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20 Analysis of synthetic macromolecules by capillary electrophoresis

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1. Introduction. Capillary electrophoresis, principles

Capillary Electrophoresis (CE) is a combination of separation techniques based on the different electrophoretic mobilities of the dissolved substances under the action of an electric field [1-3]. It could be said that this technique combines the power of separation of the conventional electrophoresis [4] with the concept of automation of High Performance Liquid Chromatography (HPLC). The main characteristics of CE are the following:

- High speed of analysis (generally under 30 minutes per run).
- High separation efficiency (usually in the interval from 10^5 to 10^6 theoretical plates per meter of column, depending on the type of analyte and the separation conditions).
- Small volumes of samples are required (only a few microliters) because the injected volumes are in the range of nanoliters.

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- It presents a great variety of applications (from metallic ions to particles including peptides, proteins, fragments of DNA, pharmaceuticals, cells, etc).
- The technique is fully automated, which makes possible the separation and analysis of samples without the constant attention of the operator.

1.1 Instrumentation

In **Figure 1** a diagram of the basic instrumentation required in a capillary electrophoresis equipment is shown. The separation of the analytes is performed inside the capillary, which is usually made of fused silica. The capillary dimensions range from 25 to 100 μm of inner diameter and from 25 to 100 cm of length. These capillaries of silica are fragile so they are externally coated with polyimide to add flexibility and resistance to them. The fused silica presents physical-chemical characteristics good enough to use UV- Vis detection, as it is almost transparent to the radiation in this part of the spectrum. Moreover, the fact that the capillary wall is thin and its thermal conductivity good facilitates the dissipation of the heat generated by the Joule effect. On the other hand, silanols created upon hydratation of silica deprotonate at pHs above 3 and the negative charges of the silanols will originate the so-called electroosmotic flow (*vide infra*). Besides, these negative charges can cause parasitary adsorption phenomena mainly in samples with high positive charge density. This could even produce irreversible adsorptions of some compounds to the silica. To prevent these problems, the inner capillary surface can be coated with polymers normally neutral or hydrophilic such as polyacrylamide, poly-vinylalcohol, polyethyleneglycol, etc.

A buffer-filled capillary and the electrodes are placed between two vials filled with the same buffer. During the injection, the inlet buffer is substituted by a vial containing the sample. A small volume of sample (nanoliters) can be introduced in the capillary by pressure, vacuum, or applying a difference of voltage (electromigration). Once the injection is done the vial containing the sample is changed by the vial with the separation buffer. After that, an electric field is applied to start the separation. In CE equipments, high voltage power supplys usually provide voltages ranging from 0 to 30 kV. The separation buffer conductivity generates a difference of potential in between the capillary extremes. The previously injected substances separate due to the difference of potential while moving to the detection point.

The detection is done on-column (in the same capillary). A small section of the outer polyimide coating is removed near the outlet end of the capillary to form the detection window. This type of continuous detection has permitted the technique automatisation, and has eliminated dead volumes by avoiding the connections. This increases the separation efficiency by decreasing band broadening and permits the quantitative analysis. On the other hand, the narrow optical pathlength of these system of detection (25 to 100 μm) provides poor detection limits.

The most frequently used detector is the UV-Vis (filters, diffraction gratings or diode array), followed by the laser induced fluorescence detectors and mass spectrometers. The detector signal is sent to an analogical-digital converter, then it is stored and processed in a computer obtaining the separation electropherogram.

The capillary is thermosthatised, dissipating the heat generated by Joule effect, to maintain a constant temperature from one analysis to another in a known range. This is the way of improving the reproducibility.

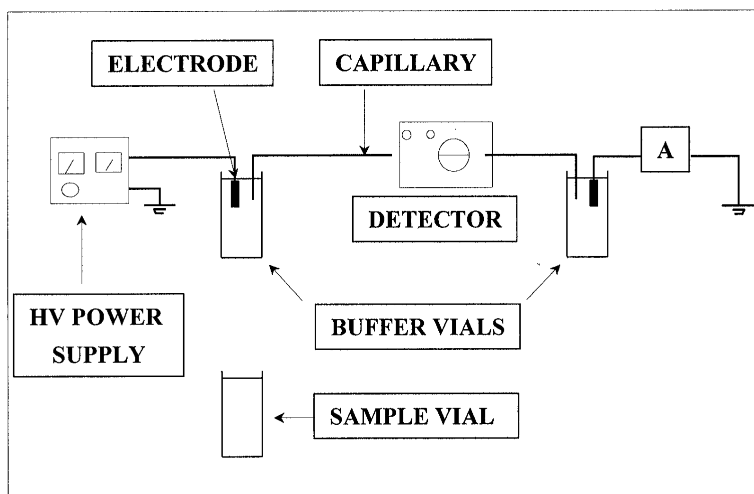

Figure 1. Basic capillary electrophoresis instrumentation.

1.2. Operation principle

The capillary inner wall contains silanol groups that get ionised gaining negative charge while in contact with the separation buffer (as shown in **Figure 2**). The ionisation degree is basically controlled by the separation buffer pH (negative charges appear in aqueous solutions with pH over 2-3). The wall, negatively charged, attracts the positive charged ions from the buffer creating a double electrical layer. This double layer has two zones; one of them *fixed* next to the capillary wall, where the interactions between the negatively charged silanol groups and the positive ions of the buffer are so strong that

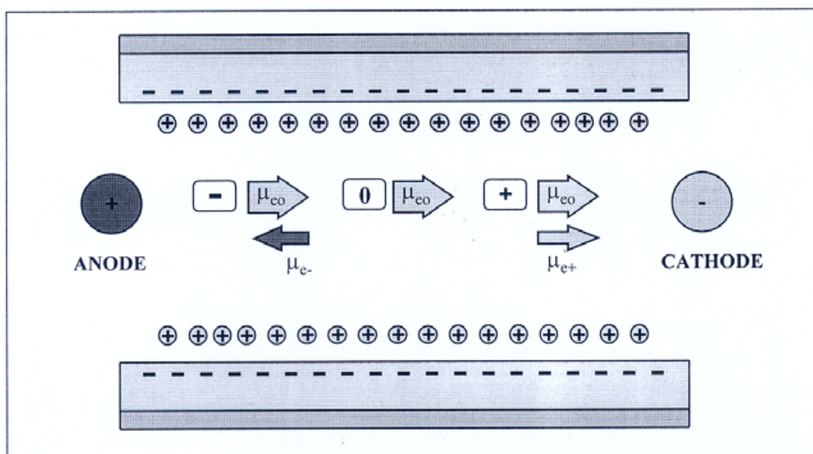

Figure 2. Electrophoretic separation by FSCE of three types of substances: with positive electric charge (+), with negative electric charge (-) and neutral substances (0).

they compensate for the thermal agitation; and another zone, further away from the wall, named *diffuse*, with weaker interactions with the charged silanols, capable of producing thermal agitation.

Under the action of the electric field the positive charges of the diffuse zone move to the cathode and drag with them the associated solvatation water. The result is a global movement of the buffer inside the capillary towards the cathode and it is defined by the electroosmotic mobility, μ_{eo}:

$$\mu_{eo} = \frac{\varepsilon \zeta}{4 \pi \eta}$$

where ε is the buffer dielectric constant, η is the buffer viscosity, and ξ (zeta potential) can be approximately defined as the potential generated between the negative charge excess at the capillary surface and the positive charge excess at the double layer. This last factor will determine the electroosmotic flow magnitude. This electroosmotic flow will move all the substances in the interior of the capillary at the same speed because it is a system property, that is to say, it will not introduce selectivity and therefore it will not permit the separation of the substances. One of the most important characteristics of this flow is that the flow profile is nearly flat and it provides high separation efficiency as will be seen below.

Moreover, under an electric field, the charged substances suffer the electromigration process, in which the charged analytes in the interior of the capillary tend to move to the opposite pole. These ions undergo two opposite forces: one of them due to the electric field and the other due to the friction. Using Stokes approximation, where the particle is considered as a rigid sphere, the friction force (F_r) for a substance in any media is given by the ecuation $F_r = 6 \pi \eta r_p v_e$, where r_p is the particle radii, η the media viscosity and v_e the movement velocity.

On the other hand, the charged particle in an electric field undergoes an electric force: $F_e = q E$, where q is the charge of the particle under the electric field E. The electric field is the result of dividing the applied voltage by the total capillary length.

Both forces become equal $F_r = F_e$, thus, the particle takes a linear uniform movement, where the velocity has the following expression:

$$v_e = \frac{q}{6 \pi \eta r_p} E$$

Being the termed electrophoretic mobility, μ_e, equal to:

$$\mu_e = \frac{q}{6 \pi \eta r_p}$$

The electrophoretic mobility is the parameter that controls the selectivity of the separation system through the relation q/r_p in the form of free zone capillary electrophoresis (FSCE), that is the most common. As it will be seen below, there can exist other parameters that, depending on the mode of the capillary electrophoresis that is used, control this selectivity, as for example the hydrophobicity, the isoelectric point, etc. The relation q/r_p is directly related with the ratio charge / volume of the substances. That is to say, for a group of substances with the same amount of electrical charge, the substances with a greater molecular size will have a relation q/r_p lower and their

electrophoretic mobility μ_e will be less, being able to be separated from those substances of a smaller size and, therefore, with higher electrophoretic mobility.

In the capillaries without an internal neutral coating that eliminates the effect of the negative charges of the capillary and therefore μ_{eo}=0, both electrophoretic and electroomotic migration are simultaneous in a sample component, as shown in **Figure 2.** Frequently the electroosmotic mobility due to the capillary wall is higher than the electrophoretic mobility of the analytes. Thus, the velocity that the substances are going to adopt inside the capillary will be the addition or subtraction (according to whether they go in the same or in the opposite direction, depending on the electric charge of the substances) of these factors:

$$\nu = (\mu_{eo} \pm \mu_e)\mathrm{E}$$

The migration time of a charged substance will be given by the expression:

$$t_m = \frac{l}{(\mu_{eo} \pm \mu_e)\mathrm{E}}$$

Where l is the capillary length from the injection to the detection point.

1.3. Characteristics of capillary electrophoresis related to other techniques

Capillary electrophoresis differs from gel electrophoresis in the applied voltages which can be very high in the former, since the dissipation of heat generated by Joule effect is much more efficient, due to the high rate outer surface/inner volume in the silica capillaries. Another characteristic of CE is that the on-column detection avoids intermediate steps existing in gel electrophoresis, such as dying. Thus, the speed and simplicity of the analysis, as well as the accuracy in quantitation are increased in CE.

The main analytical technique of separation comparable to capillary electrophoresis is HPLC. In general, the times of analysis are shorter and efficiency is higher in CE than in HPLC. This higher efficiency is mainly due to the fact that the flow profile is nearly flat in CE, rather than parabolic, as in HPLC. The flow profile is essentially flat in CE because it is generated by an electric field instead of by hydrostatic pressure as is the case in HPLC. **Figure 3** shows a scheme of both flow profiles and the respective peak shapes. As can be seen, in CE the flow does not contribute significantly to band broadening the way hydrostatic flow does in HPLC.

Finally, in CE, the reproducibility in quantitative analysis is lower, but smaller samples volumes are employed.

In general, these two techniques being based on different separation mechanisms, are considered complementary.

2. Modes of CE

There are different forms of capillary electrophoresis, mainly based in the nature of the separation media that is introduced in the capillary and also in the characteristics of the analytes that will be separated with this technique. Interestingly, the instrumentation is practically the same (**Figure 1**) for all of them. In the following sections a short description of all the currently existing capillary electrophoresis methods can be found.

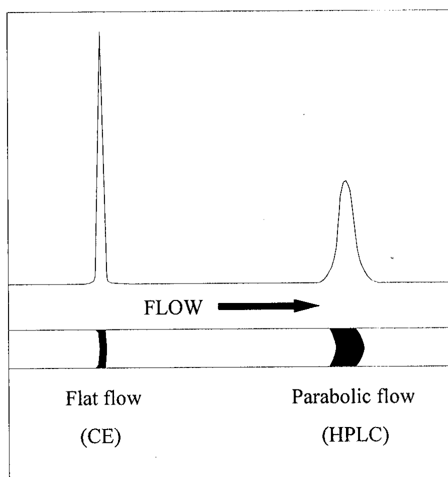

Figure 3. Flow profiles in CE and HPLC.

2.1. Free solution capillary electrophoresis (FSCE)

It was the first electrophoresis mode developed, and, nowadays, it is the most frequently used [5-7]. Inside the capillary there is only the separation buffer, therefore, it is possible to separate neutral and positively and negatively charged substances when the magnitudes of the electrophoretic (in the case of the charged ones) and electroosmotic flows are suitable, as has already been mentioned in the section "Operating principles".

Following these criteria, compounds with higher positive charge density and smaller radius, will be eluted in a shorter time. When the electric field increases, the migration times of the compounds will decrease. There is an optimum for this field giving the shortest analysis time with a suitable efficiency. Over this value, phenomena related to heat generated by Joule effect appear and separation efficiency decreases.

FSCE presents several limitations, which frequently can be overcome by using other different CE modes:

1. Generally, separation of uncharged species or with the same charge to mass ratio (as for example, DNA fragments or protein-sodium dodecylsulfate complexes, etc) can not be accomplished by using FSCE.
2. Compounds with high positive charge density can be adsorbed onto the capillary wall. This adsorption will influence negatively on the separation process.
3. Coefficients of variation for peak areas are in the range from 2 to 5% in real samples analysis. This is common for all the CE modes.
4. Finally, the sensitivity of the technique does not permit the trace analysis.

2.2 Capillary isotacophoresis (CITP)

The sample is injected between the frontal buffer, with higher mobility than the fastest compound in the sample and the terminal buffer with lower mobility than the slowest component in the sample. In CITP the substances are separated due to its electrophoretic mobility. With the application of a difference of voltage the analytes get

distributed in bands that move between the frontal and terminal buffer inside the capillary [8], all of them with the same velocity ν (hence the prefix *isotaco*, which means equal velocity).

$$v_{ITP} = E_1\mu_1 = E_2\mu_2 = E_i\mu_i$$

An equilibrium is achieved between the effective mobility of each analyte and the electric field in the corresponding electrophoretic band. Before reaching a stationary state, the substances are found outside their electrophoretic bands, and therefore, with an electric field different from that in equilibrium. As a result, the velocity of the analytes changes until they arrive at a zone in the capillary where there is a potential which makes them obtain the equilibrium velocity.

This electrophoresis mode, in spite of being less used, finds its main application area in sample pre-concentration protocols used together with other CE modes. When a diluted sample is injected and the voltage is applied, the zone is concentrated in the interface with the separation buffer. The pre-concentration depends on the characteristics (e.g., concentration and ionic strength) of the analytes, sample matrix and separation buffer.

2.2. Capillary isoelectricfocusing (CIEF)

The technique CIEF is an important tool in the analytical biochemistry area. It has been mostly applied to the separation of peptides and proteins, as shown in the first works published by Hjerten and Zhu [9].

Usually, a mixture of anfolites with different pH values are introduced in the capillary together with the sample (the peptides and proteins to be separated). When an electric field is applied, a pH gradient inside the capillary is firstly stabilised due to the anfolites, that are distributed from the anode (with low pHs) to the cathode (with high pH values) [10,11]. Peptides or proteins with positive or negative charge, under the influence of the electric field, move through the capillary to the anode or cathode until they reach the zone of the capillary in which the pH of the buffer is the same as their isoelectric point, that is to say, they get a pH value in which the number of their positive and negative charges is the same. At this pH value, analyte migration stops, as its global electrical charge is zero. When all the compounds have achieved their isoelectric point within the capillary, elution is generally performed by aplying a low pressure (keeping on the run voltage) in the anodic end moving in that way the focused bands towards the detection point. The capillaries used in this mode have an internal coating that decreases or eliminates the electroosmotic flow, because that flow would prevent in most cases the formation of the pHs gradient.

As already commented, this type of CE has, as a fundamental application, the separation of proteins and peptides (i.e., amphoter compounds) that present isoelectric point [12].

2.3. Micellar electrokinetic chromatography (MEKC)

This CE mode was initially developed to solve the separation of non charged compounds [13,14], although it can also be applied to the separation of charged substances.

MEKC involves the addition to the separation buffer of a surfactant at a concentration level at which micelles form. Detergents or surfactants are molecules that have a hydrophilic/ionic moiety on one end of the molecule and a hydrophobic moiety on the other. Micelles, aggregation of individual detergent molecules, form in aqueous solution when a detergent is present at a concentration higher than its *critical micelle concentration* (*cmc*). Micelles are generally spherical in shape being the hydrophilic groups of the detergent on the outside of the micelle, toward the aqueous buffer. The hydrophobic hydrocarbon molecules are in the center of the micelle. Detergents can be anionic, cationic, zwitterionic, or non ionic and their external charge will determine their migration towards the anode or the cathode or whether they are just moved by the electroosmotic flow.

Micelles constitute a stable second phase, that, in chromatographic terms, act as a pseudo-stationary phase which moves into the capillary [13,14]. Neutral analytes will interact with the micelles depending on their specific partition coefficient, which depends on their chemical characteristics. The time spent by the analyte inside the micelle will retard it in relation to the other neutral substances (which will interact with the micelles specifically too). Therefore, the mechanism of separation depends upon differences in distribution coefficients for the analytes between aqueous and the hydrocarbon pseudo-stationary phase.

Figure 4 shows the separation with three neutral substances with different affinity for the micelles. The compound T irreversibly interacts with the one in the buffer (Its distribution coefficient is, therefore, very high). The migration time of compound T (t_m) will be the same as that of the micelles. It will depend upon the electroosmotic flow and the electrophoretic mobility of the micelles (μ_{eo} and μ_{em}). Compound P partially interacts with the micelles. Its migration time (t_p) will depend as much upon the electrophoretic and electroosmotic mobilities as upon the compound partition coefficient between the aqueous buffer and the micelles. Compound N does not interact with the micelles. As it

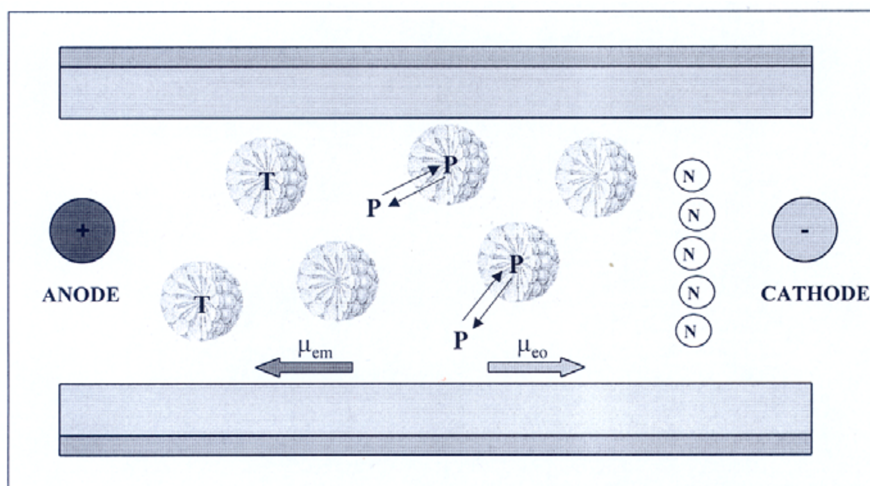

Figure 4. Electrophoretic separation by MEKC of three neutral compounds (T, P and N) with different hydrophobicities.

has no charge, the only driving force to the detector will be the electroosmotic buffer mobility. Therefore, it has a migration time (t_o) corresponding to the electroosmotic flow (which can be considered as a factor similar to the dead volume in HPLC). The difference between t_m and t_o is the so called separation window. The compounds to be separated will have migration times within this window and this fact limits the separation power of the technique.

2.4. Capillary Electrochromatography (CEC)

This type of CE has a great similarity with liquid chromatography. In CEC, the capillary is filled with silica particles (3 to 10 µm of diameter and derivatised or not) which act as a stationary phase. The buffer acts as a mobile phase that moves when an electric field is applied. Its velocity is proportional to the electroosmostic flow (i.e., $\nu_{eo}=\mu_{eo}E$). Neutral compounds are drived by the electroosmostic flow and they interact specifically with the stationary phase (in the same way as in HPLC) what originates their separation.

As happened with the MEKC technique, CEC was developed mainly due to the research works directed to the separation of non charged compounds in CE [15]. This technique is currently under development being one of its main limitations the short life of the filled capillaries. These capillaries, apart from being time consuming to prepare and/or expensive, frequently cause the formation of bubbles in the interior as a result of the application of the electric field. This makes the capillaries useless for further applications. Moreover, the employ of CEC to “real life” samples has still to be proved.

2.5. Capillary gel electrophoresis (CGE)

In this type of CE, the capillary is filled with a gel that will act as a molecular sieving. The most important application of this technique will be the separation of compounds with the same charge/mass ratio, but with different molecular mass [16,17], as for example DNA fragments, polysacharides, SDS-protein complex, ionic polymers.

In this way of CE, the molecules with a smaller molecular size are able to pass through the pores and elute first, whereas larger molecules are retarded by the gel and elute later, as can be seen in **Figure 5.**

The first gels to be used in the latter 80’s were made of crosslinked polyacrylamide, but they showed problems related to low reproducibility, resistance and stability. Nowadays, they have been substituted by the polymeric networks. They are hydrophilic non-crosslinked polymers that are dissolved in the buffer solution in a concentration usually higher than the so called *entanglement concentration*, over which a net that acts like a molecular sieve is formed (although according to Barron *et al* [18] it is not necessary to reach that concentration to obtain the effect of a molecular sieve). The most frequently used polymers are: linear polyacrylamide, polyethyleneglycol, poly-vinylalcohol, methylcellulose, etc.

3. Polymer analysis by capillary electrophoresis

Separation of biopolymers such as proteins, peptides or DNA frangments by capillary electrophoresis (CE) has nowadays become widespread due to the good posibilities of this technique in terms of analysis speed, high efficiency and low sample consumption [19-23]. Moreover, CE allows the separation of these substances according

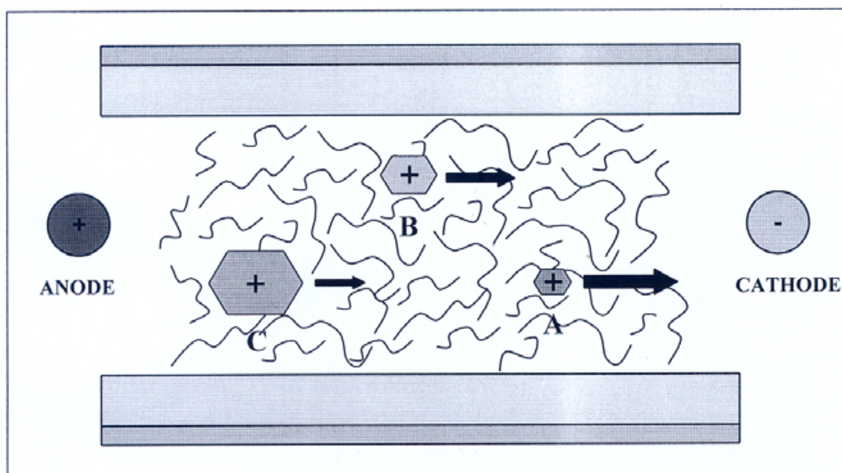

Figure 5. Electrophoretic separation by CGE of three substances (A, B and C) with different molecular size and the same charge/mass ratio.

to their different molecular size, charge/mass ratio, isoelectric point, etc. Logically, these aspects can be of utility for synthetic polymer characterization [24,25], mainly considering that properties like size and molecular dispersion of these synthetic macromolecules have a great effect in their usefulness in certain aplications. CE has demonstrated to be a very valuable technique for the fast obtention of helpful information for the characterization of this kind of polymers. However, since syntethic macromolecules come in a wide variety of forms differing in shape (branched, cross-linked, linear, etc), sizes (from molecular masses of hundreds to over a million) and chemical characteristics (neutral, ionic, hydrophobic, hydrophilic, etc), no one CE technique is universally applicable to all polymers. In order to deal with the very different polymer separations three CE modes have been basically used: free zone capillary electrophoresis, capillary gel electrophoresis and micellar electrokinetic chromatography.

3.1. Polymer analysis by free solution capillary electrophoresis (FSCE)

In this mode of CE, the synthetic polymers must be electricaly charged (i.e., bearing a negative or positive charge) since in FSCE compounds are going to be separated based on their differing charge/size ratio. One of the first application of this capillary electrophoresis mode has been the analysis of polymeric particles. An example of this is given in **Figure 6**, in which the separation of sulfated and carboxilated polystyrene nanospheres ranging from 39 to 700 nm is shown [26]. Separation of these nanoparticles is done by FSCE using a 50 μm internal diameter bare fused silica capillary [26]. Following this idea, the separation of chemically different latex particles (also bearing different numbers of attached carboxylate or sulfate groups) with 0.03 to 1.16 μm particle size has been also carried out by FSCE using a 75 μm internal diameter bare capillary [27]. In both applications an UV absorption detector working at 254 nm and

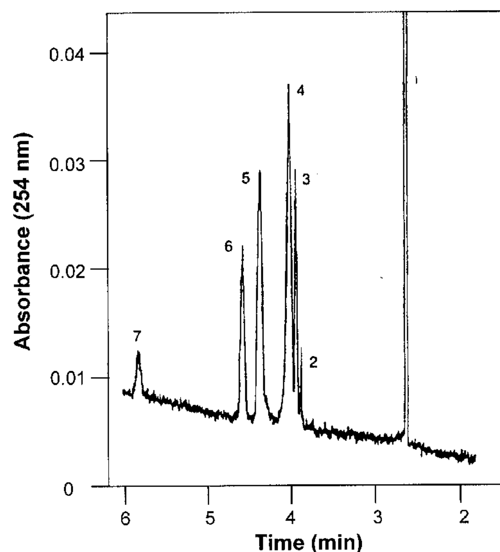

Figure 6. FSCE separation of polystyrene nanoparticulates with different sizes. **1** = riboflavin (neutral marker), **2** = 39 nm particle, **3** = 72 nm particle, **4** = 132 nm particle, **5** = 308 nm particle, **6** = 488 nm particle, and 7 = 683 nm particle. Separation conditions. Running buffer: 1 mM ACES (pH 5.80). Injection: 30 kV for 1 s. Capillary dimensions: 50 μm (i.d.) x 47.6 (l_d) cm fused silica. Field stregth: 382 V/cm. Detection: UV at 254 nm. Redrawn from ref [26].

separation voltajes about 30 kV were used. These conditions provided analysis time less than 5 minutes for most of the separations.

As above, Mc Cormick [28] also carried out the analysis of polymeric particles by FSCE. Specifically, he demonstrated the separation of colloidal silica particles ranging in size from 5 to 500 nm. According to the author, the speed of these separations ($t < 20$ min) prevents analysis interferences due to agregation reactions of these colloidal systems. On the other hand, the efficiencies obtained during these separations were very low. On the other hand, the separation of composite particles formed by colloidal polyaniline (PANI) and poly(N-vinylpyrrolidone (PVP)) [29] has been also demonstrated by FSCE. However, the authors in this case mention that the possible agregation between particles could be the responsible of the low reproducibility obtained between separations. In this work, the authors couldn't separate the composite particles formed by colloidal silica and PVP of the particles formed by polypyrrol (PPy) and poly(vinyl alcohol) (PVAL).

The need of working with electrical charged compounds for FSCE separations involves very often the reaction of the neutral polymers with substances which provide them electric charge prior to their analysis. This is the case of the poly(ethylene glycol) (PEG) separations carried out by Bullock [30]. PEG oligomers with molecular masses from about 1000 to over 3500, lack of charge as well as a UV chromophore. To overcome these limitations they were derivatized with phthalic anhydride, which allows the separation by FSCE in analysis time less than 30 minutes as shown in **Figure 7.** Also, it has been developed some procedures that allow the monitorization or the

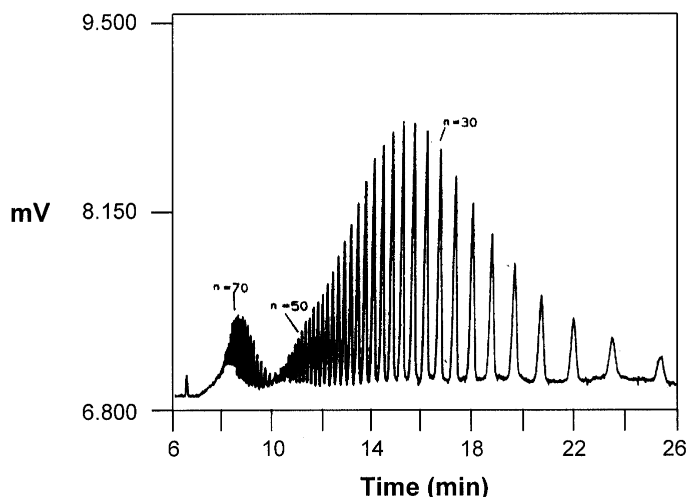

Figure 7. CE separation of PEG oligomers derivatized with phthalic anhydride. Separation conditions: 57 mM boric acid, 35 mM 1,3-diaminepropane, pH 9.7 (acetonitrile-water, 70-30) as running buffer. Injection time: hydrodynamic for 1s. Capillary dimensions: 50 µm (i.d.) x 37 (l_d). Voltage: 25 kV. Detection: UV at 205 nm. Redrawn from ref [30].

fluorescence emission of several poly(oxyalkylene)diamine (Jeffamine) polymers derivatized with a fluorescent dye as 2,3-naphthalenedialdehyde (NDA) [31]. By means of this procedure it has been possible the separation in less than 15 minutes of 30 poly(oxyethylene)diamine oligomers of average molecular mass 600 differing in one -CH_2- group between them.

Another interesting application of FSCE for synthetic polymers characterization is the work of Pesak et. al. [32]. They showed the separation of phenylacetylene dendrimers terminated with *tert*-butyl esters on their periphery from phenylacetylene dendrimers terminated with carboxilic acids. FSCE allowed both the characterization of the dendritic macromolucules studied and monitoring the transformation between these compounds through a solid-state thermolytic process. The information provided by FSCE was very helpful to finally prepare more homogeneus dendrimers.

FSCE has also been used for the monitoring of drugs (e.g., growth hormone, GH) released from different copolymeric devices (i.e., films and slabs) made of vinylpyrrolidone-hydroxyethyl methacrylate (VP-HEMA). It was observed that GH released rate is controlled by copolymer composition, and it was possible to simultaneously monitor by FSCE the release of GH and the polymer dissolved during experiments. Interestingly, it was demonstrated that both data were connected [33].

3.2. Polymer analysis by capillary gel electrophoresis (CGE)

The separation in CGE is based on the different molecular mass of the compounds being also necessary to work with electrical charged compounds. As mentioned above, the sieving effect can be generated by a gel covalent linked to the capillary wall, or due to a dissolved polymer into the running buffer. The covalently bonded gels (usually

cross-linked polyacrylamide) on the capillary wall were the first used in CE for the molecular sieving formation. However, their application for polymer analysis is very rare. One of these applications was the separation of a mixture of poly(acrylic acids) by CGE coupled to Mass Spectrometry [34]. However, these kind of gels have fallen into disuse due to the numerous stability and reproducibility problems originated by these gels. Actually these gels are being susbtituted by non cross-linked polymers (for example cellulose, polyacrylamide, poly(vinylalcohol) derivatives, dextrane), that dissolve into the running buffer generate the same molecular sieving phenomenon. One of the first applications of CGE with a dissolved polymer into the separation buffer was presented by Poli and Schure [35]. In this work, the authors employed the addition of hydroxyethyl cellulose to the carrier solution for the CGE separation of eight poly(styrenesulfonates) according to their molecular weight from 1800 to 1200000. The analysis time obtained was lower than 10 minutes. The authors compared CGE with size-exclusion chromatography (SEC), clasically used for polymer characterization. They found that results provided by CGE were better in terms of resolution and efficiency. Moreover, CGE provided a higher molecular weight range analyzable than SEC. Also, the analysis velocity achieved by CGE was three times higher than that supplied by SEC.

Bullock in 1993 [30] developed a procedure that allowed the separation of several poly(oxyalkylene)diamine (Jeffamine ED series) polymers without derivatization using FSCE and indirect UV detection. In this case the CGE separation buffer contained poly(ethylene oxide) 86000 as additive. Optimal separations for the Jeffamine polymers of average molecular mass 600 and 900 were obtained in this case in less than 10 minutes [30].

In a similar way as FSCE, CGE has been used for particle analysis made of synthetic polymers. Thus, in the work carried out by Radko et. al. [36], it was achieved the molecular sieving of polystyrene carboxylate (PSC) particles with sizes ranging between 2.8 and 10.3 μm using a buffer solution containing uncrosslinked polyacrylamide. PSC particles were derivatized with fluorescein isothiocyanate for their detection by laser induced fluorescence. It is interesting to mention that in this case the limits of particle diameter lies close to 10 μm, as a result of the fluctuation of the fluorescence observed due to the progresive light scattering of the particle.

As mentioned above, CGE separations (like FSCE separations) require to work with electrically charged compounds for their analysis. Many synthetic polymers have not electrical charge, therefore they can not be analyzed in this CE mode. To solve this limitation, polymers are usually modified prior to their analysis. Thus, Wallingford [37] carried out the separation of poly(ethylene glycol) (PEG) oligomers and ionic and nonionic ethoxylated surfactants. To do this, the neutral compounds (i.e., PEG oligomers and nonionic surfactants) were derivatized with phthalic anhydride in order to provide them charge and detectability by UV at 280 nm. The effectiveness of this procedure is demostrated by Wallingfod [37] through the separation of phthalate-derivatized PEG oligomers of PEG 1000, 3350 and 4600 by CGE. An example of the different selectivity that can be achieved by using FSCE and CGE for the separation of synthetic polymers is shown in **Figure 8.** As can be seen, FSCE fails to resolve the anionic oligomer distribution of the phosphated alkylphenol ethoxylate containg 40 mol of ethylene oxide (AP40P), while CGE was successful in baseline resolving and detecting more than 54

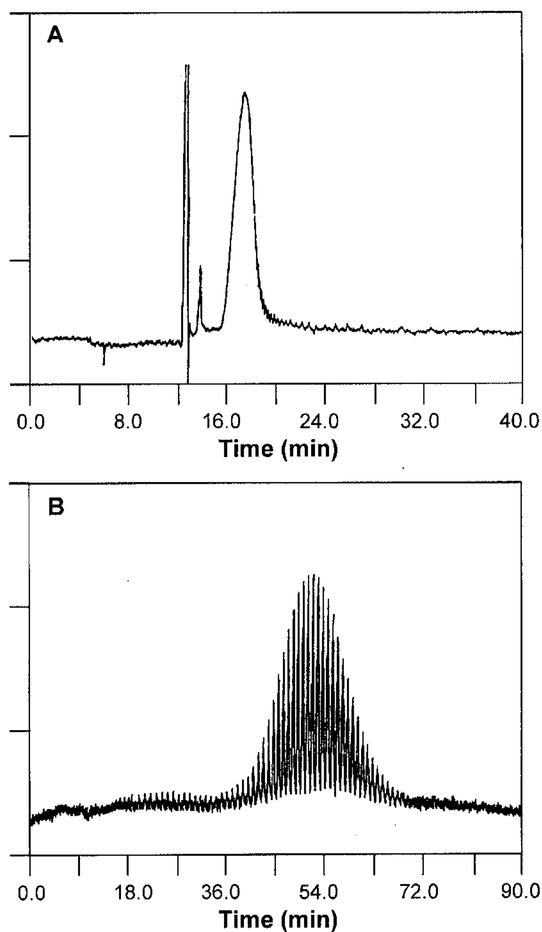

Figure 8. Separation of **AP40P** (Phosphate alkylphenol ethoxylate containing 40 mol of ethylene oxide). (A) FSCE separation conditions: Running buffer: 6 mM $Na_2B_4O_7$, 10 mM NaH_2PO_4, 20 % CH_3CN, 30% methanol, pH 7. Injection: gravity, 25 s at 25 mm. Capillary dimensions: 75 μm (d.i.) x 80 cm. Applied potential: 30 kV. Detection: UV at 206 nm. (B) CGE separation conditions: Tris-borate, pH 8.3 running buffer:. Injection: electrokinetic at –5000 V for 5 s. Column: μ-PAGE-3 without urea (from J & W Scientific), 75 μm (i.d.) x 50.1 cm. Field stregth: -220 V/cm. Detection: UV at 230 nm. Sample concentration: 20 730 μg/mL in water. Redrawn from ref [37].

oligomers of AP40P. In this work [37], PEG 4600 represented the highest molecular weight detected because of the decreasing of the signal with the molecular size in both CGE and SEC. Moreover, Wallingfod used cross-linked polyacrylamide gel-filled columns, so the method showed two main drawbacks: long analysis time (almost 90 min) and low stability and durability of the gel-filled columns used. These drawbacks were overcomed by Barry et. al.' work [38] using dextran dissolved in the runnig buffer as molecular sieving. Besides, Barry et. al. [38] used 1,2,4-benzenetricarboxylic anhydride (BTA) as derivatizing reagent to impart to PEG and ethoxylated surfactants

both charge and detectability at 210 nm. They found that under their separation conditions, migration time was linearly dependent on analyte molecular mass.

CGE has also allowed the separation of inorganic polymers highly charged. Namely, polymers of condensed phosphated [39] or 2-vinyl pyridine polycations [40] using linear polyacrylamide gel and pullulan (polymaltotriose) respectively in the running buffer as molecular sieving were separated by CGE. The analysis of the polyphosphated polymers was made using indirect photometric UV detection adding pyromellitic acid as UV absorbing background electrolyte.

3.3. Polymer analysis by micellar electrokinetic chromatography (MEKC)

As discussed above, polymers must bear an electrical charge in order to be analyzed by FSCE or CGE. In this way, the application of the electric field will result in the separation of such polymers (according to their different charge/mass ratio or molecular size, respectively). However, MEKC allows the separation of non-charged polymers depending on their different hydrophobicity. This effect is achieved adding to the running buffer a surfactant (usually sodium dodecyl sulfate, SDS) that will interact with the polymers in a different extent depending mainly on the polymer hydrophobicity. The different interaction between the surfactant and polymers causes their separation.

Although nowadays the potential of MEKC to characterize synthetic macromolecules has been proved in several applications, it is not a very extended CE mode in polymer analysis. In 1993, Bullock [30] showed the ability of MEKC for the separation of neutral Triton X series oligomers (from 1 to 46 units) in less than 20 min using SDS. Our group has used MEKC [41] for monitoring and characterizing copolymers prepared from free radical polymerization of *N*-vinylpyrrolidone (VP) and 2-hydroxyethyl methacrylate (HEMA) at different conversion degrees. As an example **Figure 9** shows the MEKC monitoring of this polymerization reaction. As can be seen, this reaction shows a bimodal behavior. Thus, copolymers rich in HEMA are initially formed (peak 1), while after 24 hours a second peak comes out which corresponds to copolymers rich in VP. These results were confirmed by the kinetc analysis of this reaction. These MEKC results were compared with those obtained by size exclusion chromatography (SEC). It was observed that MEKC and SEC provide valuable complementary information with respect to the average composition of copolymer chains and their macromolecular size and size distribution. Moreover, this was the first report of MEKC applied to the characterization of high molecular weight copolymer systems. These results allowed, in two following works, the application of MEKC for the control of cyclosporine released from VP-HEMA copolymer systems. These works proved using *in-vitro* [42] and *in-vivo* [43] assays, the dependence of release velocity of the drug on copolymer composition.

In a recent work [44] the usefulness of MEKC to monitor the radical copolymerization reactions of (2-hydroxyethylmethacrylate)-(2-acrylamide-2-methyl-propane sulfonic acid), HEMA-AMPS, and (N,N-dimethyl acrylamide)-(2-acrylamide-2-methylpropane sulfonic acid), DMAA-AMPS, was demonstrated. In this work, MEKC procedures were developed to monitor the monomer consumption together with the copolymer synthesis for the two systems, i.e., HEMA-AMPS and DMAA-AMPS. The effect of the conversion and composition on the chemical composition distribution as

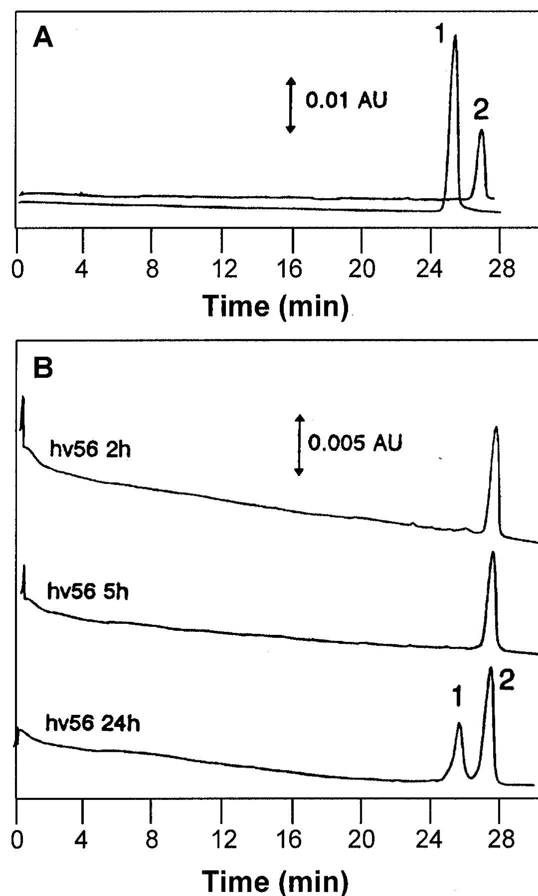

Figure 9. MEKC electropherograms of **(A)** homopolymer standards, PVP (1) and poly-HEMA (2), and **(B)** copolymer samples of the reaction hv56 (Feed molar composition of HEMA = 0.56) at different reaction time (2h, 5h and 24h). Separation conditions: 1:1 methanol:50 mM boric acic/sodium tetraborate at pH 9.5, SDS 35 mM running buffer. Injection: 5 s at 0.5 p.s.i. Capillary dimensions: 75 µm (i.d.) x 47 cm (l_t) x 40 cm (l_d). Separation voltage: 12 kV Detection: UV at 214 nm. Redrawn from ref [41].

well as on the molecular weight were analyzed by using this analytical technique. The large possibilities of MEKC for obtaining interesting information about synthesis progress, nature and composition of the formed ionic copolymers were demonstrated. Moreover, it was shown that capillary electrophoresis instrumentation can be used to monitor the electrical conductivity of the reaction product obtained at different copolymerization stages of the HEMA-AMPS system in order to clarify the polymerization mechanism.

Gyorffy et. al. [45] demonstrated that the combined effect of hydrophobicity and charge/mass ratio in MEKC has a positive influence on the separation of polyanionic macromolecules made of poly(N-vinylpyrrolidone-co-maleic acid). The authors proved

the lack of separation of the polymers by FSCE even at very different running buffer pHs, as a result of the similar charge/mass ratio of these macromolecules. The adition of SDS to the running buffer (i.e., MEKC analysis) was shown to improve the separation due to the hydrophobic effect on selectivity.

3.4. Polymer analysis by other electrokinetic procedures

Recently, a new separation technique for macromolecular compounds based on capillary electrochromatography has been developed [46,47]. The technique is called electrically driven size exclusion chromatography (ED-SEC) or size-exclusion electrochromatography (SEEC). This technique employs capillary columns (typical i.d. 30-100 µm) packed with bare silica particles (typically 3-10 µm), together with high dielectric constant solvents such as water, acenotrile or dimethylformamide (DMF). Under these condition, after aplying the high voltage, an strong electroosmotic flow is generated and with it the macromolecules move within the capillary. Polymers are separated based on their different size due to the differential exclusion from different fractions of the mobile phase in the column. According to the authors, plate numbers in SEEC can be 2-3 times higher than in standard, pressure-driven size exclusion chromatography [25,46,47].

SEEC of polystyrenes in packed capillaries using DMF as solvent was demonstrated in reference [46]. In that work [46], an improvement of the efficiency obtained for polystyrenes polymers was found compared to that obtained for standard pressure driven SEC analysis of the same solutes. Unfortunately with SEEC the retention window is smaller than under pressure conditions and moreover appeared to depend strongly on the ionic strength of the mobile phase. This phenomenon was attributed to the occurrence of pore flow that was further sutudied in reference [47]. To do this, the applicability of SEEC for the separation of polystyrenes was investigated in capillary columns packed with 5 µm particles with different pore sizes using DMF as the mobile phase. It was found that under SEEC conditions, a significant intraparticle pore flow was generated. Besides, the relative intraparticle velocity with respect to the average interparticle velocity increased with the pore size and ionic strength. It was also observed that with increasing pore flow the plate height of polymers decreased considerably. On the other hand, the intraparticle velocity impaired the selectivity of the separation. These effects could be described well with a theory that was also developed in that work [47].

Recently, the use of rigid polymer monolithic capillary columns for the separation of polystyrenes in CEC was reported [48]. However, the reported chromatogram shows an extremely low selectivity and only polymers with a very large difference in molecular mass could be separated on these columns.

4. Conclusions

The complexity of the chemical composition of synthetic macromolecules has increased the need for more reliable analytical methodologies for characterizing these materials. CE has emerged as a powerful analytical tool able to provide useful information about the chemical properties of these complex molecules. Interestingly, such information can be in some cases complementary to that provided by other classical techniques. Thus, in this revision it has been demonstrated that CE is being used in their different modes (FSCE, CGE, MEKC, etc.) to successfully face the tremendous diversity

that can be found analyzing synthetic polymers. One of the main characteristics of CE is that this technique makes possible to develop uniquely tailored separation procedures to analyze synthetic macromolecules of very different nature.

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