The interaction between the organic dye, methylene blue and DNA has been studied by MCE with electrochemical detection. Interaction produces two different signals, one corresponding to free methylene blue and other, for the complex methylene blue–DNA. The hybridization between a ssDNA and a complementary sequence, specific to the severe acute respiratory syndrome virus, has been performed and studied in a thermoplastic olefin polymer of amorphous structure CE-microchip with an end-channel gold wire detector. Moreover, studies with a longer dsDNA, an expression vector involved in the transitory or stable expression in mammals cells, pFLAG-CMV4, has also been performed.

Keywords: DNA intercalators / Electrochemical detection / Gold end-channel detector / MCE / Topas

1 Introduction

The analysis of biopolymers such as proteins, RNA and DNA has been widely accomplished by using their interaction with different indicator molecules. The nature of the interaction may vary depending on the structures and medium conditions. Even being non-covalent, the strength is enough for allowing its determination based on the proportionality between biopolymer and ligand molecules. Electroactive molecules have been widely used as indicators in the analysis of these biopolymers. Commonly, the molecule remains electroactive after interaction. Then, separation between the free ligand and the complex ligand–biopolymer is necessary. This is the reason why this methodology has been specially developed in the field of biosensing with heterogeneous assays [1]. Once the interaction took place, washing or medium exchange allows measurement of the bound fraction. Other possibility is the use of a separation technique, such as CE. Microchips are ideal for a fast separation between both fractions. Differences in charge/radius ratio are usually notorious between the free ligand and the biomolecule.

MCE, since they were introduced by Manz et al. [2, 3], have demonstrated to be a powerful and useful (bio)analytical tool. These microsystems are characterized by high-speed, high-throughput, small sample and reagent requirements as well as integration and compactness. Comparing with the relatively mature CE, microchips can be considered as an emerging technology with potential for novel designs and new applications [4].

A sensitive detection system compatible with miniaturization and microfabrication technology is demanded. Electrochemical detection (ED) provides an alternative to bulky and expensive equipment apart from being simple and low cost [5]. Apart from conductometric [6], the amperometric mode following in- or end-channel approaches is the most common. Different in- and end-channel designs have been developed for the integration of several electrodes such as thin-films of platinum [7, 8], gold [9–11], copper [10] or carbon [12], thick-films [13, 14] as well as metal disks or wires [15–21].

In the genomic and post-genomic era, sensitive and fast DNA analysis remains a priority goal. The study of the interactions between ss or dsDNA with ligands is of paramount importance in order to analyze both as well as the hybridization phenomenon. Different labels including redox-active ones [22], metal nanoparticles [23] or enzymes [24] have been employed. Redox-active molecules that contain a planar aromatic structure that could insert itself between two adjacent base pair of DNA or interact with DNA grooves are commonly employed. In this case, previous labeling of reagents is not necessary. Here metal complexes [23], anticancer agents [22] or organic dyes [25] are used. All these kind of molecules have allowed the
indirect study of the hybridization reaction between ssDNA and the cDNA.

The interaction between ssDNA and methylene blue (MB) as ligand has been previously studied [26]. Among all possible redox-active ligands, MB is a commercial organic dye that presented an excellent electrochemical behavior. Moreover, as MB is positively charged, its interaction with DNA is easier and more rapid than for anionic ligands. Thus, MCE-ED was applied to ssDNA detection for the first time using this electroactive dye based on the electrostatic interaction between cationic MB and anionic ssDNA obtaining reproducible calibration curves. The next step, that is the goal of this work, is the study of the interaction between MB and dsDNA. As in the case of ssDNA, bibliography related to the use of indicators with the aim of detecting DNA in MCE, especially with ED is scarce. Taking into account that this detection fits properly with miniaturized devices, related studies are demanded.

2 Materials and methods

2.1 Reagents

MB, Tris and boric acid (99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). EDTA was supplied by Fluka (Buchs, Switzerland). Potassium chloride, hydrochloric and perchloric acid (70%) and ethanol were obtained from Merck (Darmstadt, Germany).

A synthetic 30-mer ssDNA with sequence 5’-CTT TTT CTT TTT GTC TTT AGG CTC TGT-3’ and the complementary (cDNA) with sequence 5’-ACA GAG CCT AAA AAG GAC AAA AAG AAA AAG-3’ were purchased from Eurogentec (Belgium). This sequence is complementary to the portion of the severe acute respiratory syndrome (SARS) genome [27] comprised between bases 29 218 and 29 247, both included. Oligonucleotide solutions were prepared in TE buffer pH 8.0 (0.1 M Tris-HCl buffer solution, 1 mM EDTA). Aliquots were prepared and stored at −20°C. Working solutions of ssDNA were prepared from aliquots in the running buffer and conserved at 4°C. A double strand of DNA pFLAG-CMV4 with 6229 base pairs and a Mw of 6 kDa was kindly provided by Dr. Sánchez-Lazo from the Group of Cellular Regulation of the University of Oviedo.

All other solutions were prepared in the running buffer and filtered through Nylon syringe filters (Cameo 30 N, 0.1 μm, 30 mm) obtained from Osmonics (Minnetonka, MN, USA).

Water was purified employing a Milli-Q plus 185 equip from Millipore (Bedford, MA). All other reagents were of analytical reagent grade.

2.2 Instrumentation

A polymeric microchip in which ED is integrated was employed. Topas (thermoplastic olefin polymer of amorphous structure) microchips were purchased from Microfluidic-ChipShop (Jena, Germany). Microchips consisted of a plate (58.5 mm × 13.5 mm) of 2 mm thickness with a 54 mm long separation channel (between the running buffer reservoir, A, and the waste/detection reservoir, B) and 9 mm long injection channel (between the sample reservoir, C, and the sample waste reservoir, D). The two channels crossed each other halfway between the sample (C) and sample waste (D) reservoirs and 4.5 mm from the separation channel buffer reservoir (A). The channels were 20 μm depth with 32 μm width at the bottom and 60 μm width at the top. A cover plate of similar dimensions but 200 μm-thick is adhered for closing the channel at the top. Holes of 2 mm diameter that act as reservoirs were situated on the 2 mm-thick plate at the end of the channels. Micropipette tips were cut for obtaining 0.5 cm long pieces with a diameter of 0.3 cm at the top. They were adhered concentrically to the chip holes with Araldit (Vantico AG, Basel, Switzerland) forming reservoirs of approximately 100 μL volume. A methacrylate holder (18 cm × 13 cm × 2 cm) was fabricated for accommodating the chips. Once inserted the chip on the holder, a small rectangular piece (5 cm × 1 cm × 0.8 cm) was fixed with screws with the aim of securing the chip.

The amperometric detector was situated in the waste reservoir (B) with a three-electrode configuration and was previously described [26]. Briefly, the reference (Ag/AgCl/ saturated KCl) and counter (Pt wire) electrodes were coupled in a 230 μL micropipette tip that was introduced in the detection reservoir for performing the measurements. A gold wire (Alfa Aesar, Germany) with 100 μm diameter was used as working electrode with an end-channel configuration. It was manually aligned at the outlet of the separation channel after removing with the aid of a cutter a little portion of the cover plate. This leaves free a part of the separation channel that after widening acts as a guide channel for the wire. Alignment is made with the aid of adhesive tape and a microscope (Swift Optics, USA). Final fixation with epoxy resin (Araldit) and adhesive tape is made. In this way, only the gold disk in front of the outlet of the channel is in contact with the solution meanwhile the rest of the gold wire, since it is covered with the epoxy resin and adhesive tape, is not in contact with the solution decreasing the capacitive current and noise.

The microchip was joined to a methacrylate block consisting of two pieces (see scheme of Fig. 1). In the one that contacts the microchip, a circular cavity (5 mm diameter and 2 mm deep), that includes the initial 2 mm diameter hole, was practiced in order to make the final detection reservoir. All the pieces were maintained together with Araldit. Finally, a copper cable was fixed to gold wire with a conducting silver epoxy resin (CW2400, RS Components, UK) for electrical connection. Amperometric detection was performed with an Autolab PGSTAT 10 (ECO Chemie, Netherlands) potentiostat interfaced to a Pentium Celeron, 333 MHz, 64 MB RAM computer system and controlled by Autolab GPES software version 4.9 for Windows 98.
2.3 Electrophoresis procedure

Capillary zone electrophoresis separations were carried out in uncoated channels using two high-voltage power supplies (HVPS, MJ series) with a maximum voltage of +5000 V from Glassman High Voltage (High Bridge, NJ, USA). They were interfaced to a Pentium Celeron, 333 MHz, 64 MB RAM computer system and monitored by a DT300 Series Board, DT Measure Foundry 4.0.6 software for Windows 98. High-voltage electrodes consisting of 0.3 mm diameter, 1 cm long platinum wires (Goodfellow, UK) were inserted into each of the reservoirs (except in reservoir B) and connected by means of crocodile clips to the high-voltage power supplies.

Prior to electrophoresis, Topas microchips were rinsed with 0.1 M HClO4 and running buffer for 15 and 10 min, respectively. Washing was made with the aid of a simple vacuum system and reservoirs were filled with the running buffer solution. The microchip was fixed in its holder and a Faraday cage was used for housing it in order to minimize electrical interferences. After baseline stabilization, reservoir C was filled with the sample solution and injections were performed by applying the desired voltage at C (usually +1500 V for 5 s) with B grounded. Separation was performed by applying the corresponding voltage (usually +2000 V) to the running buffer reservoir (A) with the detection reservoir (B) grounded. Then, the detection potential was applied and the electropherogram was recorded. All experiments were performed at room temperature.

Safety considerations: High-voltage power supplies should be handled with extreme care to avoid electrical shock.

3. Results and discussion

The interaction between DNA and MB was performed employing conditions previously optimized. A microchip made of Topas, a polymeric material with promising characteristics, was employed. Easy integration of ED is made through the inclusion of a gold wire (100 µm diameter) at the end of the separation channel [26]. Among several indicators checked, MB was chosen because it presents a well-defined reversible redox process with a potential of −0.171 V versus Ag/AgCl in TBE (50 mM Tris-boric acid pH 7.0, 1 mM EDTA). The amperometric detection of MB implies a cathodic process. Thus, after performing the corresponding hydrodynamic voltamogram, an optimal potential of −0.3 V has been chosen. The solution is introduced in the sample reservoir and an unpinched injection at +1500 V for 5 s and a separation at +2000 V are performed for recording electropherograms. When MB interacts with ssDNA in solution and the mixture is injected in a MCE-ED device, two different signals are obtained, one for free MB and other (less reproducible) for the complex MB-ssDNA, around 35 and 90 s respectively, as can be seen in Fig. 2B. Determination of ssDNA is based on the decrease of the free MB signal (Fig. 2A). Interaction is now checked with dsDNA through hybridization assays. A 30-mer single strand (ssDNA, with a sequence that is specific from the SARS virus) reacts with the complementary one (cDNA) producing a double strand. Two different methodologies for the hybridization were checked. In the first case (Fig. 3A) both strands are mixed together with the electroactive intercalator. The mixture is performed off-chip in an Eppendorf tube by adding the single strands on an MB solution. After mixing, the solution is deposited on the sample reservoir for performing successive injections in the separation channel. Electropherograms are recorded with time in order to follow hybridization and MB interactions at real time. In the second methodology (Fig. 3B) time is given for previous hybridization (15–30 min) and then the solution is added to a MB solution. The final mixture is deposited on the sample reservoir for recording the electropherogram corresponding to the MB interaction.

Results are shown in Fig. 4. With the aim of comparison, signals corresponding to the interactions with single strands: MB-ssDNA and MB-cDNA (in both cases a mixture
between a 50 μM MB solution with a 10 ng/μL ssDNA solution were also recorded. In all the cases two peaks were obtained in the electropherograms, one corresponding to free MB (approximately at a migration time of 35 s) and other that stands for the complex MB-DNA (at around 90s). Figure 4 shows the decrease obtained in the first one (free MB) when compared with that of a 50 μM MB solution injected previously. A decrease for MB-ssDNA and MB-cDNA of a 16 and 24%, respectively, is obtained. The higher value obtained after adding cDNA is attributed to the higher number of guanines (6 instead of the 4 that are present in ssDNA). It has been reported that MB interacts preferently with guanines from single strands of DNA [28]. Therefore, the higher the number of this base, the higher the amount of MB that associates with strands of DNA and in turn, the higher the decrease in the signal of free MB. In the case of the hybridization between single strands (5 ng/μL ssDNA and 5 ng/μL cDNA), the decrease is slightly higher, indicating that more MB is involved. It seems that there is no difference between both modes of hybridization methodology (29.6 and 28.6% when hybridization occurs before or simultaneously to MB interaction, respectively).

After injecting a DNA solution (ssDNA, cDNA or dsDNA) MB was injected and the signal increases again. The relative standard deviation between MB signals recorded after DNA injections is 4.9%.

Differences seem to be more notorious for the signal corresponding to the complex MB–DNA. A similar signal is obtained for ssDNA (−2.3 nA) and cDNA (−1.8 nA). When hybridization takes place (dsDNA) signals are lower and differences can be seen from the two methodologies. A higher signal is obtained when the hybridization takes place before interaction with MB (data not shown). In this case more interaction can occur with the double strand.

Figure 3. Scheme for the MB–DNA interaction when it occurs simultaneously (A) or after (B) hybridization.
In order to ascertain the causes for these differences, interaction with a dsDNA is checked. An expression vector involved in the transient or stable expression in mammalian cells, pFLAG-CMV4, is employed as a model. A 50 μM solution of MB is situated in the injection reservoir, and after applying the injection voltage and introduced in the separation channel, the corresponding electropherogram is recorded. Then, a 5 ng/μL of dsDNA is added and let to react. The ligand-biomolecule occurs then off-channel by adding DNA to the dye. This mixing order, employed before, was determined to affect the distribution of dye molecules on DNA fragments [29]. Moreover, in this way, a signal of MB before interaction with dsDNA can be obtained. The resulting mixture of dsDNA, MB and dsDNA–MB complex was injected on the separation channel at different times until 120 min. Similar to what happens for a ssDNA, there is a slight decrease in the signal for free MB and a second signal, supposedly caused by the complex MB-dsDNA appears. However, clear differences between ssDNA and dsDNA can be observed in Fig. 5 where electropherograms recorded for interactions at different times (up to 120 min) are shown. In Fig. 5A (MB-ssDNA) it can be seen that there is not an observable change in the migration time with time, for either the first or the second signal. The current of the first peak maintains similar and can decrease slightly for the second. However, in the case of MB–dsDNA (Fig. 5B), the second signal increases with time and there is a significant decrease on the migration time. The interaction of MB with ssDNA is supposedly electrostatic in nature and an interaction of the dye with guanine bases is reported as well [28]. In the case of dsDNA, intercalation of the aromatic ring of MB between bases can occur apart from electrostatic-binding. In fact, MB intercalation is employed in several applications, resulting better than ruthenium complexes [30]. Therefore, in the variation of the process corresponding to MB–dsDNA, two different phenomena can be observed. The first relating to a decrease in the migration time can be due to electrophoretic causes (charge/size ratio or channel modification). On the other hand, an odd shape appears with interaction time. This can be due to capacitive phenomena in the electrodic double layer. DNA can be adsorbed on the electrode surface, and MB can be adsorbed as well when DNA is present. In this way, the electrode area decreases. Thus, the capacitive current, that is proportional to this area, decreases too. This is more marked when MB–dsDNA arrives to the electrode producing a positive peak. When reduction of MB associated with dsDNA occurs, a cleaning of the electrode occurs and the base line (and then the capacitive current) returns to its original value (Fig. 5B).

The decrease of the migration time (s) for the second signal (related with the charge/ratio of the complex) is almost linear with the interaction time up to 90 min (slope of −0.44 s/min, r = 0.996, n = 6) where a plateau is reached.
Similarly the peak current increases up to 90 min. Both
effects are shown in the graphs of Fig. 6. However, both
parameters remain constant for the first signal that corre-
sponds to free MB. This can be indicative of a primary
interaction between MB and DNA that stands for the
decrease in the first signal and a reorganization with time
for the complex MB–dsDNA.

4 Concluding remarks

Studies on the interaction between MB and dsDNA have
been performed employing an MCE-ED device. Hybridiza-
tion between a single stranded 30-mer sequence (ssDNA)
corresponding to the SARS virus and the complementary
(cDNA) is performed. Two different signals, one corre-
sponding to free MB and the second for the complex
MB-DNA appears in the electropherograms. A decrease in
the signal of free MB is always obtained after adding ssDNA
and seems to be related to the number of guanines. Interac-
tion with MB has been checked when it occurred simultane-
ously or after hybridization. No significant
differences were found and then the interaction between
MB and a dsDNA, pFLAG-CMV4, was checked. In
this case, the second signal increases with interaction time
and there is a significant decrease on the migration time,
which can be due to a reorganization of MB–dsDNA
complex. Work is in progress for sensitivity enhancement
of the ED and achievement of the separation between ss and
dsDNA. The integration of the hybridization reactions on
microchip as well as real sample analysis is going to be also
studied.

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