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Electrochemical determination of carbohydrates: Enzyme electrodes and amperometric detection in liquid chromatography and capillary electrophoresis

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Abstract

In recent years, electrochemical detection (EC) methods have become increasingly important for the determination of carbohydrate compounds in a variety of biological and pharmaceutical samples. In this work, recent advances in the design and application of EC approaches are reviewed, with the goal of providing the non-electrochemist with a basic understanding of the most important EC approaches to carbohydrate detection and an overview of their current applications. Two specific EC detection strategies are considered in detail: enzyme electrodes and electrodes used for HPLC or capillary electrophoresis detection.

Keywords: Carbohydrate analysis; Electrochemical detection; Enzyme electrodes; Capillary electrophoresis; High performance liquid chromatography

1. Introduction

Without question, the past 2 decades have seen a great increase in the use of electrochemically based methods for the solution of practical analytical problems. In order to accomplish this, electrochemistry has shifted its traditional inorganic focus on metal ions to include a broad range of organic analytes and, in particular, organic species that have important biochemical functions. As a result, numerous organic compounds, not previously viewed as good candidates for electroanalysis, are now routinely determined by electrochemically based approaches. One of the families of organics for which this is the case is the carbohydrates which, although readily oxidized by chemical agents, has not traditionally exhibited useful redox behavior at most electrodes. However, with recent advances in electrode materials and design, attractive electroanalytical techniques for carbohydrates have been developed, many of which have become the methods of choice for important applications in the biomedical and pharmacological arenas. In this work, we will survey the principal approaches that have evolved for the electrochemical detection (EC) of carbohydrate compounds.

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A major driving force for the development of EC assay methods for carbohydrates is the fact that these compounds do not generally possess a native chromophoric group that absorbs strongly at accessible UV-visible wavelengths. Thus, without prior conversion to a highly absorbing or fluorescing derivative, they are poorly suited to the usual optical detection approaches. However, EC techniques, if properly designed, permit direct detection of carbohydrates without the need for derivatization and thereby offer the possibility of simpler, faster, and more efficient assay procedures. In addition, EC systems are generally among the most economical and most flexible detection techniques. For example, the instrumentation required to carry out potential control and current measurement is relatively unsophisticated and inexpensive; and electrodes can usually be miniaturized for applications where size is important e.g. for in vivo sensing or capillary electrophoresis detection.

Another complicating factor for carbohydrate analysis is that carbohydrate samples can be extremely complex—considering the large number of different monosaccharides that occur naturally, the large number of ways in which they can be linked together to form oligo- and polysaccharides, the virtually identical chemical reactivities that different carbohydrates exhibit, and the variety of noncarbohydrate substituents with which they can be coupled. Because of this, stand alone detectors, whether electrochemical or spectroscopic in nature, seldom offer adequate selectivity for the analysis of real samples. One solution for EC detection involves coupling the electrode with a highly selective chemical agent, most often an enzyme. In these cases, the carbohydrate of interest is usually the enzyme substrate; and the product of the enzymatic reaction is monitored electrochemically with a selectivity that is essentially the same as that of the enzyme. Here, of course, each detection system typically responds to only one carbohydrate so that mixture analysis is generally not possible. A second solution, targeted to multicomponent analysis, is to couple the electrode with an efficient separation technique such as high performance liquid chromatography (LC) or capillary electrophoresis (CE). Fortunately, with EC methods, this can usually be accomplished in a straightforward fashion; and such LCEC and CEEC systems have been shown to offer high selectivity for analytes that are easily oxidized or reduced and low detectability that rivals the most sensitive fluorescence and mass spectrometric detection techniques.

Both of these approaches, involving the use of either enzymatic processes or separations techniques to impart selectivity to the EC detection operation, have had significant success in carbohydrate analysis over the past 2 decades and will be considered in turn in the discussion below. There are currently many excellent reviews available on the general subjects of enzyme electrodes and LCEC/CEEC, and we will not attempt to duplicate the large quantity of useful background information contained therein. Rather, we will refer the reader back to these reviews as appropriate and, as much as possible, focus our attention on specific developments and applications for carbohydrate detection that have taken place in these areas within the past 2–3 years.

2. Enzyme-based detection

2.1. Background

The prototypical example of an amperometric enzyme electrode is, of course, the glucose electrode which was first reported in 1967 [1] and has subsequently become, by virtue of the hundreds of papers continuing to appear on the subject, what is certainly one of the most well studied electrodes ever. In the basic version of this system, glucose is oxidized in an immobilized layer of glucose oxidase and then determined indirectly at an underlying Pt or carbon electrode by measuring the current resulting from oxidation of H_2O_2 produced (or reduction of O_2 consumed) by the enzymatic reaction. Thus, the glucose oxidase enzyme serves two essential functions that are important for all enzyme-based carbohydrate sensors. First, it converts an analyte (glucose) which is not readily oxidized at a Pt or carbon surface to an alternative compound (H_2O_2) which has much more favorable electrochemical behavior. Second,

it performs this conversion with a high degree of selectivity.

Two practical issues common to all biosensors must be taken into consideration in the design and implementation of enzyme-based electrodes for carbohydrates. These are enzyme immobilization and electron transfer mediation. As far as the former is concerned, virtually all immobilization approaches developed for biosensors have been employed for glucose oxidase and other carbohydrate-directed enzymes. These methodologies, summarized generally for enzyme electrodes by Pantano and Kuhr [2], have included, most notably, placement beneath a dialysis membrane, adsorption or covalent attachment to the bare electrode surface, reaction with cross-linking agents, addition to carbon paste, and entrapment within a gel layer or polymer film. As far as the latter is concerned, a wide variety of electron transfer mediators have also been employed, including ferrocenes, quinones, phthalocyanines, viologens, and phenothiazines among others [3–5]. The intended function of these mediators can be either to decrease the potential required to monitor the enzyme product (e.g. H_2O_2 or NADH) or to facilitate direct electron transfer between the electrode and the enzyme and thereby remove the need for any cofactor (e.g. O_2 or NAD^+).

The principal reason for the extensive activity in developing a glucose electrode has been the analytical importance of glucose itself and the need for a convenient and effective glucose monitoring device for use by diabetics. An additional factor facilitating this work has been the fact that glucose oxidase offers nearly ideal properties with respect to activity, selectivity, stability, and commercial availability. Of course, there are many alternative enzymes that are suitable for construction of analogous electrodes for other carbohydrate compounds in addition to glucose [5,6]. These include not only oxidases, which share a common dependence on O_2 as co-substrate, but also dehydrogenases, which rely instead on nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$) as cofactor. In most cases, the resulting electrodes are specific in their response and, like the glucose oxidase electrode, respond essentially to only one carbohydrate. However, in a few instances, enzymes possessing broader substrate specificity have been employed. For example, a single electrode containing oligosaccharide dehydrogenase has been shown to respond down to the 1 μM level to maltooligosaccharides up to maltohexaose as well as to xylose, glucose, galactose, mannose, and lactose [7,8].

A related approach by which enzymes have been employed with broad success to assist the EC detection of carbohydrates involves their use in postcolumn reactors in HPLC. In these systems, an appropriate enzyme or combination of enzymes is immobilized onto a porous support material, usually high-silica glass, and then packed into a small column that is placed between the separation column and the HPLC detector. When the carbohydrate analyte elutes from the HPLC column and passes through the reactor, the enzyme reaction occurs; and the starting carbohydrate may be detected indirectly by downstream EC monitoring of the reaction products—e.g. H_2O_2 for glucose and glucose oxidase. This approach, which has been recently reviewed by Nagels and Maes [6], has been applied to a variety of mono-, di-, and oligosaccharide species with subsequent detection of either H_2O_2 (for oxidase enzymes) or NAD(P)H (for dehydrogenases). In these applications, composition of the downstream electrode at which the EC detection is carried out depends on the species that it must monitor. Thus, for H_2O_2, a Pt or glassy carbon electrode is most often employed while, for NAD(P)H whose oxidation normally has a large overvoltage, carbon electrodes with electrocatalytic modifiers or even second enzyme systems have usually been used [6]. A drawback of this approach is that often the enzyme cofactor must be continuously added to the mobile phase.

2.2. Recent developments

As indicated above, glucose is easily the most important and most frequently investigated carbohydrate for enzyme-based EC detection. Not only does its determination represent a high priority for the monitoring and treatment of diabetics, but also advances made in the design and construc-

tion of glucose electrodes are often directly applicable to electrodes targeted toward other analytes. Accordingly, it is not surprising that the most interesting recent innovations in enzyme electrode systems for carbohydrate analytes have involved the glucose electrode. The most important of these will be described below.

The basic operating principle used for the glucose oxidase electrode has remained fundamentally unchanged over its 30-year life span. Nevertheless, it is only recently that electrode performance seems to be approaching the level needed to realize the 'Holy Grail' of such electrode applications, in vivo glucose monitoring with a long-term implantable glucose sensor. In such applications, the greatest difficulties have consisted of avoiding interferences due to electroactive species common to physiological systems, minimizing irreproducibility and calibration difficulties due to low or fluctuating O_2 levels, and maintaining stable, long-term sensor response.

Probably the most advanced glucose sensor in terms of actual in vivo and evaluation is that developed for subcutaneous implantation by Reach and Wilson [9,10]. This 10-cm-long, 0.45-mm-o.d. device, which has been shown to be capable of accurately tracking blood glucose levels in both animals and human volunteers, consists of a Pt–Ir anode coated with layers of glucose oxidase, cellulose acetate, and Nafion and an outer layer of polyurethane. In operation, the various polymer layers serve to prevent access of ascorbic acid, acetaminophen, and other oxidizable species to the Pt surface, to modulate transport of O_2 and glucose oxidase, and to limit protein adsorption and associated decreases in sensor response.

An alternative approach to problems associated with insufficient O_2 levels in the subcutaneous tissue involves providing the glucose oxidase with an electron acceptor or mediator chosen for its ability both to restore the enzyme to its active, oxidized state and to undergo reversible electrochemistry itself [11]. Conventionally, this mediating species has simply been added to the immobilized glucose oxidase layer where it can diffuse freely to the underlying electrode surface. An important improvement on this approach has been pioneered by Heller's group who have covalently 'wired' glucose oxidase, the mediator, and the electrode together in a single conductive unit [12–15]. This has been done most successfully by co-immobilizing the enzyme along with Os $(bipyridyl)_2$ relays on a poly(vinylpyridine) backbone that has been deposited onto a glassy carbon electrode. The Os-containing redox centers make possible direct electrical communication between the glassy carbon surface and the glucose oxidase, thereby providing a glucose-oxidizing system which is practically independent of the prevailing O_2 concentration. Most recently, glucose oxidase electrodes exhibiting greater operating stability at physiological temperatures have been constructed by including an additional layer of a thermostable Os-wired peroxidase [15].

Finally, the O_2-dependency problem has been attacked by a completely different route in a new variation of glucose oxidase electrodes reported by Wang and Lu [16]. Here, the enzyme is incorporated into a carbon paste electrode made from a mixture of graphite particles and a water insoluble liquid binder. However, unlike previous carbon paste enzyme electrodes which typically have used mineral oil as the pasting liquid, Wang substituted poly(chlorotrifluoroethylene) (also known as Kel-F) which has a very high O_2 solubility and can thereby provide a substantial internal source of O_2 for the glucose oxidase reaction. The resulting electrodes gave identical response to high glucose concentrations in both the presence and the absence of O_2 in the sample solution over a 7-h period of continuous operation. This is a very simple electrode design which will be seriously considered for in vivo applications in the near future.

3. LCEC/CEEC

3.1. Background

In virtually all of the LCEC/CEEC applications reported to date, EC detection of carbohydrates has involved oxidation under amperometric or voltammetric conditions for which many popular electrode materials are not well suited. For exam-

ple, Hg electrodes cannot withstand oxidizing potentials themselves; and carbon electrodes do not permit carbohydrate oxidation at potentials low enough for practical use. Thus, for LCEC and CEEC of carbohydrate compounds, a critical issue has been the selection of electrode material. To this point, the electrodes which have been employed successfully have consisted of either a noble metal or a transition metal, each of which will be considered in turn below.

3.2. Noble metal electrodes

In 1981, Hughes and Johnson [17] first reported the use of pulsed amperometric or pulsed electrochemical detection (PAD or PED, respectively), at Pt electrodes for the detection of carbohydrates following HPLC separation. Since then, this approach has been developed instrumentally and characterized mechanistically, largely by the Johnson group, and applied extensively, by numerous groups, to a wide variety of carbohydrate analysis problems. In the process, PED has also been employed at Au electrodes which are now the electrode of choice for this technique. PED instrumentation expressly directed toward the determination of carbohydrates has been available commercially for more than a decade, and there is little doubt that this approach is currently the most widely used for direct EC detection of carbohydrate compounds.

The processes involved in the oxidation of carbohydrates of Au and Pt electrodes and the operating principles involved in PED are thoroughly treated in several recent reviews on this subject [18–21] and consequently will be described only summarily here. It has long been known that alditols, monosaccharides, and oligosaccharides all undergo electrocatalytic oxidation at these electrodes triggered by specific interaction of the carbohydrate with surface oxides generated electrochemically at modest potentials. Subsequent O-transfer reactions occur that lead to multi-electron oxidation of the carbohydrate to formate and smaller aldaric acids. However, the practical utility of this oxidation for analytical or other applications has been limited because of the adsorption of free radical intermediates and rapid fouling of the electrode surface.

PED alleviates this problem by a controlled program of potential steps designed to remove the adsorbed species and return the electrode surface to its active state. A complete measurement cycle typically requires the application of three different potentials: the detection potential for carbohydrate oxidation, a more positive cleaning potential for desorption of adsorbed species, and a cathodic reactivation potential for removal of the oxide film and regeneration of an active surface. Typical potential values for the three steps at a Au electrode are $+0.2$ V versus AgCl for detection, $+0.8$ V for desorption, and -0.3 V for surface regeneration, with the entire sequence requiring no more than 1 s and permitting detection frequencies of 1 Hz or greater. Traditionally, the potential is held constant during each of the three steps. However, more recently, the potential during the detection stage is cycled over a small window and the current integrated in order to obtain better background rejection.

At Au electrodes, maximum sensitivity requires strongly alkaline conditions (Pt functions well over a wider pH range). Therefore, with PED, a pH >12 is normally employed for the mobile phase in LC or for the electrophoresis medium in CE. This generally does not present any problem for CE where the high pH actually proves useful in deprotonating the carbohydrates which would otherwise be uncharged and thus unsuitable for electrophoretic separation. However, for HPLC where most stationary phases are not stable above pH 9, PED must be carried out either with post-column addition of NaOH to the mobile phase or with a specialized stationary phase designed for high pH operation. In most cases, the latter approach is employed with anion-exchange columns specially designed for this application [21]. Under favorable circumstances, flow injection and HPLC PED applications employing this methodology are able to claim detection limits for mono- and disaccharides at picomole levels. For CE with its much smaller sample volumes, mass detection limits usually reach the femtomole range.

3.3. Transition metal electrodes

Carbohydrate oxidation has also been carried out at electrodes made from several transition metals including, most notably, Cu [22–24], Ni [25,26], Co [27,28], and Ru [29]. Unlike Au and Pt, these electrodes are capable of stable, long-term operation at constant applied potential without the need for cleaning and reactivation by continuous potential pulsing. Consequently, they are fully compatible with simple potentiostatic control instrumentation and do not require the more specialized pulse equipment. Like Au and Pt, the transition metal electrodes also require strongly alkaline conditions which produce oxide or hydroxide surfaces that have both an electrocatalytic function for carbohydrate oxidation and a passivating function that allows operation of the electrodes at potentials where the bare metals would undergo rapid oxidation. Because of this high pH requirement, these electrodes are also usually paired with base-compatible anion-exchange columns for HPLC separations.

In practice, these electrodes can take several different forms. For Cu and Ni, the simplest procedure involves the use of wires, disks, or films of the metals themselves. Such electrodes can be obtained commercially in a wide range of shapes and sizes as needed or can readily be generated by routine electroplating, sputtering, etc. In a few cases, alloys have been used in place of metallic Ni in order to provide increased stability of response in flow injection and LCEC operation [30–32]. Alternatively, electrodes have been prepared for all four metals by incorporating particles of the insoluble metal oxides into carbon paste or into polymer films [29,33–40]. Finally, chemically modified electrodes containing one of the metals in a specific molecular form e.g. Co as cobalt phthalocyanine [41,42] or Ni as Ni tetramethyl-dibenzo-tetraaza[15]annulene [43]—have also been employed.

For most of these electrodes, the carbohydrate oxidations are thought to be electrocatalytic in nature, initiated by the electrochemical generation of a high oxidation state of the metal substrate such as Ni(III) or Co(III). Beyond this, the specific electrode reactions involved are generally not known; and most of the time, the reaction products have not been characterized. Although it has often been postulated that the initial oxidation of the carbohydrate occurs at the carbonyl group, the fact that most of these electrodes oxidize alditols in a similar fashion suggests that this may not be the case. Of the different metals, the one whose reactions have been investigated most thoroughly is Cu where the carbohydrate oxidations have been shown by large-scale coulometry to entail many-electron processes ($n = 14$ for glucose) and to yield as final product single carbon units such as formate and carbonate [44]. Of course, knowledge of electrolysis mechanisms and products is not absolutely necessary for useful analysis applications to be designed and put into practice.

3.4. Recent developments

At this point, LCEC of simple carbohydrate compounds can certainly be described as a mature technology. Instrumentation required for both PED at noble metal electrodes and constant potential detection at transition metal electrodes is commercially available as are the high-pH anion-exchange columns normally used for the related separations. The carbohydrate analytes routinely determined via this approach include not only mono- and disacharides but also related derivatives such as alditols, aldonic, uronic, and aldaric acids, and amino sugars. Although some technical advances in LCEC continue to appear (e.g. the development of faster and more effective pulse sequences for PED [45]), the most innovative new work has generally been occurring in two alternative areas: (1) CEEC, which was initially reported for carbohydrates only in 1993 [46] and (2) the extension of EC techniques, both LC and CE, to more complex samples. Each of these will be considered in turn below.

3.5. CEEC of carbohydrates

CEEC itself is a relatively new technique, now only approximately 10 years old. Accordingly, technical innovations in CEEC are still commonplace; and the range of analytes to which CEEC

has been applied is still somewhat limited. Nevertheless, carbohydrates represent one of the analyte groups that have received the most attention to date from CEEC practitioners.

ln general, the approaches used for carbohydrates in CEEC have largely paralleled those used previously for these compounds in LCEC in that both PED at Au electrodes and constant-potential detection at Cu and Ni electrodes have been employed. Certainly, as with LCEC, a variety of different carbohydrates in a variety of different kinds of samples has been examined with success by both detection modes. As summarized in Table 1, these analytes include, in addition to simple mono- and disaccharides, oligo- and polysaccharides, carbohydrate derivatives such as alditols, sugar acids, and amino sugars, and carbohydrate conjugates such as glycopeptides and nucleotides. For most of these, the high efficiency CE separation has proven to be a good match with the high sensitivity of the EC detection. For example, the high pH solutions required for optimum electrode response with Au and Cu provide very effective electrophoresis media for carbohydrate separations. In this regard, Ye and Baldwin [55] showed that related carbohydrate compounds—e.g. glucose, glucitol, and gluconic, glucaric, and glucuronic acids—are ionized to different degrees in the strongly alkaline media used and can be easily resolved by CE. Furthermore, many traditionally difficulty problems e.g. the separation of different alditols from one another or the resolution of complex polysaccharide mixtures—can be accomplished by simple measures such as optimization of the pH of the electrophoresis buffer [55] or adjustment of the electroosmotic flow by addition of surfactants [60]. Most important, in CEEC, these problems are solved directly on the samples of interest without the formation of derivatives as required when optical detection methods are used.

However, in contrast to LCEC, implementation of EC detection in CE requires that two unique instrumental problems, electrical decoupling of the CE and EC electronics and physical alignment of the electrode with the capillary exit, be addressed and overcome. The need to isolate or 'decouple' the CE and EC systems in CEEC arises from the fact that the separation voltages in CE typically are in the 5–30 kV range and generate μA-level background electrophoresis currents. This is in contrast to typical EC detection potentials of 1 V (or less) and currents on the order of pA's. Thus, for reliable CEEC operation, it is imperative that electrical overlap of the CE and EC systems is controlled and minimized. The need to provide suitable capillary/electrode alignment arises from the fact that the ID of the capillaries used in CEEC range from 5 to 100 μm and the electrodes used are typically microelectrodes possessing diameters as small as 5–10 μm. Thus, optimum sensitivity dictates that the electrode is positioned very close to the capillary exit while good reproducibility requires that these positions, once initially established, are maintained throughout the entire analysis process. These issues, which are common to all CEEC systems irrespective of analytes, have recently been reviewed by Voegel and Baldwin [66] and are described briefly below.

Electrical decoupling in CEEC has been carried out by either of two general approaches. In the first scheme (termed 'off-column' detection), decoupling is accomplished by creating a small opening or fracture in the capillary wall 1 cm or so before the exit [67]. The fracture, which is typically covered with a porous coating such as Teflon or Nafion to inhibit solution flow and to stabilize the capillary, is then immersed in an electrolyte solution which also contains the high voltage CE electrode. With this arrangement, the CE voltage and current are dropped across the capillary only up to the fracture and should have little effect on EC electrodes placed downstream at the physical end of the capillary. In the second decoupling scheme (referred to as 'end-column' detection), the EC electrode is simply positioned at the end of the capillary in the CE buffer reservoir. If the width of the capillary is small (i.e. 25 μm ID or less), its ohmic resistance is very high. Therefore, the CE current is low in magnitude; and nearly all of the CE high voltage is dropped across the capillary itself [68,69].

In the earliest CEEC designs, capillary/electrode alignment was carried out by attaching the microelectrode, in the form of a wire or fiber, to an x, y, z-micropositioner and then moving it as

close as possible to the capillary tip [67]. In most cases, increased operational stability is achieved by partially inserting the electrode into the capillary opening which is sometimes chemically etched to create a wider gap that can accommodate larger electrodes [70]. These approaches are termed 'in-capillary' and 'optimized in-capillary' detector configurations. Subsequently, several

Table 1
CEEC of carbohydrates

Carbohydrate	Sample	Electrode	Configuration	Reference
Simple sugars	Mono- to tetrasaccharides in soft drinks	Cu	In-capillary	Colon, Dadoo, and Zare, 1993 [46]
	Mono- and disaccharides	Cu	Wall-jet	Ye and Baldwin, 1993 [47]
	Glucose	Carbon paste/ GOD[a]	Walljet	O'Shea nad Lunte, 1994 [48]
	Mono- to trisaccharides	Cu_2O in C cement	Wall-jet	Huang and Kok, 1995 [49]
	Mono- and disaccharides	Ni, Cu	Wall-jet w/ holder	Fermier, Gostkowski, and Colon, 1996 [50]
	Mono- and disaccharides in soft drinks and urine	Ni	Wall-jet w/ holder	Fermier and Colon, 1996 [51]
	Glucose	Pt/GOD[a]	On-capillary	Voegel, Zhou and Baldwin, 1997 [52]
Sugar derivatives	Glucosamines and glucose in blood	Au/PED	In-capillary	O'Shea, Lunte and LaCourse, 1993 [53]
	Alditols and mono- and disaccharides	Au/PED	—	Lu and Cassidy, 1993 [54]
	Alditols, sugar acids, and mono- and disaccharides in apple juice	Cu	Wall-jet	Ye and Baldwin, 1994 [55]
	Alditols and mono- and disaccharides in blood	Au/PED	Optimized in capillary	Roberts and Johnson, 1995 [56]
	Alditols and mono- and disaccharides in urine	Cu	Wall-jet	Voegel and Baldwin, 1996 [24]
	Glucosamine and glucose	Au/PED	Optimized in-capillary	LaCourse and Owens, 1996 [57]
	Alditols, sugar acids, and mono- and disaccharides	Cu	On-capillary	Voegel, Zhou, and Baldwin, 1997 [52]
	Alditols and mono- and disaccharides in urine	Cu	Wall-jet	Hong and Baldwin, 1997 [58]
	Glucosamine, glucosaminic acid, and glucosamine-6-sulphate	Au/PED	On-capillary	Zhong and Lunte, 1996 [59]
Polysaccharides	Maltooligosaccharides, starch hydrolysates and dextrans	Cu	Wall-jet	Zhou and Baldwin, 1996 [60]
Sugar conjugates	Glycopeptides in tryptic digests	Au/PED	In-capillary	Weber, Kornfelt, Klausen, and Lunte, 1995 [61]
	Adenosine and uridine	Carbon paste/ CoPc[b]	Wall-jet	Zhou and Lunte, 1995 [62]
	Glycopeptides in tryptic digests	Au/PED	In-capillary	Weber and Lunte, 1996 [63]
	Aminoglycoside antibiotics in urine	Ni	Wall-jet	Fang, Ye, and Fang, 1996 [64]
	Aminoglycoside antibiotics in pharmaceutical preparations	Cu	On-capillary	Voegel and Baldwin, 1997 [65]

[a] GOD, glucose oxidase.
[b] CoPC, cobalt phthalocyanine.

simpler and more reliable alignment strategies have been devised. One such approach is the 'wall-jet' arrangement in which a flat, disk-shaped electrode is used in place of a cylindrically shaped wire or fiber [47]. Here, the electrode is simply pushed up against the capillary outlet so that the exiting solution impinges directly onto and then flows radially across its surface. Because the wall-jet electrode is large enough to be seen and worked with fairly easily, optimum electrode placement is far less critical to establish and is somewhat easier to maintain over an extended series of CEEC experiments. This advantage can be extended further by fixing both the capillary and the wall-jet electrode onto a support device that then holds both more rigidly in place [50]. Finally, the design of 'on-capillary' electrodes in which both the capillary and the electrode are incorporated into a single integrated unit has been most recently reported [52,59,65]. Here, a 25 μm diameter Au wire was glued onto the exit end of the capillary [59]; or Au, Pt, and Cu were simply sputtered onto the capillary tip in the form of thin films [52,65]. In both cases, alignment is no longer an issue as the integrated capillary/electrode unit is simply inserted into the terminating electrophoresis buffer reservoir at the start of the CE experiment. As a result of these developments, not only has the convenience of CEEC alignment been enhanced but the reproducibility and stability as well. For example, both the supported wall-jet [50] and the on-capillary [59,52] configurations have shown peak height variations of only 2% for repeated glucose injections with the same capillary/electrode arrangement and of only 5% for injections made with different electrodes or after capillary/electrode realignment. Typically, the same electrodes have been used for continuous day-long experiments and for discontinuous experiments spanning several weeks. Although this may not always be the case, on-capillary electrodes have often proven to be longer-lived than the capillary [59].

At this point, CEEC unfortunately is not yet a commercially available option from CE manufacturers and thus has been the domain primarily of experienced electrochemists. However, the equipment needed to carry out EC detection is not difficult to assemble. Furthermore, the advances described above have begun to alleviate the most important decoupling and alignment problems that have previously discouraged non-electrochemists from more rapidly adopting EC techniques for their separation and detection goals. In particular, the use of small ID capillary systems, which avoid decoupling altogether, and the use of on-capillary electrodes, which avoid the microscope/micropositioner approach to alignment, promise to make CEEC a user-friendly technology. It seems apparent that, with these advances, CEEC will rapidly experience far greater application in carbohydrate analysis.

3.6. Applications to complex samples

Technical innovations aside, the most telling test of an analytical methodology is the range and importance of the applications to which it is successfully directed. In this regard, EC detection is already well established for the determination of simple carbohydrate compounds including mono- and small oligosaccharides. This is certainly true for LCEC using PED at Au and Pt electrodes and, to a lesser extent, constant potential detection at Cu and other transition metal electrode systems [21,71]. As described above, analogous CEEC techniques have also had their initial successes with analysis systems consisting of simple carbohydrate analytes [72]. Thus, it is not unrealistic to argue that EC techniques, in conjunction with HPLC and CE separations, represent the approach of choice for the analysis of mixtures of such carbohydrate compounds. However, in recent years, a significant extension of EC detection to more complex carbohydrate samples has occurred. This extension has taken two different forms—the determination of larger and more complex carbohydrates and the detection of various carbohydrates in more complex sample matrices. In particular, numerous practical applications related to pharmaceutical and biotechnological problems have begun to be addressed by both LCEC and CEEC. One advantage of EC detection in these applications is that, with the LC and CE formats in which there is only transient exposure to minute sample vol-

umes, the electrodes usually do not suffer from the surface fouling and passivation often seen with many other EC techniques in which the electrode is placed directly into the sample solution for relatively long periods of time.

In general, EC detection of polysaccharides does not inherently suffer from any of the molecular size limitations that are often seen with proteins or other macromolecules in which the electroactive site may be inaccessible to the electrode surface or analyte adsorption may lead to electrode fouling and passivation. Rather, polysaccharide samples containing from 20 to 100 monosaccharide units have been shown to give large oxidation currents with both PED at Au electrodes and constant-potential detection at Cu electrodes. This is due, presumably, to the fact that each monosaccharide contains its own oxidizable groups which contribute cumulatively to the current observed. Thus, the real challenge in polysaccharide analysis is more the separation operation than the actual detection step. For HPLC-based approaches which utilize high pH anion exchange columns (HPAEC), a great deal of work on polysaccharide separations has already been carried out. This area has recently been reviewed by Johnson and LaCourse [21] who described several examples where gradient elusion techniques with NaOH and NaOH/NaOAc mobile phases provided impressive separations of polysaccharides containing different chain lengths or positional isomerization. In all cases, the HPAEC separation was compatible with high sensitivity PED. For CEEC, far fewer analogous studies have been reported to date. Most to the point, Zhou and Baldwin [60] demonstrated that good CE separations of oligo- and polysaccharides can be achieved through the addition of cationic surfactants such as cetyltrimethylammonium bromide to the electrophoresis medium. This serves to reverse the direction of the electroosmotic flow and thereby allow the larger polysaccharides to migrate off the capillary last. The approach was characterized by the separation of maltoses containing up to seven glucose units and then demonstrated on commercial starch hydrolysates and dextrans nominally containing 90–100 glucose residues. In this work, EC detection was carried out in a constant-potential mode at Cu electrodes.

Concurrent with LCEC and CEEC applications directed toward the detection of larger carbohydrate analytes has been the application of these EC based techniques for the solution of new and more complex biochemical problems. Once again, the LCEC methods have led the way, with recent samples including HPAEC/PED (Au electrode) assays of polyglucose metabolites in the plasma of patients undergoing dialysis treatment for end stage renal disease [73] and of glucose, mannitol, and lactulose levels in urine to evaluate intestinal permeability of HIV-infected patients [74]. Similar CEEC studies are only beginning to appear. Best illustrating the unique capabilities of this approach is the use of CEEC at Cu electrodes to profile clinically important metabolic markers in human urine [58]. In a single CE run comprising < 60 min, alditol, carbohydrate, amino acid, and other urinary constituents were separated and detected at physiologically relevant levels with no sample pretreatment or derivatization.

One of the specific applications in which EC detection is currently making its most important contributions is the elucidation of the carbohydrate structures present in glycoproteins, including both identification of the glycosylation sites on the protein and determination of the composition of the attached oligosaccharides. In its entirety, this latter task requires characterization not just of the sequence of the glycosyl residues present but also of the positions of the glycoside bonds, the anomeric configurations (α or β), the absolute configurations (D or L), the ring forms (pyranose or furanose), and the points of attachment of any non-carbohydrate substituents (e.g. phosphate or sulfate). Consequently, glycoprotein analysis typically requires the use of several complementary biochemical and instrumental techniques including enzymatic hydrolysis, NMR, and mass spectrometry. In addition, various forms of chromatography are also routinely employed. Of these, HPAEC/PED, which was first applied to glycoprotein analysis in 1988 [75,76], has become one of the most widely used. The reason for this is that the carbohydrate separations achieved by HPAEC are quite sensitive to the molecular size,

sugar composition, and linkage mode of the species involved and are often able to resolve the mono- and oligosaccharide species present in the hydrolyzed carbohydrate chains. At the same time, the sensitivity of the PED detection is such that the carbohydrate analyses can be carried out at the picomole levels often required. Specific applications of HPAEC/PED in glycoprotein analysis have been reviewed by Spellman [77] and Townsend [78] and will not be described here. As might be expected, the first analogous CE/PED applications with glycoproteins have only recently been reported by the Susan Lunte group [61,63]. In this work, four glycopeptide fractions isolated from a tryptic digest of recombinant coagulation factor VII were analyzed by both HPAEC/PED and CE/PED with Au electrodes. While similar structural information was obtained from both techniques, the HPAEC approach proved applicable at somewhat lower concentrations. However, the CE approach gave better separations and required much less sample per run.

Finally, the first-time EC detection of two new and intriguing carbohydrate analytes has been reported. Lewinski et al. used glassy carbon electrodes modified with an oxide film of either Ru or Cu to oxidize and detect heparin, a highly sulfated glucosamine/glucuronic acid copolymer administered during surgery as an anticoagulant [79]. The heparin samples employed had molecular weights as high as 13–15 kDa and, at the Cu electrode, were detected at the 10-nM level. Singhal and Kuhr used sinusoidal voltammetry at Cu electrodes to detect underivatized nucleotides [80], oligonucleotides [81], and DNA [81]. Unlike most other DNA detection methods, the electrode reaction involved oxidation of the molecules' ribose backbone and not just the purine and pyrimidine bases and therefore was equally applicable to all nucleic acid species. In view of the fact that 10-kb segments of both single and double stranded DNA could be detected in the pM range, this approach seems to hold considerable promise for DNA analysis. It should be noted that, as of the time of this writing, both of the above detection schemes have been carried out only in flow injection format. However, in both cases, extensions involving LCEC or CEEC separations should be straightforward.

4. Conclusion

EC-based detection methods have often been slow to be embraced fully by the non-electrochemical community when more familiar optical methods are available as an alternative. To a large extent, this has been true for carbohydrates. However, it is clear that EC detection approaches have now developed to the stage where the possibilities that they offer for carbohydrate analysis are truly unique. First and foremost, EC methods generally permit direct detection of carbohydrate compounds without the need for conversion to more volatile or more highly absorbing derivatives. In addition, EC methods have been designed to provide both adequate selectivity and sensitivity to meet the challenges posed by the complex nature of carbohydrate samples of real, practical interest. And finally, the accessibility of EC methods has increased to the extent that a highly specialized background in electrochemistry is no longer required for their implementation. Some EC techniques—most notably, the glucose oxidase electrode and amperometric detection in HPLC—now represent mature analytical methods with their own well-developed areas of application for carbohydrates. At the same time, newer techniques such as CEEC continue to evolve with respect to both instrumentation and applications. Thus, it is apparent that the considerable contributions that EC detection is currently making in carbohydrate analysis in the biochemical and pharmaceutical arenas will only increase and intensify in the years to come.

References

[1] S.J. Updike, G.P. Hicks, Nature 214 (1967) 986–988.
[2] P. Pantano, W.G. Kuhr, Electroanalysis 7 (1995) 405–416.
[3] J.E. Frew, H.A.O. Hill, Anal. Chem. 59 (1987) 933A–944A.
[4] J. Wang, Analytical Electrochemistry, VCH, New York, 1994, pp. 133–145.
[5] L. Gorton, Electroanalysis 7 (1995) 23–45.
[6] L.J. Nagels, P.C. Maes, in: Z. El Rassi (Ed.), Carbohydrate Analysis, Elsevier, Amsterdam, 1995, pp. 577–606.
[7] T. Ikeda, T. Shibata, M. Senda, J. Electroanal. Chem. 261 (1989) 351–362.

[8] T. Ikeda, T. Shibata, S. Todoriki, M. Senda, H. Kinoshita, Anal. Chim. Acta 230 (1990) 75–82.
[9] D.S. Bindra, Y. Zhang, G.S. Wilson, R. Sternberg, D.R. Thevenot, D. Moatti, G. Reach, Anal. Chem. 63 (1991) 1692–1696.
[10] V. Thome-Duret, G. Reach, M.N. Gangnerau, F. Lemmonier, J.C. Klein, Y. Zhang, Y. Hu, G.S. Wilson, Anal. Chem. 68 (1996) 3822–3826.
[11] A.E.G. Cass, G. Davis, G.D. Francis, H.A.O. Hill, W. Anston, I.J. Higgins, E.V. Plotkin, L.D.L. Scott, A.P.F. Turner, Anal. Chem. 56 (1984) 667–671.
[12] B. Gregg, A. Heller, J. Phys. Chem. 95 (1991) 5970–5975; 5976–5980.
[13] A. Heller, J. Phys. Chem. 96 (1992) 3579–3587.
[14] E. Csoregi, D.W. Schmidtke, A. Heller, Anal. Chem. 67 (1995) 1240–1244.
[15] G. Kenausis, Q. Chen, A. Heller, Anal. Chem. 69 (1997) 1054–1060.
[16] J. Wang, F. Lu, J. Am. Chem. Soc. 120 (1998) 1048–1050.
[17] S. Hughes, D.C. Johnson, Anal. Chim. Acta 132 (1981) 11–22.
[18] D.C. Johnson, W.R. LaCourse, Anal. Chem. 62 (1990) 589A–597A.
[19] W.R. LaCourse, D.C. Johnson, Carbohydr. Res. 215 (1991) 159–178.
[20] D.C. Johnson, W.R. LaCourse, Electroanalysis 4 (1992) 367–380.
[21] D.C. Johnson, W.R. LaCourse, in: Z. El Rassi (Ed.), Carbohydrate Analysis, Elsevier, Amsterdam, 1995, pp. 391–429.
[22] S.V. Prabhu, R.P. Baldwin, Anal. Chem. 61 (1989) 852–856.
[23] P. Luo, S.V. Prabhu, R.P. Baldwin, Anal. Chem. 62 (1990) 752–755.
[24] P.D. Voegel, R.P. Baldwin, Am. Lab. 28 (1996) 39–45.
[25] K.G. Schick, V.G. Magearu, C.O. Huber, Clin. Chem. (Winston Salem, NC) 24 (1978) 448–450.
[26] R.E. Reim, R.M. Van Effen, Anal. Chem. 58 (1986) 3203–3207.
[27] T.R.I. Cataldi, I.G. Casella, E. Desimoni, T. Rotunno, Anal. Chim. Acta 270 (1992) 161–171.
[28] T.R.I. Cataldi, A. Guerrieri, I.G. Casella, E. Desimoni, Electroanalysis 7 (1995) 305–311.
[29] J. Wang, Z. Taha, Anal. Chem. 62 (1990) 1413–1416.
[30] J.M. Marioli, T. Kuwana, Electroanalysis 5 (1993) 11–15.
[31] P.F. Luo, T. Kuwana, Anal. Chem. 66 (1994) 2775–2782.
[32] P.F. Luo, T. Kuwana, D.K. Paul, P.M.A. Sherwood, Anal. Chem. 68 (1996) 3330–3337.
[33] J.M. Zadeii, J. Marioli, T. Kuwana, Anal. Chem. 63 (1991) 649–653.
[34] Y. Xie, C.O. Huber, Anal. Chem. 63 (1991) 1714–1719.
[35] S. Mannino, M. Rossi, S. Ratti, Electroanalysis 3 (1991) 711–714.
[36] Q. Chen, J. Wang, G. Rayson, B. Tian, Y. Lin, Anal. Chem. 65 (1993) 251–254.
[37] M.E.G. Lyons, C.A. Fitzgerald, M.R. Smyth, Analyst 119 (1994) 855–861.
[38] K. Kano, M. Torimura, Y. Esaka, M. Goto, J. Electroanal. Chem. 372 (1994) 137–143.
[39] S. Mannino, M.S. Cosio, P. Zimei, Electroanalysis 8 (1996) 353–355.
[40] K. Kewinski, Y. Hu, C.C. Griffin, J.A. Cox, Electroanalysis 9 (1997) 675–679.
[41] L.M. Santos, R.P. Baldwin, Anal. Chim. Acta 206 (1988) 85–96.
[42] J. Zhou, S.M. Lunte, Anal. Chem. 67 (1995) 13–18.
[43] T.R.I. Cataldi, E. Desimoni, G. Ricciardi, F. Lelj, Electroanalysis 7 (1995) 435–441.
[44] M.Z. Luo, R.P. Baldwin, J. Electroanal. Chem. 387 (1995) 87–94.
[45] M.B. Jensen, D.C. Johnson, Anal. Chem. 69 (1997) 1776–1781.
[46] L.A. Colon, R. Dadoo, R.N. Zare, Anal. Chem. 65 (1993) 476–481.
[47] J. Ye, R.P. Baldwin, Anal. Chem. 65 (1993) 3525–3527.
[48] T.J. O'Shea, S.M. Lunte, Anal. Chem. 66 (1994) 307–311.
[49] X. Huang, W.T. Kok, J. Chromatogr. A 707 (1995) 335–342.
[50] A.M. Fermier, M.L. Gostkowski, L.A. Colon, Anal. Chem. 68 (1996) 1661–1664.
[51] A.M. Fermier, L.A. Colon, J. High Resolut. Chromatogr. 19 (1996) 613–616.
[52] P.D. Voegel, W. Zhou, R.P. Baldwin, Anal. Chem. 69 (1997) 951–957.
[53] T.J. O'Shea, S.M. Lunte, W.R. LaCourse, Anal. Chem. 65 (1993) 948–951.
[54] W. Lu, R.M. Cassidy, Anal. Chem. 65 (1993) 2878–2881.
[55] J. Ye, R.P. Baldwin, J. Chromatogr. A 687 (1994) 141–148.
[56] R.E. Roberts, D.C. Johnson, Electroanalysis 7 (1995) 1015–1019.
[57] W.R. LaCourse, G.S. Owens, Electrophoresis 17 (1996) 310–318.
[58] J. Hong, R.P. Baldwin, J. Cap. Elec. 4 (1997) 65–71.
[59] M. Zhong, S.M. Lunte, Anal. Chem. 68 (1996) 2488–2493.
[60] W. Zhou, R.P Baldwin, Electrophoresis 17 (1996) 319–324.
[61] P.L. Weber, T. Kornfelt, N.K Klausen, S.M. Lunte, Anal. Biochem. 225 (1995) 135–142.
[62] J. Zhou, S.M. Lunte, Anal. Chem. 67 (1995) 13–18.
[63] P.L. Weber, S.M. Lunte, Electrophoresis 17 (1996) 302–309.
[64] X. Fang, J. Ye, Y. Fang, Anal. Chim. Acta 329 (1996) 49–55.
[65] P.D. Voegel, R.P. Baldwin, Electroanalysis 9 (1997) 1145–1151.
[66] P.D. Voegel, R.P. Baldwin, Electrophoresis 18 (1997) 2267–2278.
[67] R.A. Wallingford, A.G. Ewing, Anal. Chem. 59 (1987) 1762–1766.

[68] X. Huang, R.N. Zare, S. Sloss, A.G. Ewing, Anal. Chem. 63 (1991) 189–192.
[69] W. Lu, R.M. Cassidy, Anal. Chem. 66 (1994) 200–204.
[70] S. Sloss, A.G. Ewing, Anal. Chem. 65 (1993) 577–581.
[71] Y.C. Lee, J. Chromatogr. A 720 (1996) 137–149.
[72] R.P. Baldwin, in: H. Shintani, J. Polonsky (Eds.), Handbook of Capillary Electrophoresis Applications, Blackie, London, 1997, pp. 617–626.
[73] R.A. Burke, M.G. Hvizd, T.R. Shockley, J. Chromatogr. B 693 (1997) 353–357.
[74] Y. Bao, T.M.J. Silva, R.L. Geurrant, A.A.M. Lima, J.W. Fox, J. Chromatogr. B 685 (1996) 105–112.
[75] M.R. Hardy, R.R. Townsend, Proc. Natl. Acad. Sci. USA 85 (1988) 3289–3293.
[76] L.-M. Chen, M.-G. Yet, M.-C. Shao, FASEB J. 2 (1988) 2819–2824.
[77] M.W. Spellman, Anal. Chem. 62 (1990) 1714–1722.
[78] R.R. Townsend, in: Z. El Rassi (Ed.), Carbohydrate Analysis, Elsevier, Amsterdam, 1995, pp. 181–209.
[79] K. Lewinski, Y. Hu, C.C. Griffin, J.A. Cox, Electroanalysis 9 (1997) 675–679.
[80] P. Singhal, W.G. Kuhr, Anal. Chem. 69 (1997) 3552–3557.
[81] P. Singhal, W.G. Kuhr, Anal. Chem. 69 (1997) 4828–4832.