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Review

Recombinant drugs-on-a-chip: The usage of capillary electrophoresis and trends in miniaturized systems – A review

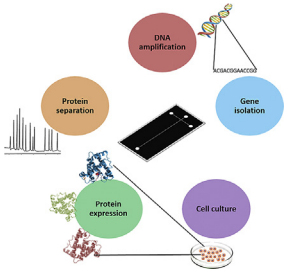
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HIGHLIGHTS

- Principal analytical tools for analysis of recombinant drugs are presented.
- A critical comparison among different substrates for fabrication of miniaturized systems is made.
- Applications of miniaturized systems to produce recombinant drugs are shown.
- Future trends of miniaturized systems capable of integrating analytical and proteomic tools in a single device are envisioned.

GRAPHICAL ABSTRACT



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ABSTRACT

We present here a critical review covering conventional analytical tools of recombinant drug analysis and discuss their evolution towards miniaturized systems foreseeing a possible unique recombinant drug-on-a-chip device. Recombinant protein drugs and/or pro-drug analysis require sensitive and reproducible analytical techniques for quality control to ensure safety and efficacy of drugs according to regulatory agencies. The versatility of miniaturized systems combined with their low-cost could become a major trend in recombinant drugs and bioprocess analysis. Miniaturized systems are capable of performing conventional analytical and proteomic tasks, allowing for interfaces with other powerful techniques, such as mass spectrometry. Microdevices can be applied during the different stages of recombinant drug processing, such as gene isolation, DNA amplification, cell culture, protein expression, protein separation, and analysis. In addition, organs-on-chips have appeared as a viable alternative to testing biodrug pharmacokinetics and pharmacodynamics, demonstrating the capabilities of the miniaturized systems. The integration of individual established microfluidic operations and analytical tools in a single device is a challenge to be overcome to achieve a unique recombinant drug-on-a-chip device.

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1. Introduction

1.1. Recombinant protein drugs

Recombinant proteins are polypeptides produced from cloned genes by recombinant DNA techniques, generally in a heterologous expression system. The process begins with DNA fragments containing the gene of interest. Once it is identified, isolated, and characterized, that gene is inserted into a vector by recombinant DNA technology. Gene structures used as vectors, such as plasmids, bacteriophages, and artificial chromosomes, receive the insertion of the donor fragment with the aid of restriction and binding enzymes. These vectors are then inserted into a host cell and have the ability to self-replicate by using the synthetic machinery of the host cell [1–3].

Host cells range from bacteria, yeast, and filamentous fungi to mammalian cell cultures, with bacterial hosts being the most common [3–9]. The gram-negative bacteria *Escherichia coli* is a valuable system and one of the most used to date because it *i*) has the ability to grow rapidly, *ii*) reaches high cell concentrations, *iii*) allows the use of inexpensive substrates, and *iv*) is the organism with the best-known genome and metabolism amongst living organisms [2,3,7]. *Saccharomyces cerevisiae* yeast is another host cell that is extensively used. This organism is the most popular eukaryotic cell model, and it has been used to produce insulin and other recombinant proteins at a large scale. Since these recombinant drugs need post-translational modifications—such as glycosylation—to be bioactive, eukaryotic cells have the necessary machinery to perform such bioactivation [6,8,9].

Recombinant protein products are commonly stored in the cytoplasm as inclusion bodies, where, in some cases, they can be directed to the periplasmic space, and in some rare events, to the extracellular matrix. Choosing the ideal mechanism for recombinant protein expression depends on the target protein, as well as its function and application. Recombinant protein applications include the food industry, gene therapy, genetically modified organisms, development of diagnostic recombinant antibodies or antigen kits, and the manufacture of new drugs [6,10–12].

Several recombinant drugs are currently being produced by recombinant DNA techniques, with hormones such as insulin, human growth hormone (hGH), and erythropoietin (EPO) being amongst the most utilized [8,13–17]. Monoclonal antibodies (mAbs) and vaccines produced by recombinant techniques are on the rise and present great potential in clinical applications over synthetic drugs [10,18,19]. Those biopharmaceuticals are glycosylated forms of proteins, which means that the expression vectors must be eukaryotic cells rather than less complex bacterial systems [20]. When dealing with more complex eukaryotic systems, effective separation and characterization techniques are needed in order to concentrate, separate, and identify the product of interest from the complex matrix composed of other abundant proteins and organelles [21,22]. These types of analysis require heavy sample preparation and manipulation, which can contaminate the sample and introduce errors into the analysis. However, this step could be facilitated through the use of online/inline systems.

Synthetic drugs cannot perfectly perform the biological role of proteins in an organism, but recombinant protein drugs can. Their higher specificity greatly decreases the side effects on the biological system. Recombinant drugs are less likely to stimulate an organism's immune response, due to the similarity to the body-synthesized proteins. The use of recombinant drugs, however, requires the identification and complete characterization of the biotherapeutics, as well as their quantification, which are performed by different analytical tools to obey the regulations of each country [23–25].

To illustrate the current and trending technologies in recombinant drugs, we show in Fig. 1 a summarized description of the steps involved in recombinant protein production workflow.

1.2. Challenges in recombinant protein characterization

Recombinant proteins are susceptible to undergoing chemical and physical changes during all the production steps [26]. Among the most common chemical changes are: *i*) modifications of the primary structure of the peptide, such as mutations and insertions [27]; *ii*) the failure of amino acid incorporation during the translational process [26]; and *iii*) post-translational modifications (PTMs), which include glycosylation, phosphorylation, oxidation, sulfation, deamidation, acetylation, carboxylation, hydroxylation, disulfide linkage formation, isomerization, and proteolytic processing [20,27–29]. The physical changes include mostly aggregation and the denaturation process, which involves tertiary and quaternary structures, and interactions between proteins [26,28]. The true analytical challenge is the heterogeneity in recombinant protein expression alongside the numerous changes and degradation pathways that may occur during biological processes [30].

The expression system of choice will depend on the protein to be expressed, as well as the desired function, since most of the recombinant protein biopharmaceuticals only become active after PTMs [20,28]. For example, non-glycosylated proteins are commonly expressed in *E. coli* systems [31], but when produced in yeast-based systems, they can link sugar moieties in amino acid

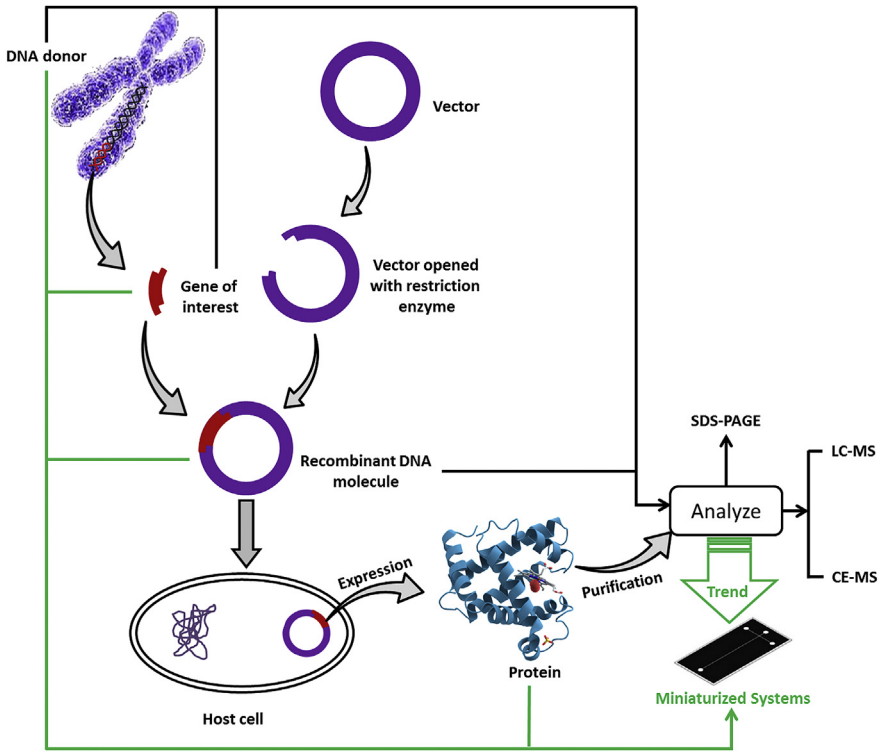


Fig. 1. Scheme of production of recombinant proteins and the analysis of the products of each step by conventional analytical tools (black line), and the trend towards using miniaturized systems to perform such characterizations (green line).

side-chains, thus affecting the half-life of the product in human serum, which is an undesirable effect [20]. If these proteins were expressed in plant cell systems, however, this may result in immunogenic proteins for humans, which would induce an immune response [20]. Thus, mammalian cell lines are preferred for the production of glycosylated proteins due to the availability of PTMs. Commonly used cells include immortalized Chinese hamster ovary (CHO), mouse myeloma (NS0), baby hamster kidney (BHK), human embryo kidney (HEK-293), and human retinal [32] cells.

1.3. Regulations

In terms of assuring the global quality and safety of biological medicines under development, drug testing requires the approval of regulatory agencies such as the Food and Drug Administration (FDA) in the USA, the European Medicines Agency (EMEA) in Europe, the Pharmaceutical and Medical Devices Agency in Japan, and the Brazilian Health Surveillance Agency (ANVISA) in Brazil [33–40]. Each regulatory agency has the common objective of assuring quality, safety, and efficacy of recombinant drugs, as suggested in their guidelines [33–39,41–44].

Furthermore, the Brazilian regulatory agency (ANVISA) establishes some classical techniques, such as the Kjeldahl, Bradford or Biuret analyses, to provide information about protein concentrations in recombinant protein drug production [35,37]. The Kjeldahl analysis is not a specific test for proteins, but is rather a quantitative method for the determination of total nitrogen in a sample [45–47], and this test can result in increased values of protein concentration. Establishing the Kjeldahl test as one of the official techniques for protein analysis allows the adulteration of products with, for example, milk and other protein-derived products [47–50]. The most commonly used compounds in protein adulteration are urea and melamine, which are nitrogen-rich compounds that are known to be harmful when consumed [47,48,50]. Therefore, such classical tests should be banned from regulatory affairs to avoid product falsification, and this is where systems capable of analyzing proteins and peptides that utilize robust separation and detection techniques could be beneficial.

Moreover, the use of modern analytical tools to evaluate recombinant protein drugs is essential for providing detailed information about these biomolecules and possible contaminants, thus helping to assure the efficiency and safety of biotherapeutic products. The coupling of different analytical techniques is an alternative to empower the characterization of biological products [33]. The importance of capillary electrophoresis in the characterization of such biologicals has been recognized by most of the important regulatory agencies, and appears in monographs in Europe, Japan, and the United States [40].

The quality of biotherapeutic products must be evaluated in each step of the manufacturing and commercialization process, along with continuous comparison to already licensed products, also known as Reference Biotherapeutic Products (RBPs) [23,36,41–43,51,52]. Comparative studies can be carried out by testing the biochemical, physicochemical, and biological properties of the new biomolecules, which must present similar efficacy and safety of the RBPs to proceed with the clinical trials [1,23,33,37,41–43,53], and the express approval of the FDA (FDA biological license application approval list; data available at http://www.fda.gov/BiologicsBloodVaccines/DevelopmentApprovalProcess/BiologicalApprovalsbyYear/default.htm). Only a few recombinant protein drugs have been approved in recent years in the USA, and the reason for this could be due to the limited capacity of the FDA Center for Biologics Evaluation and Research, as stated by Kinch [54]. This lack of production shows the need for improved, fast analytical tools to analyze and characterize biopharmaceuticals, which would speed up license approvals from the regulatory agencies. The use of such analytical tools could also aid in the comparison of efficacy in biodrugs between different

regulatory agencies, which could speed their approval in some countries.

2. Analytical tools for recombinant protein analysis

Analysis of proteins can be performed by a variety of techniques that combine distinct separation and detection methods. The most common separation methods for protein separation are liquid chromatography and electrophoresis using different detectors, such as UV–vis spectroscopy, fluorescence spectroscopy, and mass spectrometry (MS) [55–57].

Chromatographic methods are robust, presenting less dependence on the matrix containing the analytes, and liquid chromatography (LC) coupled with an MS detector enables intact protein analysis with a high-resolution separation and mass accuracy detection, in addition to its ability to evaluate the pharmacokinetics of recombinant drugs [58]. LC coupled with electrospray ionization time-of-flight mass spectrometry (LC-ESI-TOF-MS) allows analysis of protein glycosylation, such as the identification and quantitation of glycoforms of recombinant IgG monoclonal antibodies [59]. However, there has been a delay in the use of liquid chromatography techniques at the microscale level and few groups are developing the technology, mainly due to the difficulty of efficiently introducing stationary phases into microchannels [60].

Electrophoretic methods, on the other hand, present higher-resolution separation than the chromatographic methods, and are preferred for top-down proteomics [55]. Electrophoretic methods also have more potential to be miniaturized into microchip technologies, and thus are becoming the preferred separation technique for miniaturized applications.

The electrophoretic methods in gels, especially sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), are essential to determining protein characteristics, such as molecular weight, and to check protein purity [61]. One of the disadvantages of SDS-PAGE is the relatively poor resolution in the characterization of proteins with similar molecular weight, which includes protein isoforms and/or post-translational modifications [62]. Two-dimensional gel electrophoresis (2-DGE) can overcome some of the problems of SDS-PAGE because it can perform the separation in two dimensions. The first dimension is responsible for protein separation by isoelectric focusing (IEF), according to the protein charge, while the second dimension is based on the differences in the protein molecular weights. The 2-DGE technique has the ability to separate protein isoforms and to identify post-translational modifications, making it a powerful tool in protein studies [63]. Coupled with a high sample capacity, 2-DGE has become the most common technique in protein analysis for biochemistry laboratories [55]. However, some limitations are associated with 2-DGE and SDS-PAGE techniques due to the following: differences in the solubility of proteins; low abundance of proteins of interest in comparison with the whole proteome; high dynamic range and pI values (isoelectric point) of proteins (2-DGE); highly acidic or basic proteins pI (2-DGE); and molecular mass limitations, for both upper and lower limits [55,64].

2.1. Capillary electrophoresis

Despite the positive characteristics of gel-based electrophoresis techniques for characterization of recombinant drugs, *e.g.* erythropoietin [65], some of the limitations can prevent their use in a quality-control laboratory, for example: *i*) laborious and long steps, *ii*) intense sample preparation, *iii*) generation of large amounts of toxic waste, *iv*) lack of automatization, and *v*) they do not yield quantitative evidence of impurities in a batch process. These limitations clearly demonstrate the need for changes in the analytical tools used for quality-control analysis [61]. Capillary electrophoresis (CE), on the other hand, *i*) involves a short analysis time, *ii*) allows for automatization and coupling with other techniques, and *iii*) requires only small sample quantities, thus overcoming the analytical limitations of gel-based techniques. CE also allows the analysis of intact proteins, without conformational changes [56,66], which is an essential tool for the analysis of vaccines, monoclonal antibodies, recombinant drugs, and other biological products [67,68].

The most common CE methods for protein analysis are capillary zone electrophoresis (CZE) and capillary isoelectric focusing (cIEF). In CZE, a small sample plug is introduced into the equipment and the separation occurs by differences in the electrophoretic mobility of the analytes in a background electrolyte, whereas in cIEF, the entire capillary is filled with sample and ampholytes and the analytes are separated by differences in their isoelectric points. It is important to prevent protein precipitation during the isoelectric focusing since it can provide false positive or false negative results, and this precipitation is one of the problems associated with this technique [55]. Another problem related to protein analysis by CE is the adsorption of protein molecules to the surface of fused-silica capillaries in a non-specific manner, which interferes with the separation and reproducibility [55,66,69,70]. Coating of the capillary in a dynamic or static mode has been adopted as a strategy for decades to counter the effects of protein adsorption in fused-silica capillaries [71].

When applying the dynamic coating method, coating agents are continuously flushed into the capillary with electrolyte, and these agents interact with the capillary silica walls and prevent protein adsorption during the separation process. When using mass spectrometry detection, however, the coating agents can suppress the ionization efficiency, raise the background noise in the mass spectrum, and can contaminate the ion source chamber, thus affecting the results since ionic additives are typically used in conjunction with the background electrolyte (BGE) [71]. When applying a static coating, it does not present the same issues, and it actually improves the separation process, in addition to not suppressing ionization efficiency when coupled with MS once the coating agents are bonded (covalently or not) to the silica surface [56,66,71]. Static coating does have its weaknesses, however, such as the suppression of electroosmotic flow (EOF) when much of the coating consists of neutral molecules [71].

Various existing detection methods can be used in CE. Ultraviolet (mostly diode array detectors), fluorescence, and electrochemical detection methods have been some of the most popular [40,68]. CZE and CIEF have been shown to be the most suitable methods for CE-MS coupling in the analysis of intact proteins [56]. The combination of CE with MS allows the separation and the identification of different proteins forms, and this exceptional method is of great interest in research laboratories and industrial applications [72].

CE-MS seems to be an appropriate method for recombinant drugs in development due to its sensitivity, selectivity, and high-resolution information [72]. Other analytical tools, such as capillary electrophoresis coupled with nuclear magnetic resonance spectroscopy (CE-NMR), which provides structural and dynamic details about protein folding, can be applied [73], as well as CE-SDS [74,75]. CE-NMR analysis is a versatile technique that also allows for its application in miniaturized systems [72], and CE-SDS on a microchip could be used to perform direct analysis involving protein folding *in situ* [74,75], a relevant parameter to be evaluated in biodrug characterization.

Another example of CE application to recombinant drug analysis is the use of CE-SDS gels by biopharmaceutical laboratories, which are used as the gold standard to assess protein purity and

heterogeneity. Its prominent position within CE applications is due to its *i*) better process automation, *ii*) higher separation resolution, *iii*) faster analysis time, and *iv*) better reproducibility results than other laborious slab-gel techniques. The CE-SDS gel kits are also widely available [55,56,65–67,73].

2.2. CE modes applied to recombinant protein characterization

Distinct separation modes in capillary electrophoresis have been used to study physicochemical properties of recombinant molecules. For example, CZE and cIEF have been used to characterize charge heterogeneity of mAbs, and the miniaturized form of cIEF, microchip isoelectric focusing (mIEF), produced a high-speed analysis during mAb characterization [67,76]. This miniaturized system showed great efficiency when separating four or more charge variants of mAbs, such as bevacizumab, trastuzumab, and cetuximab, and also can be used to identify N-glycan-attachment sites in human transferrin, showing the versatility of these microdevices [76]. Table 1 presents a comparison of the principal modes of separation in capillary electrophoresis and applications in both conventional and miniaturized forms.

Due to variability in protein production, a complete characterization of recombinant proteins in all production steps is necessary from gene isolation and host system selection to biodrug safety and efficacy evaluations [30]. Capillary electrophoresis allows for distinct separation modes (Table 1) that can be used to evaluate protein heterogeneity and PTMs [92], in addition to ensuring the quality and efficacy of biopharmaceutical products [26,30], thus showing the versatility of CE techniques and their usefulness in protein analysis. Table 2 presents the use of electrophoretic techniques to assess heterogeneity and PTMs in proteins.

Most separations can be done by miniaturized systems, except for modifications such as oxidation and deamination, which have not been performed yet. The Caliper LabChip GXII system may soon, however, be able to perform these types of modifications [122], thus further showing how miniaturization can be used for the characterization of recombinant proteins and their modifications.

3. Future trends

3.1. Why miniaturization?

Recombinant protein production at the industrial scale started at the end of 20th century, and today it is a billion-dollars market [123,124]. European countries invested about 2% of their gross domestic product (GDP) in research towards this area, whereas the USA leads the development of new biotechnology products, owning 81% of patents of biotechnology in the world, two-thirds of the

Table 1
Comparison of different CE modes applicable to characterization of biologicals.

Mode	Principle	Selectivity	Features	Application in a conventional system	Application in a microfluidic system	Reference
CZE	Effective molecule charge determined by pH of the running buffer	Distinct electrophoretic mobility of analytes	CZE does not separate neutral molecules. The flow rate is low, affecting the analyte migration caused by the strength of the electric field. The use of a buffer gradient is not necessary	CZE was used to analyze charge heterogeneity of recombinant monoclonal antibodies	CZE was used to separate DNA fragments in a low-cost microdevice made by polyester-toner (PeT)	[67,77–81]
CIEF	pH gradient generated by ampholytes in the running buffer	Difference of isoelectric point of the analytes	Determines isoelectric point of analytes. It can be used to separate complex mixtures of peptides and intact proteins	cIEF–ESI–MS/MS was used to analyze and identify protein digests of a three-protein-mixture, and also host cell impurities of recombinant monoclonal antibodies	Microchip isoelectric focusing (μIEF) was used to analyze and evaluate heterogeneity in mAbs	[24,76,77,80–82]
CITP/ITP	Different buffer systems create different separation zones	Distinct electrophoretic mobility of the analytes at constant velocity between a discontinuous electrolyte system	Separation only occurs between two buffer systems: a high mobility buffer and a low mobility buffer. Can be used to pre-concentrate analytes.	cITP was used to evaluate the degree of oxidation of low-density lipoprotein (LDL) by $CuSO_4$	Transient ITP on a chip was developed for DNA sizing	[77,80,81,83–85]
MEKC	Analyte partition between running buffer and charged micelles with a hydrophobic interior	Partition coefficient of the analyte between the micelles and the electrolytes	Permits the separation and analysis of neutral and hydrophobic components through chromatographic methods	MECK coupled with DLIF detector was used to characterize fluorescent labelling reaction between BSA and a cyanine fluorescent dye	Two-dimensional separation of tryptic digests based on MEKC and CZE on-a-chip	[77,80,81,86–88]
CGE	Gel network introduced in a capillary format, covalently attached to capillary walls or not.	Different mobility of analytes in a gel media. Analytes with smaller molecular size travel faster through gel; Conversely, larger analytes migrate slower.	Diminishes band broadening and prevents analyte adsorption on capillary walls. Can eliminate EOF.	CE-SDS coupled with a PDA detector was used to evaluate the purity of light chain, heavy chain and non-glycosylated monoclonal antibodies.	Separation of fluorescently labeled proteins on-a-chip, with a pre-concentration step, with a porous silica membrane	[80,81,89–91]

CZE: Capillary zone electrophoresis; cIEF: Capillary isoelectric focusing; CITP/ITP: Capillary Isotachophoresis; MEKC: Micellar electrokinetic chromatography; CGE: Capillary gel electrophoresis.

Table 2
Analysis of post-translational modifications in proteins using capillary electrophoresis.

Type of protein modification	Description	Separation technique	Detection Mode	Reference
Post-Translational Modifications (PTMs)	Glycosylation	CZE	LIF	[93–95]
		CZE	UV–vis	[96]
		[a]Glass microchip/CZE	Fluorescence	[97,98]
	Phosphorylation	CZE	UV–vis	[99,100]
		cIEF	Chemiluminescence	[101]
		[a]Glass microchip/CZE	Fluorescence	[102]
	Oxidation	CZE	UV–vis (DAD)	[103]
	Deamidation	CZE	LIF	[104]
	Acetylation	CZE	ESI-MS	[105]
		CZE	UV–vis (DAD)	[106]
		[a]Commercial fused-silica microchip	LIF	[107]
	Carboxylation	CZE	LIF	[108]
	Hydroxylation	CZE	MS	[109]
	Disulfide linkage formation	CZE	UV–vis	[110]
	Isomerization	CZE	UV–vis	[111,112]
		CZE	MS	[113]
		[a]Glass microchip/MEKC	Fluorescence	[114]
	Proteolytic processing	CZE	UV–vis (DAD)	[115]
		CZE	UV–vis	[116]
Mutations	–	CZE	ESI-MS	[117]
		CZE	LIF	[118]
Aggregation	–	CZE	UV–vis	[119,120]
		CZE	LIF	[121]
		[a]Caliper LabChip GXII	Fluorescence	[122]
Denaturation	–	CZE	UV–vis	[119,120]

CZE: Capillary zone electrophoresis; cIEF: Capillary isoelectric focusing; MEKC: Micellar electrokinetic chromatography; UV–vis: Ultraviolet–visible spectroscopy; DAD: Diode array detector; LIF: Laser-induced fluorescence; MS: Mass spectrometry; ESI: Electrospray ionization.
[a] Systems based on microchip technology.

market of recombinant drugs, and almost half of all modern biotechnology companies [125]. The recombinant drugs market rapidly expanded and is now growing continuously [123], but even with the large profits that pharmaceutical companies are making, they are concentrated in reducing cost and expenses by seeking for alternatives to research and develop products in a more effective way [126]. European and North American companies dominate the production of recombinant products due to large investments in the area, which is a trend that has not been observed in underdeveloped countries. These countries invest less than 0.5% of their GDP [125] in biodrug research and development, which justifies their less pronounced development. The contrast in technological barriers between countries can hinder the availability of recombinant drug products for some countries and thus inhibit access to biopharmaceuticals to those who really need them [125].

Miniaturized systems allow high-speed electrophoretic separations in seconds, with sample volume requirements in the order of nano-to picoliters [127]. These miniaturized systems can be used for distinct biomolecules, such as DNA, RNA, and proteins, and can also be used for drug screening, as well as cellular components and advanced therapeutics analysis [128,129]. Furthermore, smaller-scale separations can be carried out faster and more efficiently than in benchtop laboratory techniques at the macroscale [128].

The qualities associated with the simplicity, less complex analytical instrumentation, portability [30,128,129], and the ability to manufacture microchips with low-cost materials [130–132] make these microdevices attractive to large pharmaceutical companies because these devices meet the reduction of costs and expenses requirements. Moreover, miniaturized systems can be used as a research tool in in-development countries, which will allow all regions to have access to biotechnological technologies.

Even though all the individual operations in microfluidic devices are already well established (*e.g.*, fluid transport, mixing, metering, reagent storage, and release), the integration and/or interfacing between these distinct functionalities are not yet completely developed [133]. This could be due to incompatibilities between substrates and reagents, sealing problems, carryover effects (especially in separation processes), pressure drop across the channels, channel geometry, and fixed vs. mobile parts (especially in centrifugation steps, where just one part of the microfluidic system should be rotated instead of the whole structure), among other possible incompatibilities [133]. It is necessary to consider all of these possible problems during the planning stages since future problems could compromise the whole project [133]. Since there is no report of a complete system that covers all the steps of recombinant protein production and analysis in one single microchip, it is hard to foresee all the difficulties that could arise. However, it is more important to note that it is possible to build such a system, with all the advantages that it brings: low sample and reagent consumption, lower risk of contamination, faster analysis, more reliable and reproducible results, and the capacity of parallelization, which translates into more tests being performed at the same time. All these characteristics can improve biodrug development, testing, evaluation, and accreditation by the relevant agencies, which could improve population health.

3.2. Trends in bioanalysis: microchip technologies

Microdevices can be fabricated in glass, silicon, quartz, or other cheaper substrates, such as polyester-toner (PeT) or polydimethylsiloxane (PDMS) [78,132]. Glass substrates present a great similarity with the fused-silica capillaries that are used in electrophoresis, showing higher separation efficiency and greater electroosmotic flow than the polymeric substrates [134]. Table 3 provides a summary of the main substrates for low-cost microcapillary electrophoresis devices (μCE).

Glass-based microsystems present some drawbacks related to their fabrication, such as in the creation of homogeneous channels, where due to the use of a fragile substrate, the corrosion process exposes the fragility of glass by forming irregular channels and even cracking or breaking the glass itself. Glass microchip manufacturing also requires specific equipment, which raises the costs of the device's fabrication, and thus does not meet the requirements for inexpensive devices [139]. On the other hand, glass

Table 3
Alternative substrates to the fabrication of the low-cost microcapillary electrophoresis devices.

	Glass	PDMS	PeT
Advantages	• Similarity with fused-silica capillary • Mechanical strength • Optical transparency • Excellent electrical insulator • Chemical inertness • Reusable device	• Flexibility • Optical transparency • Efficient channel sealing • Ability to produce pumps and valves	• Reduced cost devices • Short analytical time • Low sample consumption • Fast production, when compared to other methods • High reproducibility
Drawbacks	• Time-consuming process • Low reproducibility • High failure rate during etching • Most expensive fabrication process	• Interaction with analytes due to hydrophobic character • Channel clogging from non-cured monomer • Lower resolution in comparison with glass substrates	• Lower resolution than other substrates • Disposable device (single use)
Fabrication process	• Photolithography combined with chemical etching	• Master mold coated with photoresist and exposed to UV radiation • Elastomer along with curing agents added to master mold and cured in oven	• Toner printing over polyester sheets • Thermal treatment to seal microchips
Fabrication time	24 h	4 h	10 min
Cost per chip	~$ 40	~$ 5	~$ 0,15
Reference	[78,130,131,134–136]	[78,130,131,136,137]	[78,130,131,136,138]

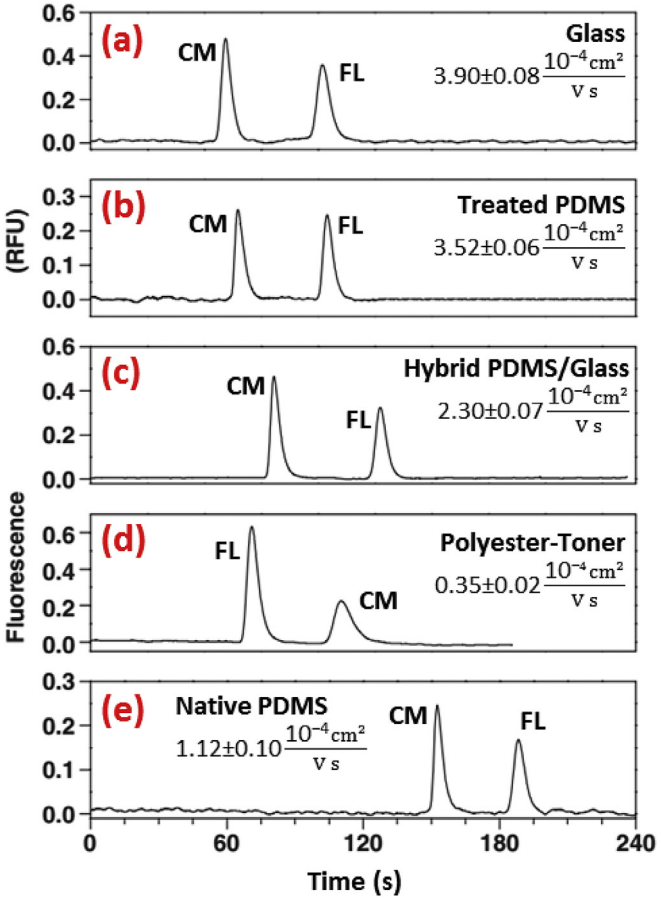


Fig. 2. Microchip capillary electrophoresis separation of fluorescein (FL) and coumarin (CM) (25 μmol L^{-1} for both) using (a) glass, (b) treated PDMS, (c) hybrid PDMS/glass, (d) polyester-toner, and (e) native PDMS microdevices. All separations were performed in normal polarity, with the exception of (d) polyester-toner, which was performed with negative polarity. In each panel, the electroosmotic flow is noted. Modified from Coltro, W.K.T. et al. [131], with permission. Copyright 2016 John Wiley & Sons Inc.

channels present the same chemistry as fused-silica capillaries, and the same EOF for CE purposes. Fig. 2a–e shows the difference in EOF and in the separation of fluorescein and coumarin between (a) glass, (b) plasma-treated PDMS, (c) hybrid PDMS-glass, (d) polyester-toner, and (e) PDMS microchip devices [131].

The difference in the EOF measured in each substrate can be used to tailor the separation depending on the analytes. The slow EOF on PeT chips (Fig. 2d) required an inversion of the separation polarity in order to achieve separation due to the higher electrophoretic mobility of the analytes in comparison with the EOF in normal polarity [131]. Fig. 3a–d presents a performance comparison between Pyrex and PDMS microchips for the electrophoretic separation of peptides, in particular, the peptide hormones angiotensin I, II, and III [140,141], using laser-induced fluorescence (LIF) as the detector. Lunte's group also performed this separation in a conventional system using UV–vis and electrochemical detection methods [140], which can also be used in a microchip format.

Moreover, alternative low-cost methods for microchip fabrication exist in polyester-toner devices, which present characteristics such as a simple fabrication process, low instrumental requirements, and a low cost of production [130,131]. These devices can be applied for the analysis of diverse components, such as proteins and DNA [78,130,131,136]. Fig. 4 presents a scheme of a polyester-toner based microchip device for dynamic solid-phase extraction (dSPE) and PCR amplification of DNA, which are some of the steps that are common during handling DNA for cloning of proteins.

A multilayer PeT microchip showed good efficiency for dynamic solid-phase extraction (dSPE) and PCR amplification of DNA in deep microchannels, allowing parallel extractions and compatibility with DNA amplification via PCR [136] when compared to conventional glass microchips. The authors attribute the advantages of PeT chips over glass microchips to the adsorption of the *Taq* enzyme onto the glass substrate, as well as the longer time and higher costs associated with the fabrication of glass microdevices [136] (Table 3). The PeT microdevice allowed DNA extraction and DNA PCR amplification on a single disposable microchip [136], which minimizes sample handling and diminishes the possibility of contamination in the PCR step.

Microcapillary electrophoretic devices (μCE) are versatile analytical tools that can be coupled to a mass analyzer, which is currently a trend in the analysis of biopharmaceuticals. In addition, μCE can be used with various distinct detection methods, such as optical, electrochemical, and conductivity detectors [57,142–144]. As the CE-MS system is already recognized as a powerful analytical tool for recombinant protein drug producers [72,145], we believe that the miniaturized devices should be used more extensively in the whole manufacturing process. The reduced size of devices and time of analysis in μCE combined with its low sample consumption abilities make them ideal for use in a quality-control setting

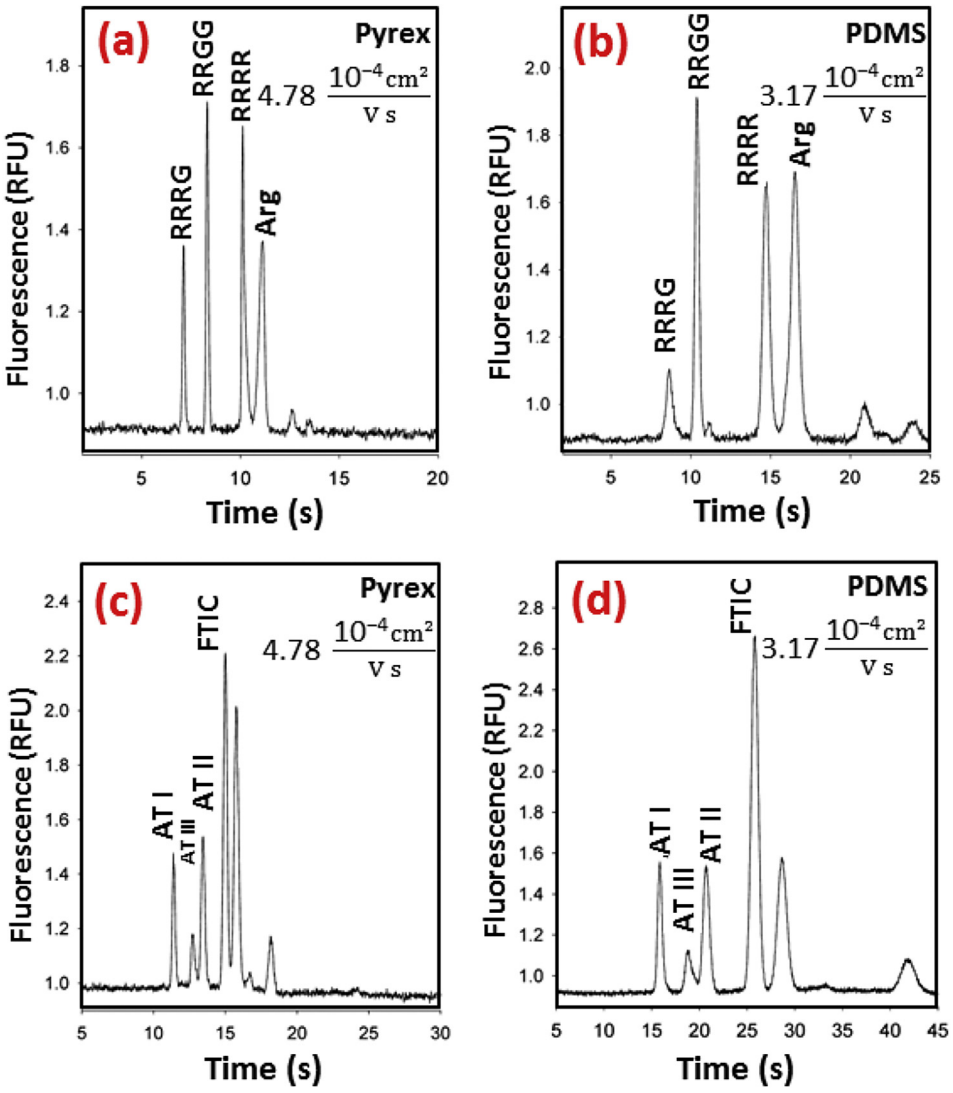


Fig. 3. Separation of fluorescently labeled peptides and arginine amino acid (500 nmol L^{-1} for both) in (a) a Pyrex microchip (separation potential of 3200 V) and (b) a PDMS microchip (separation potential of 4000 V). Separation of FITC-labeled peptide hormones angiotensin I, II, and III (500 nmol L^{-1} each) in a (c) Pyrex microchip (separation potential of 3200 V) and in (d) PDMS microchip (separation potential of 4000 V). Modified from Lacher, N.A. et al. [140]. Copyright 2016, with permission from Elsevier.

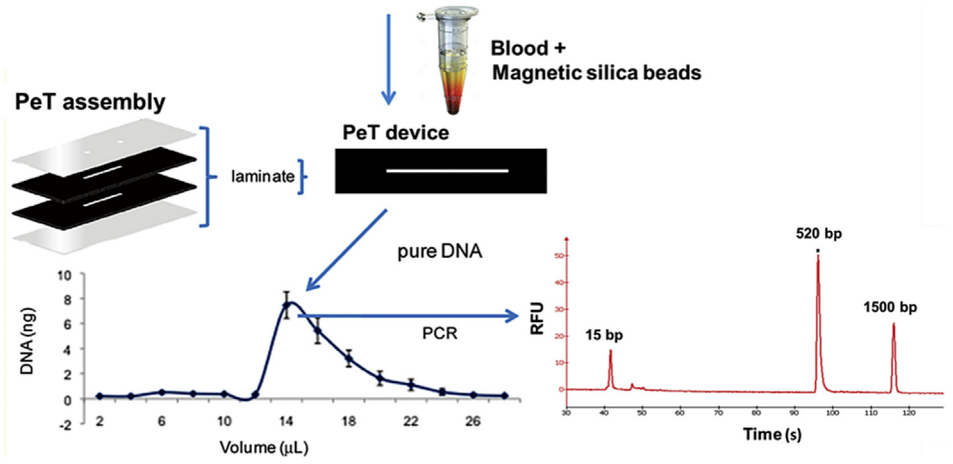


Fig. 4. Polyester-toner based microchip device for dynamic solid-phase extraction (dSPE) and PCR amplification of DNA. Modified from Duarte, G.R.M. et al. [136], with permission. Copyright 2015 American Chemical Society.

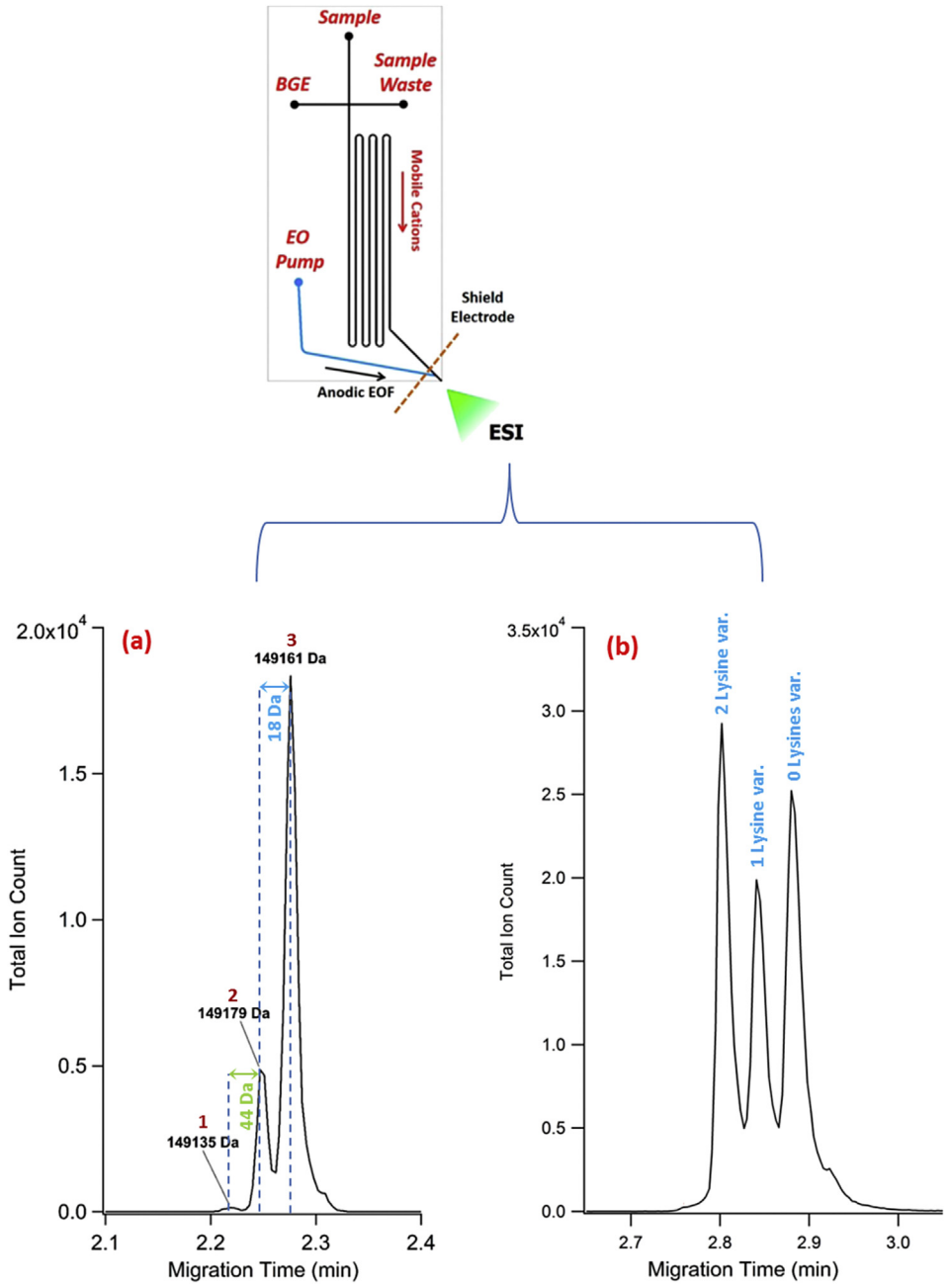


Fig. 5. Separation of mAb using μCE-ESI-MS. (a) Separation of charge variants of IgG2 type antibody. The difference between the first and the second variant is the loss of a carboxylic acid group (44 Da). The difference between the second and third variant is the formation of pyroglutamic acid at the N-terminus of the mAb heavy chain (18 Da). Reprinted (adapted) with permission from Redman E.A. et al. [148]. Copyright 2016 American Chemical Society. (b) Separation of charge variants of the Infliximab biodrug using an aminopropylsilane polyethylene glycol (APS-PEG_{450}) coated microchip. From right to left, there is an addition of a lysine residue in the variant band, which changes the mAb electrophoretic mobility. Reprinted (adapted) with permission from Redman E.A. et al. [147]. Copyright 2015 American Chemical Society.

[73,76,146]. As an example, μCE coupled with mass spectrometry proved to be a useful tool in the analysis of monoclonal antibodies [147,148]. A μCE-ESI-MS system was used to separate charge variants in monoclonal antibodies (Fig. 5) [147,148], as well as to assess post-translational modifications in antibody drug conjugates [148], demonstrating the functionality of these miniaturized systems.

Each protein therapeutic presents a pharmacological classification [149] and distinct methods of CE can be applied to the recombinant drugs analysis (Table 4).

Other applications of miniaturized systems are organs-on-a-chip microfluidic technology, which allows for the study of human disease physiology and pathophysiology in a synthetic organ, which enables the reproduction of *in vitro* disease models [161,162]. Synthetic organs can replace animal models, which present a high cost and can bring ethical implications. The artificial mimicking systems allow the study of basic human functions, as well as diseases of the organism, thus providing an understanding of the origin, evolution, and treatment of each disease [161–167]. During recombinant drug administration, one possible effect is the activation of the organism's immune response due to the intake of a foreign protein, which can lead to adverse reactions and health problems for the patient. The use of organs-on-a-chip can address the investigation of the organism's response to biodrug administration [161–164].

Table 4
Applications of capillary electrophoresis for the separation of protein therapeutics according to their pharmacological classification.

Group definition	Sub-group definition	Recombinant drug example	Description	Separation technique	Detection mode	Reference
Group I: protein therapeutics with enzymatic or regulatory activity	Ia: Replacing a protein that is deficient or abnormal	Human growth hormone	Anabolic and anti-catabolic activity	CZE	UV	[150,151]
	Ib: Augmenting an existing pathway	Erythropoietin (epoetin-α, epoetin-β)	Stimulates erythropoiesis	CZE CZE-ESI	UV IT-MS	[152]
	Ic: Providing a novel function or activity	Human hyaluronidases PH-20	Depolymerization Hyaluronic acid	CZE	UV	[153]
Group II: protein therapeutics with special targeting activity	IIa: Interfering with a molecule or organism	Rituximab	Monoclonal antibody adjuvant chemotherapy for B-cell lymphoma.	CZE	LIF ESI-MS	[154]
	IIb: Delivering other compounds or proteins	Gemtuzumab Ozogamicin	Monoclonal antibody linked to calicheamicin by chemotherapy for Acute myeloid leukemia	CZE	LiF	[155]
Group III: protein vaccines	IIIa: Protecting against a deleterious foreign agent	HPV vaccine	Immunization against human papillomavirus	CZE	UV	[156]
	IIIb: Treating an autoimmune disease	Monoclonal anti-Rh(D) antibody	Neutralizes Rh Antigens	Prep CZE	MALDI-TOF-MS	[157]
	IIIc: Treating cancer	Cytokine	Immunotherapy	Prep ITP-CZE RP-HPLC/ICE	UV ESI-MS LIF	[158,159]
Group IV: protein diagnostics	–	Secretin	Stimulation of endogenous secretion pancreatic	CZE	UV	[160]

Table 5
Recombinant protein drug fabrication steps and existing microchip technologies.

Step	Description	Microchip technology	Reference
Gene isolation	Extraction and elution of DNA using a silica-based solid phase extraction on microchip	Silica matrix introduced into borosilicate glass microchip	[172]
DNA amplification	Quantitative PCR performed in a microfluidic chip	Cyclo-olefin polymer microchip attached to 2 aluminum heaters	[173]
DNA analysis	Separation and analysis of PCR product	Polyester-toner microchip and Bioanalyzer commercial system	[78]
Cell culture	Culture and monitoring of endothelial cells	Polystyrene microchip as support to culture medium	[174]
Protein expression	Transcription and translation of proteins inside nanowells, in a cell-free process	96-well glass microplates as support	[175]
Protein separation and analysis	Isolation and enrichment of proteins on a 2-D capillary electrophoresis microchip	PMMA multi-dimensional CE microchip	[146]
Pharmacokinetics and pharmacodynamics	Evaluation of pharmacokinetic parameters of fluorescently labeled human mAb in serum	Caliper LabChip GX commercial system	[122]

The use of miniaturized systems could further address some of the issues associated with the production and development of biodrugs by speeding up processes such as license approvals from regulatory agencies. The versatility and low cost of miniaturized systems are highly attractive for industries in the recombinant drug market and research laboratories, and such systems will likely become an important sector of the biotechnology market.

Another example of microchip technology versatility is the use of these microdevices to synthesize proteins in a cell-free process on a chip [168,169]. This protein synthesis system uses vectors and a reactor to produce larger amounts of proteins in a shorter time that with *in vivo* systems [169,170], and has been used to study transcription and translation processes [170]. More than a research tool, this technology can be employed to produce personalized therapeutic biodrugs, such as lymphoma vaccines [171], which increases the possibility of integration of distinct microfluidic features to the fabrication of recombinant protein drugs.

It is worth noting that technology involving recombinant protein analysis already exists, such as the commercialized miniature capillary electrophoresis system applied to the omics area (DNA, RNA, and protein analysis), *i.e.*, the Caliper LabChip GX system [122]. The label-free backscattering interferometry technique that permits investigation of the binding events of protein-related compounds [170], which can also be performed in a microchip format, is already commercially available [171]. We envision, as the next probable step for biopharmaceutical companies, the integration of all of these functionalities in an all-in-one microfluidic device, thus minimizing sample handling and cross-contamination, while also enhancing productivity and satisfying all the requirements of regulatory agencies.

4. Concluding remarks

Miniaturized systems are a low-cost alternative in genetic and proteomic analyses, presenting high versatility in all areas of recombinant drug development and production. The microdevices can be fabricated from distinct materials, such as glass, PDMS, and PeT, or in a hybrid form including these materials [77]. μCE devices are also compatible with MS detectors and NMR spectrometers, making them a powerful tool for biopharmaceutical analysis.

Furthermore, applications of microdevices also include working with cell cultures and organs-on-a-chip, reducing the need for animal models and *in vivo* studies. Due to the diverse abilities of microchip technology, the capability of integrating conventional

analytical and proteomic tools in a single system (gene isolation, DNA amplification, cell culture, protein expression, protein separation and analysis, pharmacokinetics, and pharmacodynamics – Table 5) meets the requirements for a quality-control system for recombinant drugs.

Various applications brought by microchip technology are already being applied by various research groups; however, for recombinant drugs-on-chips to become a reality, it is necessary that the features mentioned herein be applied in a single device, thus creating one powerful and complete analytical tool for the recombinant drug industry.

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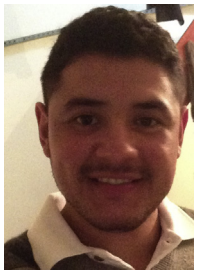
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