group at York University. His current scientific research involves development of a highly sensitive technique for identification of cancer subtypes through the quantitative analysis of miRNA signatures.

Prof. Sergey N. Krylov obtained his M.Sc. degree in Physics in 1987 and Ph.D. degree in Biophysical Chemistry in 1990, both from Lomonosov Moscow State University. His postdoctoral training was with Prof. H. Brian Dunford and Prof. Norman J. Dovichi at the University of Alberta. In the year 2000, he accepted a position of Associate Professor in the Department of Chemistry at York University in Toronto, where he was promoted to Professor in 2006. From 2003 to 2013, he held the Canada Research Chair in Bioanalytical Chemistry. He is a founder and Inaugural Director of the Centre for Research on Biomolecular Interactions at York University. He is recognized internationally for his pioneering work in the fields of

chemical cytometry, kinetic analysis of affinity interactions, and selection and applications of oligonucleotide aptamers. He recently became interested in development of innovative technologies for cancer subtyping and continuous-flow small-scale chemical manufacturing. His research contributions have been recognized with a number of awards including the Petro Canada Young Investigator Award, Premier's Research Excellence Award, and two recognitions from the Chemical Society of Canada: the McBryde Medal and the Maxxam Award.

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