Parkinson's disease (PD) is a frequent degenerative disorder that is diagnosed based on clinical symptoms. When the first symptoms appear, more than 70% of the dopaminergic cells are already lost. Therefore, it is of utmost importance to have reliable biomarkers to diagnose much earlier PD. In this context, alpha-synuclein (aSyn) is a protein of high interest because of its tendency to form oligomers and amyloid fibrils. The oligomeric forms seem to play a critical pathological role in PD. To date, most of studies aiming at detecting and quantifying aSyn oligomers were performed by immunoassays, mainly by ELISA using specific antibodies. In this study a capillary gel electrophoresis (CGE) coupled with fluorescence detection method was developed to detect and quantify the oligomeric forms of aSyn formed in vitro. All the results obtained were supported by SDS–PAGE analysis, a widely used and well-known technique but exhibiting a main drawback since it is not an automated technique. The repeatability and the intermediate precision of the method were evaluated, as well as the stability of the labeled and non-labeled aSyn samples. After careful screening and optimization of various labeling reagents, 4-fluoro-7-nitrobenzofurazan (NBD-F) was selected and used to establish a calibration curve with monomeric fluorescently-labeled aSyn. Finally, the method was used to study the effect of doxycycline on the oligomerization process. Altogether, our results show that CGE is a very promising automated technique to analyze aSyn monomers, as well as small oligomers.

**Keywords:** aSyn / CGE / Labeling / SDS–PAGE

**DOI** 10.1002/elps.201800224

Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

Parkinson’s disease (PD) is a frequent movement disorder, the second most common adult-onset degenerative disease of the central nervous system. Initially, the disease was described by James Parkinson in the “Essay on the shaking palsy” (1817), in which he reported the major motor symptoms of PD, that are the hallmarks of the disease: bradykinesia, rigidity and tremor [1]. In the absence of validated diagnostic biomarker, PD diagnosis remains clinical but this approach suffers from a lack of specificity to discriminate patients with PD from normal aging, people with non-parkinsonian tremor, or those presenting with a parkinsonian syndrome not caused by PD. Because of the overlapping clinical features, the differential diagnosis between these conditions is difficult on clinical grounds, especially in the early stages. Another important drawback of the clinical diagnosis is that 50% of the dopaminergic neurons are already lost when the first symptoms appear. Even if PD is the center of much attention to find one or multiple biomarkers (BMs), no validated marker is available until now. In the context of PD, this would be of tremendous importance for different reasons. First, one or more reliable BMs would provide an earlier and more accurate diagnosis of the disease, leading to a better and earlier treatment, even before the emergence of the first symptoms. Second, BMs would be very useful
in clinical trials, as they would allow one to test the efficacy of different treatments. Third, they would also be helpful to monitor objectively the disease progression [2].

Synucleinopathies, including PD, are mainly characterized by the accumulation and the deposition of alpha-synuclein (αSyn) protein into amyloid fibrils. These deposits form inclusions called Lewy Bodies are present mainly in neurons and glial cells. αSyn is a small acidic protein, constituted of 140 amino acid residues. αSyn, together with beta- and gamma-synuclein, constitute the family of synucleins. αSyn is physiologically expressed at high level in the central nervous system under a monomeric form, but it can also be detected in the periphery. The physiological function of αSyn remains unclear, but it may play a role in neuronal plasticity [3] and in normal SNARE-complex assembly [4]. The primary structure of αSyn is characterized by three distinct regions, the amphiphatic N-terminal region (1-60), the central NAC region (61-95) and the acidic C-terminal region (96-140). The NAC region is extremely hydrophobic and it forms the core of the deposited fibrils. The aggregation pathway of αSyn into amyloid fibrils is complex and involves the formation of various species including oligomers, protofibrils and fibrils. In vitro and in vivo studies support the hypothesis that prefibrillar oligomers constitute the toxic species of αSyn [5,6]. Therefore, intensive researches are currently performed to characterize the properties of these species (structure, toxicity, …) at the molecular level and, to detect and quantify them in vitro or in peripheral fluids.

Total αSyn levels measurements are based on immunoassay platforms, and the most widely applied ELISA method allows the quantification of total αSyn levels, with a detection limit of down to 1 pg/mL [2,7]. Few studies quantifying specifically the oligomeric species of αSyn in cerebrospinal fluid (CSF) and peripheral fluids were also performed [8–15]. Nevertheless, all across these studies, differences in the measured levels are likely to be related to different analytical procedures, from sample collection to sample processing [16], which clearly demonstrates a real and urgent need for procedure standardization. To avoid such discrepancies in the results, a number of important peri-analytical considerations have to be taken into account when quantifying this protein in different biofluids [17,18]. These considerations correspond to variables which are important to take into account during a biomarker study in PD (based on immunoassay), and more especially for the quantification of total αSyn protein, like the addition of detergent, the aliquot volume, the temperature of sample collection, etc.

ELISA and other immunoassay techniques are well known and very sensitive, but several factors can affect the signal, thus causing bias in biomarker measurements [2]. These factors include the interference of endogenous peroxidase and heterophilic antibodies, a lack of standardization for the reference peptides, or the competition of monomers for the detection of oligomers during in vivo or in vitro assays [7,19–21]. Moreover, variations in the signal intensity can be observed depending on the detected species, as ELISA gives higher signal for the oligomeric forms compared to the monomeric forms. The development of another methodology taking into account the challenges described above would provide a complementary approach to quantify αSyn and its different oligomeric forms.

Over the last decades, SDS–CGE, also called CGE has become a powerful technique for protein analyses. Nowadays CGE methods are used daily in biopharmaceutical industries to support analytical characterization, process development and quality control of therapeutic recombinant monoclonal antibodies (mAbs). This method is based on the use of different sieving matrices as background electrolytes [22]. SDS–PAGE has been used for over four decades [23,24] and it is still commonly used for protein separation in many laboratories. This technique suffers, however, from a lack of repeatability, probably because of many manual operations (sample loading, staining …). It is also a time-consuming and labor-intensive technique. CE technology encompasses these issues by bringing advantages like an enhanced precision, the use of smaller quantities of reagent and an on-line quantitative detection by UV or by LIF detection for high sensitivity. Moreover, CGE has a high resolving power and high throughput. It also provides automated operation allowing a significant gain of time for the analyst, while avoiding the bias and errors linked to the different steps relative to SDS–PAGE technique. Using CGE it is also possible to have a more accurate protein quantification [25] and precise molecular weight (MW) determination [26–31]. Moreover the CE system can be coupled with different kinds of detectors, including the laser-induced fluorescence (LIF) detector that is particularly interesting in terms of sensitivity and selectivity [32–36].

The goal of this study was to follow a molecule of biomedical interest. To achieve this goal, a CE method coupled to LIF was developed to separate and quantify the different oligomeric species of αSyn protein formed in vitro. For this purpose, the reliability of the analytical system had to be checked, so repeatability and intermediate precision were evaluated. Importantly, the stability of an αSyn solutions was also investigated. It is worth noting that the robustness of the CE method was already demonstrated [37–39]. But in the context of this study devoted to the analysis of potentially unstable molecules having a biomedical interest, it was important to demonstrate that the conditions were appropriate. Then, different labeling agents were screened in order to obtain sensitive LIF detection, and the labeling process was optimized by testing different derivatizing agent/protein ratios, among other parameters. All the results obtained by CE–LIF were compared to those acquired by SDS–PAGE using the same samples. Since the developed CE–LIF method is able to separate the different species of the protein, it also allows to calculate the oligomeric/total form species ratio. Finally, this method was applied to study the impact of compounds on the oligomerization process. As doxycycline is described to decrease the number of αSyn oligomers, the effect of this compound was evaluated using the optimized CE–LIF method.
2 Material and methods

2.1 Chemicals and reagents

Ultrapure water was supplied by a Milli-Q equipment (Millipore, Bedford, MA, USA) and Chromasolv syringe filters (0.20 μm) were obtained from Macherey-Nagel (Duren, Germany). Dimethylsulfoxide (DMSO), NH₄OH, borate buffer, sodium acetate and acetic acid were purchased from VWR (Leuven, Belgium). PBS was obtained from Lonza (Basel, Switzerland) and corresponds to a pH 7.4 phosphate buffer (9.5 mM). A pH 8.0 borate buffer was prepared by using chemicals from Merck (Darmstadt, Germany). 4-fluoro-7-nitrobenzofurazan (NBD-F), NHS-Fluorescein, Alexa Fluor 488 (NHS-ester) and ATTO-TAG CBQCA were purchased from Thermo Fisher Scientific (Waltham, USA). Chromeo P503 was from Santa Cruz Biotechnologies (Heidelberg, Germany), and fluorescein isothiocyanate isomer I (FITC) was from Merck (Darmstadt, Belgium). Doxycycline was obtained from Sigma Aldrich (Saint-Louis, MO, USA). Recombinant human aSyn was produced in E. coli and purified as described previously [40]. It was produced and purified at the Center for Protein Engineering (CIP, University of Liege, Belgium) and stored at –80°C under its lyophilized form.

2.2 CGE

The experiments were conducted using a G7100 CE system (Agilent Technologies, Waldbronn, Germany), equipped with an autosampler and a temperature control system. In order to use the CE-4-p mode (Capillary Electrophoresis mode with usage of higher pressure), an external pressure provided by an oil-free air supply system was added. The CGE method conditions described below were adapted from Nunnally et al. [37], for the equipment used. The detection was carried out using an online diode array detector and a laser-induced fluorescence (LIF) detector. UV detection was set at 200 and 214 nm. The LIF detector was purchased from Picometrics Technology (Labège, France), and is composed of laser diodes with a continuous constant-amplitude output (or CW, continuous wave). It was used at an excitation wavelength of 488 nm. Bare fused-silica capillaries with an internal diameter of 50 μm were obtained from Optronis (Kehl, Germany). Capillaries of 33 cm total length (8.5 and 20.5 cm effective length for UV and LIF detection, respectively) were used. Experiments were performed using the outlet injection mode unless otherwise stated. Samples were injected hydrodynamically by applying a pressure of -100 mbar during 100 s. After injection, a voltage ramp was generated during 1.6 min to reach the voltage of 16.5 kV in a normal polarity mode. During the migration (run of 30 min), a two bar pressure was applied to both inlet and outlet vials in order to avoid foam formation, and the capillary was thermostated at a temperature of 25°C. The background electrolyte (BGE, also called SDS Gel Buffer) used for all analyses was purchased from Beckman Coulter (Brea, CA, USA). Once a day, capillaries were conditioned as follows: high pressure flush at 2 bar with 0.1 M NaOH for 10 min, with 0.1 M HCl for 5 min and with water for 2 min, and high pressure flush at 4 bar with SDS Gel Buffer for 10 min. Prior to each run, capillaries were conditioned as follows: high pressure flush at 4 bar with 0.1 M NaOH for 3 min, with 0.1 M HCl for 1 min, with water for 1 min and with SDS Gel Buffer for 10 min. Under these running conditions, a MW mix composed of proteins with known sizes was injected at the beginning of each sequence. A MW calibration curve was constructed, and a logarithmic regression with a determination coefficient of >0.99 (r²) was obtained.

Prior to CGE analysis, all samples were directly transferred in polypropylene microvials, without the addition of any reducing agent, meaning they are analyzed by CGE under non-reducing conditions. Also, at the beginning of each sequence, a QC is injected to guarantee the reliability of the followed results.

2.3 SDS–PAGE

SDS–PAGE was used in parallel with the CGE separation method. NuPAGE® Bis-Tris 12% gel and Mark 12, used as MW marker, were provided by Thermo Fisher Scientific (Waltham, MA, USA). The protein migration took place in MOPS buffer (Thermo Fisher Scientific), and the electrophoretic profiles were revealed using SYPRO® Ruby protein gel stain, purchased from Thermo Fisher Scientific.

The fluorescence was detected with an Amersham Imager 600 RGB (GE Healthcare, Chicago, IL, USA), containing CCD optics from Fujifilm (Tokyo, Japan). The data were processed with ImageQuant TL Software by using the 1D gel analysis module, where a lane in the image represents each sample. The analysis assesses the amounts of separated components from the integrated intensity of bands, and the volume quantitation (integrated intensity) of the bands is calculated from the area under the average lane profile and the width of the lane. The method uses a line spanning the width of a gel lane to generate a profile from the average signal at each row of pixels perpendicular to the line. Prior to SDS–PAGE analysis, only NuPAGE LDS sample buffer (4X) was used. Ultrapure water was added if it was necessary to adjust the final concentration of aSyn at 10 μM.

2.4 aSyn stability in solution

After production, the identity of the protein was confirmed by Q-TOF mass spectrometry before the experiments performed by CGE, with a spectrum with an absorption maximum at 280 nm. Three different media were tested to evaluate the aSyn stability: a 0.16% NH₄OH solution, ultrapure water, and PBS. The aSyn solutions were prepared at a concentration of 2 mg/mL (140 μM) in Protein LoBind microcentrifuge tubes (Eppendorf, Hamburg, Germany) and left without agitation at room temperature. Aliquots were taken at different times: 4–16–28–40–216 h, and they were analyzed by CGE–UV.
To evaluate the effect of freeze-thaw cycles in 0.16% NH₄OH solution, a 2 mg/mL aSyn solution was tested over five cycles. Aliquots were then analyzed by CGE–UV and SDS–PAGE.

### 2.5 aSyn derivatization

After careful screening and optimization of various labeling reagents (Chromeo P503, NBD-F, NHS-Fluorescein, Alexa Fluor 488 (NHS-ester), ATTO-TAG CBQCA and FITC (Supporting Information Fig. 1)), NBD-F was chosen as labeling agent. A stock solution of 10 mM NBD-F in DMSO was prepared, aliquoted in amber microcentrifuge tubes (Eppendorf, Hamburg, Germany) to protect it from light, and stored at –80°C. All the reactions were performed under inactinic light in amber microcentrifuge tubes. The labeling process consisted of the addition of a 10 mM NBD-F solution and aSyn into a borate buffer (50 mM, adjusted at pH 8.0) at a ratio of 15:1 (NBD-F:aSyn), corresponding to a final concentration of 150 µM and 10 µM of NBD-F and aSyn, respectively. The solution was then heated at 30°C during 90 min under gentle agitation.

### 2.6 aSyn oligomerization

The impact of doxycycline on aSyn oligomerization was investigated. A 20 mM stock solution of doxycycline was prepared in ultrapure water. aSyn was incubated at a concentration of 5 µM in a 500 µM doxycycline solution (prepared in borate buffer), so that the aSyn:doxycycline ratio was 1:100.

### 3 Results and discussion

#### 3.1 CGE versus SDS–PAGE performance

SDS–CGE requires a sieving matrix as BGE to separate the proteins according to their size, and a commercial gel from Beckman Coulter was used for this purpose. The repeatability and the intermediate precision of the CGE method were evaluated in terms of corrected peak area and migration time RSDs obtained following the injection of a reference mixture of nine proteins markers having a wide range of MW (from 10 to 225 kDa) (Table 1). The area of each protein was corrected by the area of the 10 kDa protein. As shown in this Table, the migration times of the proteins in CGE were found to be very stable (RSDs < 0.1%). The same protein mixture was also analyzed by SDS–PAGE using SYPRO® Ruby staining, and the volume of each band was evaluated (Table 1). A better method repeatability was observed in CGE–UV (RSDs for corrected peak area from 0.4 to 2.6%) compared to SDS–PAGE (RSDs for band volume from 3.1 to 10.5%).

Regarding the intermediate precision of the CGE method, low RSD values were obtained for corrected peak areas (between 0.4 and 2.8%) and for migration times (between 0.4 and 0.6%) (Supporting Information Fig. 1). These results indicate a good repeatability and intermediate precision for the CGE method therefore based on a well characterized method, it can be applied on our protein of interest.

#### 3.2 aSyn stability in solution

The stability of a protein is an important parameter to be considered. It is particularly true for aSyn, as it is capable of self-assembly under certain conditions. Different factors can affect the stability, and the solution in which the protein is dissolved is an important one. In a previous study on amyloid β1-42-peptides, researchers found that the presence of 0.16% NH₄OH dramatically slowed down the oligomerization process [41]. As aSyn is also an amyloid protein intrinsically unfolded, we tested a 0.16% NH₄OH solution as well as ultrapure water, PBS and a pH 4.0 acetate buffer. The latter has been described to promote the oligomerization process [42]. The samples were analyzed by CGE–UV, and for comparison, the percentage of the monomeric form regarding the total amount of the protein was considered. Figure 1 reports the evolution of this percentage as a function of time. After 12 h, incubation into 0.16% NH₄OH solution,

### Table 1. (A) Evaluation of the method repeatability with CGE–UV and SDS–PAGE. (B) Evaluation of intermediate precision with CGE–UV. The areas of the CGE–UV for each protein was corrected by the area of the 10 kDa protein

<table>
<thead>
<tr>
<th>%</th>
<th>14.4 kDa</th>
<th>20 kDa</th>
<th>35 kDa</th>
<th>50 kDa</th>
<th>66.6 kDa</th>
<th>100 kDa</th>
<th>150 kDa</th>
<th>225 kDa</th>
</tr>
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<tbody>
<tr>
<td>(A) REPEATABILITY (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Volume (SDS–PAGE)</td>
<td>4.0</td>
<td>3.1</td>
<td>4.7</td>
<td>9.2</td>
<td>6.5</td>
<td>5.9</td>
<td>8.7</td>
<td>10.5</td>
</tr>
<tr>
<td>Corrected area (CGE–UV)</td>
<td>0.4</td>
<td>0.4</td>
<td>1.1</td>
<td>0.6</td>
<td>0.7</td>
<td>1.1</td>
<td>2.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Migration time (CGE–UV)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
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<tr>
<td>(B) INTERMEDIATE PRECISION (n = 3; k = 3)</td>
<td></td>
<td></td>
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<tr>
<td>Corrected area (CGE–UV)</td>
<td>0.4</td>
<td>1.1</td>
<td>2.4</td>
<td>2.1</td>
<td>1.5</td>
<td>2.7</td>
<td>2.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Migration time (CGE–UV)</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.5</td>
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</table>
ultrapure water and PBS, more than 95% of aSyn was monomeric indicating a good stability; instead in pH 4.0 acetate buffer only 82% of the protein was still monomeric. After 216 h, 88% of the protein remained monomeric in 0.16% NH₄OH solution, 80% in ultrapure water and 74% in PBS. Only 48% remained monomeric in pH 4.0 acetate buffer, which is in good agreement with the potential of this solution to promote aggregation [42]. As aSyn in 0.16% NH₄OH showed the highest stability over time, this solution was used for further experiments. Supporting Information Fig. 2 represents the overlay of the electropherograms in the different tested media, at T₀ (Supporting Information Fig. 2A), and after 9 days (216 h, Supporting Information Fig. 2B). At T₀, the only peak observed corresponds to the MW of aSyn monomer. Whatever the medium composition, the peak is sharp, without any degradation or oligomerization product, corresponding to 100% of the monomeric form. These peaks were compared to those obtained with samples after 216 hours of incubation (Supporting Information Fig. 2B). As shown in this figure, degradation products, having a lower MW, migrate faster than the monomeric peak, in all tested solutions. The electropherograms observed in the Supporting Information Fig. 2B had a range of migration time from 5.5 to 9.0 min for acidic conditions, corresponding to molecular sizes from 9.2 to 82 kDa. As can be seen in the electropherogram, the species observed directly after the monomeric peak could be composed of fragments plus monomer and/or oligomeric species of aSyn. Those products were observed in ultrapure water and especially in pH 4.0 acetate buffer, giving rise to a large peak that probably includes different MW species. A stock solution of aSyn in 0.16% NH₄OH was frozen at −80°C to avoid a too fast degradation of the protein. The stability of the protein after five freeze-thaw cycles was tested in CGE–UV and SDS–PAGE (data not shown). No significant difference in monomeric form abundance was observed using both approaches, even after five cycles.

### 3.3 Optimization of aSyn derivatization

Since aSyn has no tryptophan residue its intrinsic fluorescence is low; and furthermore the amino acid sequence is composed of 4 tyrosine, corresponding to 2.9% in terms of its entire sequence. The native fluorescence of aSyn was evaluated (at 266 nm), but the sensitivity was not sufficient to follow the oligomerization products. That is why an increased sensitivity was needed, and to do this the derivatization of the protein by the use of a labeling agent was considered in order to enable the use of LIF detection and achieve better sensitivity compared to UV detection. More than 10% of aSyn are lysine residues (15 out of 140 amino acids), corresponding to 15 primary amines. Therefore, different amine-reactive labeling agents were considered: FITC, Chromo P503, NHS-fluorescein, Alexa Fluor 488 (NHS ester), ATTO- TAG CBQCA and NBD-F. All these reagents were chosen because they have an excitation wavelength compatible with the use of a 488 nm laser.

Preliminary experiments showed that NBD-F gave the best compromise between sensitivity and background noise (Supporting Information Table 1). This compound is a fluorogenic labeling agent that reacts with primary and secondary amines in alkaline medium (pH around 8 and 9) [43,44]. The detection limit with NBD-F was previously found to be of 25 ng/mL for BSA with a labeling ratio of 1:150 (BSA:NBD-F), which is much lower than with other detection techniques.
such as Coomassie blue staining (2500 ng/mL) and UV–visible detection in SDS–CGE (500 ng/mL) [45]. It is worth mentioning that an appropriate buffer has to be used to provide a high labeling yield while avoiding labeling reagent hydrolysis [42]. Therefore, the composition of the reaction buffer had to be optimized for each tested labeling agent. Moreover, the concentration ratio of the labeling agent to the protein had also to be carefully optimized, since a too large excess of the reagent can lead to a high background noise and interferences during the separation.

In this study, as the goal was to obtain the best sensitivity, and since the peaks efficiency and the background noise values were similar for the different tests performed, only the peak areas were taken into consideration. In a first step, three buffers were tested, with different pH values: bicarbonate buffer (100 mM, pH 8.3), phosphate buffer (20 mM, pH 7.5), and borate buffer (50 mM, pH 7.5–8.5–9–9.2, see Supporting Information Fig. 3B). The borate buffer adjusted at pH 8.0 was found to be the most appropriate, as it gave the highest peak areas compared to the other buffers.

Different incubation times were tested, namely 30, 60, 90, 120 and 150 min. Compared to other conditions, the incubation time of 90 min gave higher peak areas. (Supporting Information Fig. 3C), so an incubation of 90 min was chosen. The effect of the temperature of the labeling reaction was also evaluated at room temperature, 30°C or 60°C, and compared to other conditions, the temperature of 30°C gave higher peaks areas (Supporting Information Fig. 3A), which was therefore selected. In conclusion, the labeling reaction with NBD-F had to be performed in borate buffer (50 mM, pH 8.0), during an incubation time of 90 min, at a temperature of 30°C and under 600 rpm agitation. Figure 2A illustrates the electropherograms obtained after the labeling reaction in the optimized conditions, compared with a blank. Two well-separated peaks corresponding to the free labeling agent and labeled αSyn are mainly observed in the sample solution. Small peaks present before the main peak correspond to degradation products, and the small peak observed after the peak corresponds to a dimeric form, which are often observed in all conditions. The presence of degradation and dimeric forms are also observed when samples are analyzed by SDS–PAGE. The NBD-F:αSyn ratio being also a critical parameter, different ratios from 5:1 to 25:1 were tested. Figure 3 represents the results obtained (n = 3) by comparing the peak areas of the monomeric αSyn at the different ratios. The peak area obtained at a ratio of 5:1 was much lower than those found at the other ratios, probably because of an incomplete labeling reaction due to a too low quantity of reagent. Over labelling a protein can cause quenching due to dye to dye interactions, but it can also decrease solubility leading to precipitation and to a quenching of the fluorescent signal [46]. Here, the best results in term of peak area of labeled αSyn were obtained at a 15:1 ratio.

3.4 Stability of NBD-F labeled αSyn

After the selection of NBD-F as labeling agent, the stability of the labeled samples was evaluated by taking into account the peak areas. Labeled αSyn has to be stable over time, to allow its analysis in a sequence of multiple samples.

A sample of 5 μM labeled αSyn was analyzed by CGE–LIF over a 20 h period. Six injections of the same sample
were made during this period (approximately one injection every 3 h). The values were distributed around the average, and the peak area RSD value for monomeric aSyn was found to be 6.6%. Moreover, the bias between the first and the last injection was 10%, which indicates a satisfactory stability of the labeled aSyn during at least 20 h.

### 3.5 Labeled aSyn quantitation

aSyn has been shown to be present in healthy control groups as a normal CSF constituent in the low ng/mL concentration range (from 27 to 32 pM) [16]. It is also found in blood (including plasma and serum) under physiological conditions at a level up to tenfold higher than in CSF, and in whole blood lysates at a level up to 10,000-fold higher [16, 47]. This large amount in whole blood is due to the fact that aSyn is highly expressed in the hematopoietic system [48]. aSyn was also found in neuronal synapses at a physiological concentration of about 0.7 mg/mL [49].

A calibration curve from 0.5 to 50 μM of labeled aSyn was carried out using the optimized CGE–LIF method. To perform this calibration curve, independent triplicates at each concentration were prepared and analyzed. A determination coefficient ($r^2$) > 0.99 was obtained. A RSD value of 20% was obtained at the lowest concentration (0.5 μM), whereas it was <10% at the other concentration levels.

As mentioned in Section 1, the low MW aSyn oligomeric species seem to play an important role during the development of PD. To date, only few ELISA studies [8–15] reported measurements of aSyn oligomers. Nevertheless, some factors can affect the signal obtained using this technique, resulting in variations in protein measurements. Furthermore, depending on the oligomers studied, the answer will not be the same, so for a specific oligomeric form, ELISA requires aSyn standards having the same oligomeric state, which is very unlikely.

The next step of this study consisted of evaluating the ability of the optimized CGE–LIF method to monitor the presence of low MW oligomers individually. A solution made up of different small oligomeric species was analyzed by CGE–LIF and SDS–PAGE (Figure 2B and C). The electropherogram (Figure 2B) shows that besides the monomeric aSyn (1X), other smaller peaks corresponding to oligomeric species, from dimers (2X) to hexamers (6X) are present. Their MW was assessed by the mix of MW standards. SDS–PAGE (Figure 2C) analysis was also performed on the same samples and several bands corresponding to the monomeric species and the oligomeric forms were observed. A S/N ratio of 16 was calculated for the oligomeric species having the lowest S/N for comparable background noise (corresponding to the hexameric form), indicating that the different species from monomeric could be quantified after method validation. These are very promising results, showing the potential of the CGE–LIF method to separate the different species present in a sample. Since no standard of oligomeric species exists, relative quantification was performed taking into account the normalized ratio of the oligomeric species level to the total forms aSyn concentration.

In view of the perspectives offered by this method, an interesting application is to monitor the potential effect of some compounds on the oligomerization of aSyn. More precisely, this CGE–LIF method could be used to evaluate inducers or inhibitors compounds influencing the oligomerization process. With this aim in view, a preliminary experiment was carried out by incubating a solution of aSyn with doxycycline and analyzing it by CGE–LIF after different incubation times, from 0 to 96 h. The normalized oligomers/total forms ratios were calculated, taking into account the ratios at T0 and at a given time. Figure 4 shows the normalized ratios obtained for the samples with and without doxycycline, at 0, 24, 48 and 96 h. In the presence of doxycycline, a lower ratio was observed at each incubation time, compared to the control solution. These observations indicate a decrease of the concentration of low MW aSyn oligomeric species while the monomeric
species concentration remains rather stable. These results are in agreement with those obtained by González-Lizárraga et al. [50], who observed in the presence of doxycycline a reshape of αSyn oligomers into off-pathway and high MW species that do not evolve into fibrils. These species present a less hydrophobic surface than the on-pathway oligomers, and they were found to reshape towards non-toxic parallel beta-sheet structured forms [50]. This experiment demonstrates the great interest of the developed CGE–LIF method regarding the study of αSyn behavior in the presence of potentially therapeutic agents.

4 Concluding remarks

In this paper, we described the development of a fast CGE–LIF method to monitor different αSyn species (i.e. monomer and oligomers). The use of a well-known technique such as SDS–PAGE helped us to support the results obtained by CGE. The developed method presents advantages over SDS–PAGE: it is an automated method, giving qualitative and quantitative data about αSyn species, which is very interesting since specific oligomeric species are toxic. The repeatability and the intermediate precision were evaluated based on a reference mix of proteins, having a wide range of MW from 10 to 225 kDa. Migration time RSDs <0.6% and peak areas RSDs <2.8% were observed. Nowadays, the oligomerization process is an important challenge to take up, and a better understanding of such a mechanism would be a great help, even if there is still a lot to do in that field. By following the levels of different αSyn species, or by using concentration ratios (oligomers to total forms), this innovative CGE–LIF method could be a useful tool to evaluate different potentially therapeutic agents, or to study different factors influencing the oligomerization process in vitro. Preliminary experiments were made with doxycycline, showing the potentiality of the CGE–LIF method.

M.-P. Merville is Senior Research Associate at the National Fund for Scientific Research (F.R.S.-FNRS), Belgium. This work was financially supported by the University of Liège and the Wallonia Region.

The authors have declared no conflict of interest.

5 References

