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Electrophoresis of megaDalton proteins inside colloidal silica

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Abstract

With the growth of the biopharmaceutical industry, there is a need for rapid size-analysis of proteins on the megaDalton scale. The large pore sizes needed for such separations cannot be easily reached by pushing the current limits of size-exclusion chromatography or gel electrophoresis. The concept detailed here is the formation of arbitrarily wide pores by packing nonporous colloidal silica in capillaries. This method can be called packed-capillary electrophoresis, or “pCE”. Electrophoresis of protein standards (11–155 kDa) by pCE, using 345 nm diameter particles in 100 μ m diameter capillaries, gives 2x higher resolution than a typical PAGE gel in 1/6 of the time. The electropherograms show that pCE is highly efficient, with half-micrometer plate heights for all seven standards, giving 10^5 plates for a 50 mm length. The large pore radius of 65 nm enables baseline resolution of proteins of 0.72, 1.048 and 1.236 MDa in less than 15 min. The short separation time of pCE is attributed to the absence of small pores that restrict protein migration in gels. The pCE separation is applied to the analysis of a stressed pharmaceutical-grade IgG4 sample, giving unprecedented baseline resolution of monomer, dimer, trimer and tetramer in less than 10 min.

Keywords

Aggregation; Complexes; IgG; MDa; Separation

1 Introduction

The growth in the use of proteins in the pharmaceutical industry, especially monoclonal antibodies, relies on a battery of tools to characterize such large proteins [1,2]. Heterogeneity of these proteins in amino acid sequence, post-translational modification and structure needs to be understood. Aggregation of therapeutic monoclonal antibodies is a concern due to the potential of size variants to induce an immune response [3]. Size-exclusion chromatography is currently the method of choice for characterizing aggregation, but it is unable to baseline resolve aggregates larger than dimers [4]. Analytical

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ultracentrifugation is often used to characterize larger aggregates [5], but long analysis times inhibit routine use. For high-throughput screening of new formulations to minimize aggregation, neither size-exclusion chromatography nor ultracentrifugation have sufficient throughput. Alternatively, one might consider gel electrophoresis, with its multiple lanes, but this is offset by its slower speed. Capillary gel electrophoresis, with replaceable gel media, is a potential alternative, but commercial systems indicate that 250 kDa is the size limit and resolution of aggregated species is minimal. Gels with gradients from low to high cross-linking (e.g., 3 to 12%) now allow for electrophoretic separations of native, megaDalton proteins, albeit with separation times of hours [6,7].

Silica colloidal crystals have been demonstrated to sieve proteins by virtue of the nanoscale interstitial dimensions [8–10]. These have the potential of solving the problems of limited protein size and low separation speed. To illustrate, sieving in gels is described using Ogston's model of accessible volume in random fibers [11], depicted in Fig. 1A [12]. Pore size increases with lower number density of polymer fibers, but the gel then becomes more fragile. This instability limits the sizes of proteins that can be analyzed. Another problem is the wide distribution of pore sizes, where the small pores transiently trap proteins to significantly slow separation speed. Figure 1B depicts the face-centered cubic packing of silica colloidal spheres, giving interstitial pores of uniform size for a perfectly crystalline medium. The pore sizes can be made arbitrarily large without sacrificing the ruggedness of the material. Even without perfect crystalline order, there is a much narrower pore size distribution, which enables high separation speed. The design is also simple and rational: Bird-sall et al. showed that a protein with radius of gyration of r will have an electrophoretic mobility, μ , that is determined by just two properties of the material that govern the accessible free volume: its porosity, ϵ , and its interstitial hydrodynamic radius, R [8].

$$\frac{\mu}{\mu_0} = \epsilon \cdot \left(\frac{R-r}{R} \right)^2 \quad (1)$$

The parameter μ_0 is the electrophoretic mobility in an open capillary. The hydrodynamic radius inside the medium is controlled by the choice of particle diameter, d_p .

$$R = \frac{d_p}{3} \cdot \frac{\epsilon}{1 - \epsilon} \quad (2)$$

Since the porosity range for packed spherical particles is typically 0.26 to 0.40, choice of particle diameter largely determines the molecular weight range of the proteins for which the material is applicable.

The purpose of this work is to investigate the use of packed colloidal silica for the capillary electrophoresis of large proteins up to and beyond 1 MDa. Previous studies were limited to molecular weights of <70 kDa and much shorter separation distances [8] of 5 mm or less, whereas in this work, capillaries 10-fold longer and proteins 20-fold larger are used. These

advances enable application to analysis of a therapeutic monoclonal IgG4 that is prone to aggregation.

2 Materials and methods

2.1 Silica

Uniform non-porous silica spheres of nominally 350 nm were purchased from Fiber Optic Center, Inc. (New Bedford, MA). The particles were calcined at 600°C for 12 h in a furnace (Thermo Fisher Lindberg/Blue M) to drive out volatile reagents remaining after the sol-gel reaction [13]. The particles were then annealed at 1050°C in the same furnace for 3 h [14], rehydroxylated by sonicating in boiling 1 M nitric acid for 24 h, and rinsed with ultrapure water. Rinsing was repeated by alternately suspending in deionized water and then centrifuging until the supernatant was neutral. The particles were dried in a 60°C vacuum oven. The average diameter after treatment was determined by scanning electron microscopy (Nikon Neoscope) to be 345 ± 10 nm.

2.2 Proteins

Unlabeled bovine serum albumin (BSA, 66 kDa) and unlabeled Thyroglobulin from bovine thyroid (~670 kDa) were purchased from Sigma Aldrich (St. Louis, MO). A sample of pharmaceutical-grade monoclonal antibody IgG4 was provided by Eli Lilly (Indianapolis, IN). NativeMark™ (720,1048 and 1236 kDa) unlabeled protein standards were purchased from Thermo Fisher Scientific (Waltham, MA). All of the aforementioned proteins were labeled for fluorescence detection using an Alexa Fluor™ 488 antibody labeling kit purchased from Life Technologies (Grand Island, NY). After labeling, a buffer exchange was done to transfer the proteins to the running solution without dilution. The buffer exchange used Amicon Ultra 0.5 mL centrifugal filters (MilliporeSigma, Burlington, MA). The cut-off of the spin filter used for buffer exchange of the NativeMark™ proteins removed the lower molecular weight standards, leaving the three standards of 720 kDa and above, as confirmed by PAGE. Benchmark™ protein standards (11–155 kDa), pre-labeled with Alexa Fluor™ 488, were purchased from Life Technologies (Grand Island, NY).

2.3 Capillary preparation

A 100 µm inner diameter Teflon-coated fused silica capillary (Polymicro Technologies, Phoenix, AZ) of 200 cm in length was treated with 0.1 M NaOH for 20 min, rinse with ultrapure water, and then 100% ethanol using flow by a syringe pump (Harvard Apparatus, Holliston, MA), flowing at rate of 300 µL/min. Next, the fused silica capillary was cut into 20-cm segments. The segments were dried in a 60°C vacuum oven for one hour. Slurries (35% w/w) of silica particles were prepared in ultrapure water via sonication (Cleanic Branson 2800 Ultrasonic Cleaner, Richmond, VA). Slurries were pumped by a disposable plastic syringe into the 20-cm length capillaries. The capillaries were packed against a frit at 7500 psi (545 bar) delivered by a packing pump (Series 1500 Scientific Systems Inc., State College, PA). After packing, the capillaries were stored at room temperature. Packing lengths of 4 to 5 cm were used for the pCE experiments.

2.4 Preparation of PDMS Reservoirs

Polydimethylsiloxane (PDMS) wells were prepared using 40 g of mixture of a 4:1 ratio of SYLGARD[®] silicone elastomer curing agent and base. A lab-made mold (rectangle with two circular openings) was placed on top of the mixture and allowed to gel overnight.

2.5 Packed capillary electrophoresis

For pCE experiments, two PDMS reservoirs were placed at a distance of 5 cm from one another on a 16 × 25 cm glass slide. The running solution was either 0.1 or 1% w/v sodium dodecyl sulfate (SDS), as indicated in the figure caption, and 0.1% v/v formic acid and ultrapure water (18 MΩ), with pH = 2.63. After wicking running solution for 1 min into a capillary packed with 345 nm bare silica particles, each end of the capillary was placed in a reservoir filled with running solution. Packed capillaries were conditioned at 100 V/cm using a high-voltage power supply (10A25A Series, Ultravolt, Ronkonkoma, NY) controlled by LabView until the current (typically between 5.6 and 5.9 μA) was stabilized. For injection, the capillary was removed and dipped into solution containing fluorescence-labeled proteins to diffuse into the cathode end of the capillary for 1 min. Platinum electrodes were placed in the outer PDMS reservoirs, and an electric field 100 V/cm was used in all experiments.

For detection, a Nikon Eclipse TE2000-U inverted fluorescence microscope fitted with a 2X objective (CFI Plan Apo 2X from Nikon Instruments Inc., Melville, NY) was connected to a ProEM 512 charged-coupled device camera (Princeton Instruments, Trenton, NJ). A mercury lamp (380–600 nm in wavelength, Nikon Intensilight C-HGFI, Nikon Instruments Inc., Melville, NY) was used for excitation. An excitation range of 470–490 nm and an emission range of 505–520 nm were selected with a FITC filter cube from Omega Optical (Brattleboro, VT). During CE experiments, images were acquired using the Winview 32 software package (Princeton Instruments, Trenton, NJ) and electropherograms were plotted using OriginPro (OriginLab) software.

2.6 PAGE

PAGE separations were conducted using a BIO-RAD mini-protean tetra cell (Hercules, CA). The gels consisted of 10% polyacrylamide and 3% crosslinking, 6.5 cm in length. Precision Plus protein standards, dual color (BIO-RAD, Hercules, CA) were used as the MW standards to view proteins during separation. For thyroglobulin and IgG4 sample, the gel run time was 1 hour at 190 V. To give a better separation of the larger proteins (720, 1048, 1236 kDa) in the Native Unstained protein standards, a longer gel run time (2 h) was used. Gels were then stained with Coomassie brilliant blue (Bio-Rad, Hercules, CA). Gel images were scanned with an HP Office-jet Pro 8610, and the jpg image was converted to intensity using ImageJ (National Institutes of Health).

3 Results and discussion

Figure 1C and D show electropherograms of the Benchmark[™] protein size standards for PAGE and pCE, respectively. The more familiar PAGE image is also shown to convey that this is a high quality gel. Both methods resolve the protein standards. The most extreme

molecular weights that are common to both pCE and PAGE separations are aligned in the electropherograms to compare resolution for the two methods. It is visually apparent that the resolution is higher for pCE. Comparing resolution for similar molecular weights, specifically peaks 1 and 8 for PAGE vs. peaks 1 and 7 for pCE, the resolution is calculated to be two-fold higher for pCE. The resolution between the proteins at the high end of the molecular weight scale is even greater for pCE, e.g., between 100 and 150 kDa, and this is attributed in part to a larger pore diameter of pCE. The separation times are dramatically different: 10 min for pCE and 60 min for PAGE. Some of the six-fold increase in separation speed is due to the three-fold higher electric field for pCE compared to PAGE: 100 V/cm compared to 30 V/cm, respectively. The other factor of three increase in speed is likely due to the absence of trapping pores that are inherent to the random fibers of the gel. These are absent in the packed capillary because the pores size distribution is much narrower.

The packed particles give significantly less peak broadening. This is illustrated in Fig. 2A, in which plate height is plotted as a function of molecular weight for the gel and pCE. The plate height for pCE is 0.53 μ m regardless of the molecular weight of the protein. For the gel, the plate height is already 10-fold higher for the smallest molecular weight in the plot, 20 kDa. The plate height increases with protein molecular weight at a steep slope, increasing from 25 μ m for the 20 kDa protein to 280 μ m for the 150 kDa protein. The increase in plate height is attributed to the distribution of pore sizes, where the protein is temporarily trapped in smaller pores and has to diffuse out to begin electromigrating again. Larger proteins have more of a labyrinth of smaller pores to circumnavigate, and they diffuse to the wider pores more slowly than do smaller proteins. The high efficiency of pCE also owes to the absence of smaller pores, which gives rise to higher mobilities. In support of this interpretation, the higher mobility of pCE relative to PAGE is illustrated by the data of Fig. 2B, which is a plot of log(MW) vs. electrophoretic mobility relative to that of free solution, μ/μ_0 . Note in the figure that the mobility axis for PAGE has to be multiplied by 10 to be visible on the same scale as that of pCE. The pore size distribution slows the mobility of even the smallest proteins used in PAGE, giving more than a 10x lower mobility.

The average pore radius for pCE is deliberately larger than that of PAGE to enable separation of larger proteins. The average pore radius for each medium can be determined by fitting the electrophoretic mobilities to molecular weight. For this fitting, the radius of gyration of r , of the proteins is estimated from number of amino acid residues, N , by Equation (3) [8].

$$r(A) = N^{3/5} \quad (3)$$

Using the average molecular weight of an amino acid residue of 110 g/mol, the molecular weight (MW) is converted to protein radius in nm.

$$r(nm) = \frac{(MW/110)^{3/5}}{10} \quad (4)$$

Equation (3) estimates the radius of gyration of BSA in SDS solution to be 4.0 nm, which agrees with the value of 4.0 nm measured by small angle neutron scattering for BSA in SDS solution [15].

For the gel, Rodbard and Chrambach derived the relation in Equation (3) for a protein of radius r , based its accessible free volume in the gel in Ogston's network of random fibers [16].

$$\frac{\mu}{\mu_0} = \exp(C(R + r)^2) \quad (5)$$

The unobstructed electrophoretic mobility, μ_0 , was measured for lysozyme in SDS solution in an open capillary: $\mu_0 = 3.6 \times 10^{-4} \text{ cm}^2/\text{V s}$. This was assumed to be the same for all proteins. The values of average pore radius, R , and constant, C , are empirical, fitting to $R = 21.2 \text{ nm}$ and $C = -0.0079$, respectively. This function is plotted as the dashed curve in Fig. 2B. The data fit to the equation reasonably well in light of the simplicity of ignoring molecular shape, pore shape and estimating radius of gyration.

For pCE, Equation (1) is also based on accessible free volume but it has ostensibly no empirical constants: mobility for a given molecular weight depends on porosity and pore radius. The porosity of the material was independently measured to be 0.34, as described by the method of Birdsall et al. [8], giving a hydrodynamic radius of 56 nm for the measured 345 nm diameter of the particles. Nonetheless, porosity and pore radius were used as fitting parameters since there is significant error in the particle diameter. The electrophoretic mobilities were corrected for the slight electroosmotic mobility of $4.5 \times 10^{-5} \text{ cm}^2/\text{V s}$, as measured by the method of Huang et al. [17]. The curve from fitting the data for pCE to Equation (1) using the estimated radius of gyration from Equation (4) is plotted in Fig. 2B. The data fit to the equation well, revealing a pore radius of 55 nm and a porosity of 0.35. The porosity is in good agreement with the independent estimate of 0.35, based on flow rate and the Kozeny Carmen equation. The pore size of 55 nm is similar to the hydrodynamic radius, calculated using in Equation (2) [18]. Specifically, for the particle diameter, d_p , of 345 nm and the porosity, ϵ , of 0.35, $R_{\text{hyd}} = 62 \text{ nm}$. This underscores the ability to use these equations for rational design of materials for pCE.

The extension of the curve for pCE to higher molecular weights in Fig. 2B shows that mobility would still be significant beyond 1 MDa and that the velocity would be nearly half of the maximum velocity in the medium for 1 MDa, projecting feasibility of such separations. By contrast, the curve for the gel is beginning to increase more rapidly toward high MW and the velocity is 100-fold lower than for pCE at 155 kDa. This indicates that the separation of larger proteins would begin losing resolution in addition to becoming

extremely slow. The gel was made with 10% polymer having 3% cross-linking. Larger pores can be made to increase the practical scale of molecular weights about two-fold, although the gels are more fragile and inhomogeneous, as mentioned earlier. The plot summarizes that one can expect to separate MDa proteins with pCE with much higher speed.

Calculation using the data of Fig. 2B for pCE indicates that time scale for separating megaDalton proteins for this pore size is expected to be on order of 15 min. The electropherogram obtained by pCE for proteins of 720, 1048 and 1236 kDa is given in Fig. 3A, confirming that these three proteins are indeed resolved on the time scale of 15 min. The amount of SDS was increased to 1% for reproducible results for these large proteins. The peaks are significantly wider than the small protein standards. This can either be due to protein heterogeneity or to an unrecognized broadening mechanism that comes into play for large proteins. The inset of Fig. 3A shows an image of the PAGE gel, obtained after a 2-h time. Only the 720 kDa protein leaves the stacking gel and it moves only slightly into the separation gel. The two larger proteins do not even migrate significantly into the stacking gel (5% polymer, 3% cross-linking). Again, the much higher electrophoretic mobilities of these very large proteins in pCE owes to the absence of smaller pores, and the separation is enabled by the wide pores.

An important application of technology for separating large proteins is the analysis of aggregation of therapeutic monoclonal antibodies, as discussed earlier. IgG monomers have molecular weights of 150 kDa, therefore, dimers (300 kDa), trimers (450 kDa) and tetramers (600 kDa) would fall in a range between the two sets of protein standards used here. To calibrate the material in this intermediate region, Thyroglobulin (~670 kDa) was used. Its electropherogram by pCE is shown in Fig. 3B. As with the other larger proteins, the peaks are wider for this protein. It is known to be heterogeneous due to post-translational modifications; in fact, the vendor gives a molecular weight range of 30 kDa. There are two peaks in the pCE electropherogram of Fig. 3B, and the PAGE image in Fig. 3D also shows two peaks. Thyroglobulin is a tetrameric protein consisting of monomers of 165 kDa linked by disulfide bonds. The calibration is consistent with the likelihood that the intact tetrameric protein (670 kDa) dissociates into dimers (335 kDa). Both peaks are significantly larger than the protein standard of 150 kDa, hence Thyroglobulin does not significantly dissociate into monomers. For pCE, the later peak in pCE is thus interpreted to be the intact tetrameric protein of 670 kDa, and the earlier peak is interpreted to be the dimeric fragment of 335 kDa. These fill in the gap in molecular weight between the smaller and larger protein standards.

The IgG4 sample, after being mildly temperature-stressed by storage at room temperature for a few hours, was analyzed by pCE, and the resulting electropherogram is shown in Fig. 3C. The peak detected at 5.5 min represents 98% of the total peak area, and the PAGE results in Fig. 3D show that the most intense peak lines up with the 150 kDa protein standard. This intense peak is thus interpreted to be the expected monomer peak. Three more peaks are resolved by pCE in Fig. 3C, and are denoted as aggregates. The PAGE results in Fig. 3D also show additional peaks, with the next highest MW peak of IgG4 nearly lining up with the thyroglobulin dimer of 330 kDa, hence it is interpreted as the IgG4 dimer of 300 kDa. The other peak in PAGE could be a trimer, as it is slightly lower on the gel than the

Thyroglobulin tetramer. On the other hand, pCE resolves three aggregates peaks rather than two. The missing peak in PAGE for the highest molecular weight could be due to its being too broadened for detection.

Figure 4 presents a plot of $\log(\text{MW})$ vs. normalized mobility, (μ/μ_0) , for pCE. The fit to the theoretical curve now includes the larger protein standards, Thyroglobulin and the three larger standards, but not the IgG4 species. The best fit gives the same porosity, 0.35, but it now gives a larger pore radius, 65 nm, which is in better agreement with the calculated hydrodynamic radius of 62 nm from Equation (2). This is attributed to the higher molecular weight standards more accurately indicating the pore radius due to increased sieving compared to the smaller protein standards. Figure 4 shows that the sieving from this pore size gives approximately a 50% decrease in the mobility. Smaller pores would give a greater decrease in mobility to increase resolution, although this would necessarily be at the expense of separation time. The electric field was limited to 100 V/cm due to the already high speeds of the zones; hence, one could combine the use of smaller particles with higher voltages to improve resolution without sacrificing speed.

The plot of molecular weight vs mobility in Fig. 4 also includes the IgG4 species, indicated by the filled circles. Recognizing that IgG4 tends to aggregate in multiples of 150 kDa, it is likely, a priori, that these are dimer, trimer and tetramer. This is consistent with the theoretical curve: the second peak in pCE appears to be the dimer (300 kDa) since its velocity is only slightly higher than the thyroglobulin dimer (330 kDa). Further, the third peak and fourth peaks are interpreted to be the trimer (450 kDa) and tetramer (600 kDa), respectively, since they lie close to the working curve corresponding to these molecular weights. The IgG4 tetramer (600 kDa) is similar in mobility to Thyroglobulin (670 kDa). To our knowledge, this is the first demonstration of baseline separation of these four IgG species by any separation technique.

A future goal is to couple pCE directly to electrospray-MS without SDS. In contrast to capillary gel electrophoresis, the sieving medium for pCE does not leave the capillary, thus making pCE-MS feasible in principle. Native gel electrophoresis would be needed, and to this end, linear polyacrylamide as a bonded phase on colloidal silica has been shown to negligibly adsorb proteins under aqueous conditions at neutral pH [19, 20]. With mass spectrometry, it would no longer be necessary to rely on theoretical curves, such as the one made for Fig. 4, thus providing a general tool for characterizing IgG aggregates and other noncovalent protein complexes.

4 Concluding remarks

Capillaries packed with colloidal silica of nominally 350 nm in particle diameter can be used for sieving electrophoresis to separate proteins with molecular weights exceeding 1 MDa on the time scale of 15 min. The material has a plate height of only 0.5 μm , giving sharp peaks for pure proteins. The electrophoretic mobility is described well by theory in the case of denatured proteins without disulfide bonds. A 10-fold greater length than used in previous studies enables the applicability of this type of material to the challenging separation of IgG4 aggregates. The results show baseline resolution of the monomer, dimer, trimer and

tetramer. The material can be rationally designed, projecting that larger particles would allow for another order of magnitude in size. For example, since radius of gyration goes as the $3/5$ power of molecular weight, a factor of 4 increase in particle diameter, from 350 nm to 1.4 μm , would shift the curve up one order of magnitude in molecular weight. The material has the potential of allowing coupling to mass spectrometry for native pCE-MS for a wide range of large proteins and complexes.

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Abbreviations:

IgG4	immunoglobulin 4 subclass of IgG
MW	molecular weight
pCE	packed capillary electrophoresis
PDMS	polydimethylsiloxane

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Figure 1.

Conceptual comparison of sieving media, with the empty space shown for (A) polyacrylamide gel depicted by random fibers forming random pore sizes (image is from Picu et al. [12]), and (B) ordered array of monodisperse silica spheres forming a monodisperse pore sizes. Electropherograms of protein size standards in these types of media are (C) PAGE with absorbance standards, separation time = 60 min, gel length used = 65 mm, along with image of PAGE electropherogram, aligned with plot of electropherogram, and (D) pCE with fluorescent size standard, separation time = 10 min, separation length = 50 mm. The dashed lines connect peaks for proteins of similar molecular weights between PAGE and pCE to facilitate comparison of resolution.

Figure 2.

(A) Relation between plate height and protein size. Open circles are for pCE and filled circles are for PAGE, using protein size standards for each. Both lines are least-square fits. (B) Plot of mobility in pCE, normalized by mobility in an open capillary, versus molecular weight. 0.1% SDS was used. Open circles are data points for pCE and closed circles are for PAGE, with the time axis also expanded 10x due to the very low mobility in gels. The solid curve is fit of theory to the data using pore radius, R , as the fitting parameter, giving $R = 55$ nm. The dashed curve is fit to theory for gels.

Figure 3.

Electropherograms by pCE for larger proteins. Each is plotted on the same time axis with the times aligned. (A) The MDa proteins are separated by pCE in less than 15 min. The inset shows the same proteins in PAGE. One protein is still at the top of the gel after 2 h, whereas the other two remain at the top of the stacking gel after 2 h. (B) Thyroglobulin, (C) IgG4 and (D) PAGE of thyroglobulin and IgG4, along with size standards. 1% SDS was used in all cases.

Figure 4.

Plots of MW versus relative mobility for all proteins for pCE, with 1% SDS as the running solution. Data include the smaller protein standards (11–155 kDa), larger protein standards (720, 1048 and 1236 kDa) and Thyroglobulin (670 kDa, and fragment of 335 kDa), shown as open circles (O). The solid line is a plot of the theoretical curve for the pore radius of 65 nm. Data for the IgG4 sample are shown as solid circles (•).