Journal of Pharmaceutical Sciences 110 (2021) 3541–3545

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Rapid Communication

Application of Tunable Resistive Pulse Sensing for the Quantification of Submicron Particles in Pharmaceutical Monoclonal Antibody Preparations

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ARTICLE INFO

Article history:
Received 10 March 2021
Revised 19 July 2021
Accepted 19 July 2021
Available online 22 July 2021

Key words:
Biopharmaceutical characterization
Monoclonal antibody(s)
Particle size
Protein(s)
Protein aggregation

ABSTRACT

Tunable resistive pulse sensing (TRPS, qNano Gold, IZON Ltd.) was investigated as a method to quantify submicron particles (SMPs) between 0.1 and 1 μm in solutions of biopharmaceuticals. To reduce sample dilution, a spiking-in approach was used to add the appropriate amount of electrolytes required for the measurement. For correct particle quantification, an electrolyte concentration of at least 50 mM sodium chloride was needed. Intra- and inter-nanopore variability were below 5% for size and below 10% for concentration measurements when analyzing polystyrene standard beads. Submicron particle counts in a stir stressed IgG1 monoclonal antibody formulation resulted in a non-symmetrical, almost bell-shaped size distribution with a maximum at 250 nm when using a NP300 nanopore (IZON Ltd.). It was shown that particle counts are heavily underestimated below 250 nm, and therefore it is recommended to quantify particle counts by TRPS in samples with heterogeneous particle size distributions (e.g., biopharmaceuticals) only starting from the maximum of the histogram towards the upper limit of detection.

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Introduction

Today, submicron particle (SMP) characterization and quantification in biopharmaceuticals between 0.1 and 1 μm is often recommended or requested by regulatory agencies in addition to subvisible particle (SVP) monitoring.[1] Applicable techniques for determination of SMPs include resonant mass measurement (RMM) and nanoparticle tracking analysis (NTA).[2] Recently, resistive pulse sensing (RPS) was introduced as new and orthogonal method for quantifying SMPs by two companies. Tunable resistive pulse sensing (TRPS, qNano Gold, IZON Ltd., Christchurch, New Zealand) uses a stretchable nanopore, whereas microfluidic resistive pulse sensing (MRPS, nCS1, Spectradyne LLC.) employs a microfluidic chip for particle analysis. The detection of particles by RPS relies on the Coulter counter principle, which determines the particles based on changes in conductivity when passing a capillary. Consequently, the detection depends on the ionic strength of the sample solution.[3] A dilution of the sample in phosphate buffered saline (PBS) is regularly used to overcome the lacking conductivity in samples with low ionic strength.[3–5] Accurate determination of particle sizes with high resolution, even in multimodal mixtures of three difference sizes of polystyrene beads, by using TRPS was reported allowing also qualitative comparisons of submicron particle populations.[6] Known applications of RPS include the analysis of extracellular vesicles, bacteria, viruses, nanoparticulate systems, and more.[7]

In this study, a suitable sample preparation method and measurement protocol for submicron particle counting in biopharmaceuticals by using TRPS was developed. The main focus was to avoid strong dilution of the proteinaceous sample which might further reduce a potentially low particle concentration, by utilizing a spiking-approach for introduction of electrolytes to increase conductivity. Furthermore, a suitable data evaluation method is presented for proteinaceous samples typically containing protein aggregates with a heterogeneous size distribution.[8]

Materials and methods

Materials

L-Methionine, polysorbate 80, sodium chloride (NaCl), and sucrose were purchased from Merck KGaA (Darmstadt, Germany). Dibasic and monobasic sodium phosphate, tris base and tris

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https://doi.org/10.1016/j.xphs.2021.07.012
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hydrochloride, glycine and Dulbecco's phosphate buffered saline were obtained from Sigma-Aldrich (Steinheim, Germany). Histidine monohydrochloride monohydrate was purchased from Alfa Aesar (Kandel, Germany). A monoclonal antibody (Bayer AG, Leverkusen, Germany), belonging to the IgG class 1 (referred to as mAb), in 10 mM histidine buffer pH 5.5 with 130 mM glycine, 5% sucrose, 20 mM methionine, and 0.05% polysorbate 80 was used as model protein. The identical formulation not containing the mAb was used as placebo throughout the study. Coating solution and calibration beads were purchased from IZON Ltd. (Christchurch, New Zealand). In-house highly purified water (HPW) (conductivity 0.055 μs/cm) was dispensed from an Arium®Pro purification system (Sartorius, Göttingen, Germany). All diluents used in the study were freshly filtered using a 0.02-μm Anotop 25 syringe filter (Whatman, Maidstone, UK). A 0.22 μm polyethersulfone (PES) syringe filter (VWR International GmbH, Darmstadt, Germany) was used for sterile filtration of mAb samples. A 5 μm Acrodisc® syringe filter with Supor® membrane (Pall Corporation, Port Washington, NY, USA) was used to remove large protein aggregates prior to TRPS analysis.

Conductivity measurements

Electric conductivities of samples were measured in triplicate at 20 °C by using an Inolab Cond Level 2 P conductivity meter equipped with a TetraCon 325 electrode (WTW, Weilheim, Germany) calibrated with a 100 μS/cm standard.

Preparation of polystyrene bead standards

Polystyrene (PS) standard beads with diameters of 110 nm (CPC100, $1.10*10^{13}$ particles/ml), 203 nm (CPC200, $2.17*10^{12}$ particles/ml), and 350 nm (CPC 400, Lot 1: $9.5*10^{11}$ particles/ml and Lot 2: $9.0*10^{11}$ particles/ml) were purchased from IZON Ltd. (Christchurch, New Zealand). Calibration beads CPC100 and CPC200 were diluted 1000-fold and CPC400 were diluted 10000-fold in two steps in filtered (0.02 μm) placebo solution and spiked with NaCl stock solution to a NaCl concentration of 50 mM in the sample immediately prior to analysis. To compare intra- and inter-nanopore variability of the investigated spiking-approach, the calibration beads CPC100 and CPC200 were diluted 1000-fold and CPC400 were diluted 10,000-fold in two steps in filtered (0.02 μm) PBS immediately prior to analysis.

Preparation of proteinaceous particles

A monoclonal antibody was used as model protein to generate proteinaceous particles at a concentration of 5 mg/mL. The solution was filtered by using a 0.22-μm PES syringe filter and an aliquot of 30 ml was subsequently stressed by stirring at 300 rpm for 15 min at room temperature. Prior to analysis, stir-stressed samples were filtered through a 5-μm PES membrane filter in order to remove large aggregates which could lead to blocking of the nanopore. The samples were further aliquoted (190 μl), and each aliquot was spiked with 10 μl of a 1 M 0.02-μm filtered NaCl stock solution to reach a NaCl concentration of 50 mM in the sample. Each aliquot was analyzed for SMPs immediately after NaCl addition using a NP300 nanopore.

TRPS method on IZON qNano gold

SMPs were quantified by tunable resistive pulse sensing (TRPS) on a qNano Gold system (IZON Ltd., Christchurch, New Zealand). With the TRPS instrument, particle concentration is measured in a particle-by-particle analysis and results obtained from sample measurements can be calibrated with a single-point calibration at the same measurement conditions (stretch, voltage, and pressure) using polystyrene standard beads of known size and concentration.[9] Frequency and amplitude of particles in the sample run can thereby be calibrated, which allows the calculation of particle concentration[9] and size[10] of the sample.

A nanopore NP300 with an analysis range of 150–900 nm was fitted to the qNano Gold system and a radial stretch of 47 mm was applied. A volume of 70 μl and of 35 μl of filtered (0.22 μm) coating solution was added to the lower and upper fluid cell, respectively. A pressure of +20 mbar for 30 min and -20 mbar for 15 min was applied using IZON's variable pressure unit. The coating solution was removed and both fluid cells were cleaned by rinsing with highly purified water (HPW) and blowing dry with filtered (0.22 μm) pressurized air without removing the nanopore from the qNano Gold system. A volume of 70 μl of electrolyte, in this case placebo solution spiked with a 1 M 0.02-μm filtered NaCl stock solution to reach a NaCl concentration of 50 mM, was added to the lower fluid cell. For the measurements, a volume of 35 μl of sample was added to the upper fluid cell and measurements were conducted in "monomodal" mode (single pressure setting, +10 mbar). Voltage was adjusted to reach a current of approximately 100 nA, as recommended by the manufacturer. Cleanliness of the system was checked by recording of the particle count in electrolyte (<10 particles in 10 min required). A particle read of at least 500 particles or a maximum recording time of 10 min were chosen as measurement limits for calibration beads or proteinaceous samples. The measurement was stopped when either of the limits was reached. The recording was paused when blockages occurred and the nanopore was unblocked according to the manufacturer's guidance[11] before resuming the recording.

Results and discussion

Electrolyte concentration for TRPS analysis

Different concentrations of electrolyte (20–100 mM NaCl) in HPW were used to determine the minimal electrolyte concentration required for stable measurement conditions on nanopores of different sizes (NP300 (150–900 nm), NP600 (275–1570 nm), NP1000 (490–2900 nm)). In order to keep the current constant at around 100 nA (Fig. 1A) the voltage was increased exponentially with decreasing electrolyte concentration. At low electrolyte concentrations the baseline signal became noisy and particles were no longer detectable. Larger nanopores required in general a lower electrolyte concentration than smaller nanopores. A concentration of at least 50 mM NaCl was needed using a NP300 (Fig. 1A) to reach a current of around 100 nA with a voltage below 1.0 V, as recommended by the manufacturer's user manual. TRPS therefore requires a conductivity to reach stable measurement conditions similar to microfluidic resistive pulse sensing (MRPS).[12]

Formulation buffers contribute to the sample's conductivity, and the extent was investigated by conductivity measurements and the correlation to a sodium chloride calibration curve. It was found that commonly used buffer systems in protein formulation (phosphate, tris, and histidine at 20 mM buffer salt concentration) contribute an equivalent of 5–10 mM NaCl (histidine) or 15–25 mM NaCl (phosphate, tris) to the overall electrolyte concentration (Fig. 1B). The mAb formulation, which was used for our further studies, comprised a histidine/glycine buffer with an electrolyte concentration equivalent to 7.5 mM NaCl. The low conductivity of the formulation led to the conclusion to spike a concentration of 50 mM of sodium chloride from a 1 M NaCl stock solution into the sample to facilitate measurements on a NP300. Spiking with 10 μl of a 1 M NaCl solution to a 190 μl sample (e.g. placebo or mAb) accounted for a dilution of 5.2% in comparison to dilution factors of 100- or 1000-fold in commonly used protocols.[3–5]

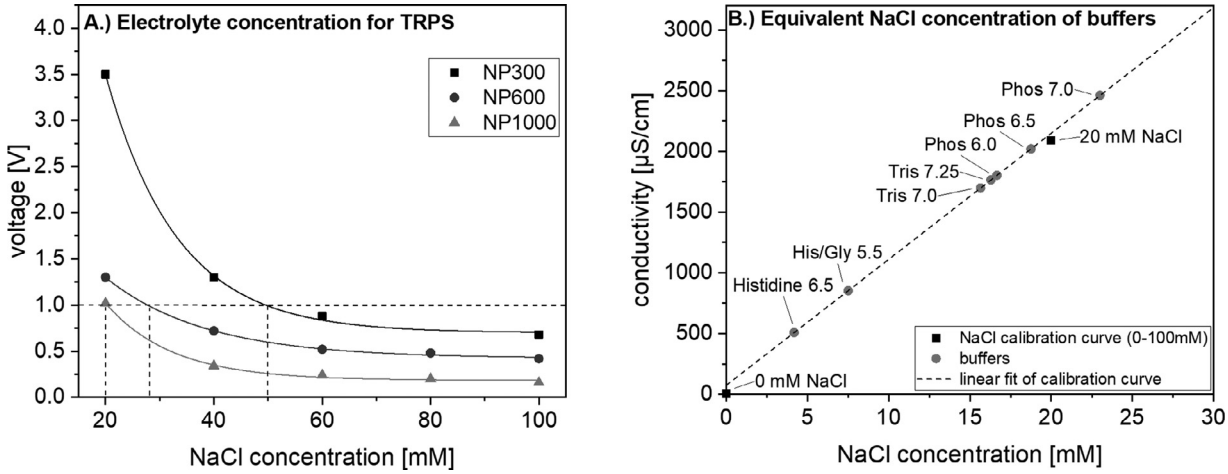

Figure 1. Determination of electrolyte concentration for TRPS measurements. A) Applied voltage to reach 100 nA current on different nanopores in dependence of the sodium chloride concentration. B) Conductivity and equivalent NaCl concentration of different formulation buffers. A sodium chloride concentration of at least 50 mM is required to stay below a voltage of 1 V on a NP300 nanopore. Formulation buffers, at a concentration of 20 mM, contribute to the conductivity at an equivalent sodium chloride concentration between 3 and 25 mM NaCl.

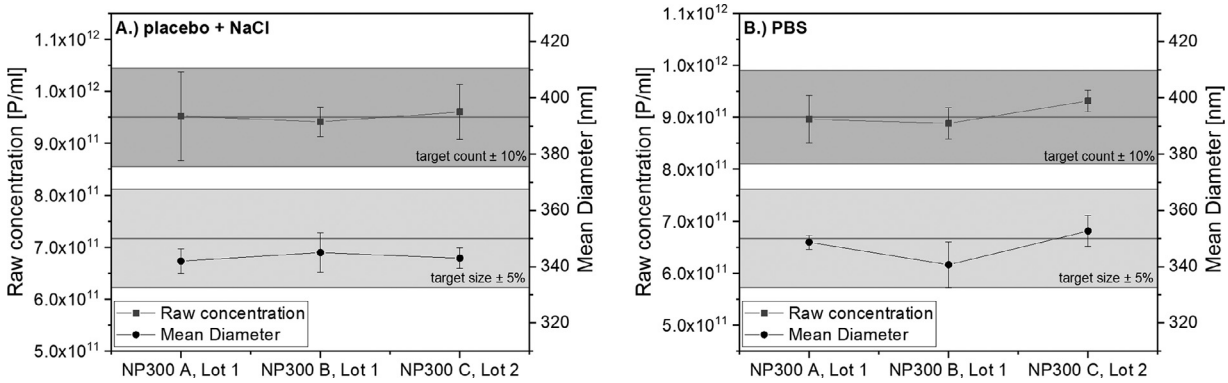

Figure 2. Intra- and Inter-nanopore variability measured in A.) placebo + NaCl, B.) PBS. Mean ± standard deviation (n=3) shown for size and concentration of a polystyrene standard beads (350 nm; placebo + NaCl 9.5*1011 Particles/ml; PBS: 9.0*1011 Particles/ml) determined with three different nanopores. Target count ± 10% marked as dark grey area. Target size ± 5% marked as light grey area. Dilution of polystyrene beads in placebo spiked with NaCl resulted in similar intra- and inter-nanopore variability compared to dilution in PBS.

Intra- and inter-nanopore variability with spiking-in approach

Counting and sizing precision was verified for the new sample preparation method by using polystyrene reference beads (CPC400) dispersed in placebo + 50 mM NaCl or in PBS. Particle concentration and size were measured in triplicate on three different NP300s at 47 mM stretch and a current of around 100 nA. Determination of particle concentration (Fig. 2) was accurate with 100.1 ± 5.6% and 100.6 ± 3.9% relative concentration using the spiking approach or dilution in PBS, respectively. Size was determined with an accuracy of 98.1 ± 1.3% and 99.2 ± 2.1% in placebo + NaCl and PBS, respectively. Intra-nanopore and inter-nanopore variability for size were in the range of ±5% and for concentration in the range of ±10% of the expected values for the spiking approach (Fig. 2A) and for dilution in PBS (Fig. 2B).

Data evaluation for submicron particle concentration in protein samples

Prior to submicron particle analysis using TRPS, large protein aggregates were removed through a 5 μm filtration, as they were identified as a main cause of blockages of the nanopore and therefore instable measurement conditions (Supplementary Fig. 1S). Particle concentration < 1 μm was not impacted by this filtration step, indicated by identical particle rates of SMPs in the range 200–900 nm in samples with and without 5 μm filtration when no blockages occurred. On the contrary, particles were prevented from passing the nanopore when regular blockages occurred. Without filtration, the probability of blockages was very likely, with blockages occurring every few seconds. After 5 μm filtration, measurement durations of several minutes were possible without blockages. In addition, particle concentrations ≥ 1 μm are typically determined using other well established techniques such as light obscuration and flow imaging.[13]

Submicron particle concentration as obtained on a NP300 nanopore for a stir-stressed sample after 5 μm filtration (Fig. 3A) indicate a non-symmetrical, almost bell-shaped distribution with a maximum around 250 nm. A decrease in particle concentration below 250 nm is considered not plausible since, based on a 10 nm mAb monomer, aggregates are formed over a wide size range from small to large sizes rather than forming a distinct particle population.[14–16] We hypothesize that particle concentration is underestimated at the lower size limit of the nanopore since the particles get too small in relation to the orifice, resulting in a weak signal. Consequently, many

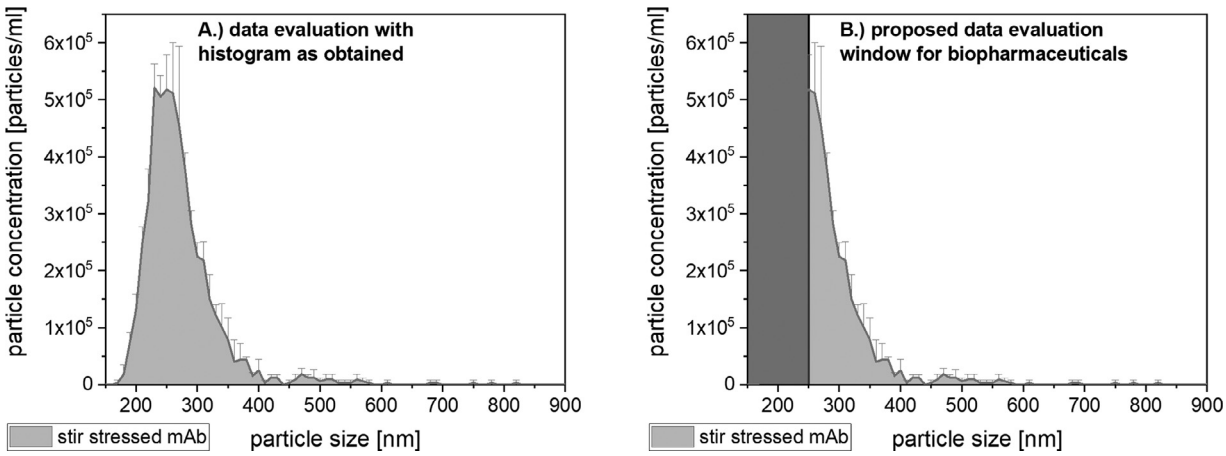

Figure 3. A) Particle size distribution histogram as obtained on a NP300 nanopore at 47 mm stretch for stir-stressed mAb. B) Proposed way of data evaluation for a sample with heterogeneous particle size distribution (e.g. aggregated IgG). The dark grey area is omitted from analysis due to underestimation of the particle concentration. Particle concentration is recommended to be reported from maximum of the histogram onwards.

Table 1
Determination of measurement range on an NP300 nanopore at 47 mm stretch using polystyrene beads of different sizes. Actual concentration is shown as mean ± standard deviation (n = 3) and relative concentration was calculated using the nominal particle concentration of the polystyrene standard. No size cutoff was applied for data analysis. Substantial underestimation of the particle concentration observed for CPC200 beads, which are close to the proposed lower quantification limit, whereas nominal concentration was determined for larger polystyrene beads (CPC400).

Polystyrene beads	Nominal size of the standard beads [nm]	Nominal Concentration [particles/ml]	Actual concentration measured [particles/ml]	Relative concentration [%]
CPC100	110	$1.10*10^{13}$	$4.12*10^{10} \pm 5.19*10^{10}$	0.4 ± 0.5
CPC200	203	$2.17*10^{12}$	$1.27*10^{12} \pm 1.33*10^{11}$	58.7 ± 6.1
CPC400	350	$9.50*10^{11}$	$9.13*10^{11} \pm 3.64*10^{10}$	96.1 ± 3.8

smaller particles remain unrecognized by the detection algorithm resulting in an underestimation of the particle concentration. A quantification of submicron particles is therefore suggested in the range from 250 nm onwards.

This hypothesis was confirmed by determining the particle concentration of count standards with different sizes below (CPC100), around (CPC200) and above (CPC400) the proposed measurement range for the used instrument setup (Table 1). Particles with a size below the measurement range were not detected, whereas particles with a size above the measurement range were counted in their nominal concentration. Particle concentration was underestimated by 40% for particles with a size around the lower limit of the measurement range of the given instrument setup. Therefore, we propose to evaluate particle concentration in samples containing a heterogeneous particle size distribution (e.g., stressed protein samples) by accumulating particle counts starting at the maximum of the histogram and towards larger sizes. Consequently, underestimation of the particle concentration for particles smaller than the maximum is avoided (Fig. 3B).

A significant increase in SMPs was observed even after a short duration of exposure to stirring stress by using the described way of data evaluation. Particle concentration increased from $1.4 \pm 0.26*10^6$ to $3.3 \pm 0.35*10^6$ particles/ml larger than 250nm through stirring for 15 min at 300 rpm. TRPS was thereby shown to be a suitable technique to reliably detect and quantify SMPs in proteinaceous samples.

Conclusion and outlook

The conductivity of commonly used buffering agents at a concentration of 20 mM was found to be not sufficient for submicron particle analysis using TRPS. The addition of electrolytes via dilution in PBS to overcome the lack in conductivity is an approach regularly reported in literature.[3–5] To avoid substantial dilution of the proteinaceous sample, which may further reduce potentially low particle concentrations, a spiking-approach to introduce electrolytes to increase conductivity was investigated in this study. Changing the sample preparation protocol from dilution in PBS to spiking-in the appropriate concentration of electrolytes from a concentrated stock solution of NaCl to low ionic-strength samples offered the benefit of minor sample dilution without affecting the data quality obtained in submicron particle counting and sizing by TRPS. The ionic strength of a 50 mM NaCl solution was sufficient for TRPS measurements with a nanopore size of NP300 or larger, thereby covering the particle size range from 0.15 to 2 μm. Intra- and inter-nanopore variability was good with <5% deviation for sizing accuracy and <10% deviation for counting accuracy on three different nanopores, which can be considered remarkably low for counting in the nanometer size range.[17] The spiking-in approach resulted in an accuracy of 98.1 ± 1.3% for size and 100.1 ± 5.6% for concentration determination, compared to the target size and concentration of polystyrene standard beads. The spiking-in approach offers the advantage of a minor dilution factor of 5.2% when spiking with NaCl stock solution to a NaCl concentration of 50 mM in the sample without compromising data accuracy compared to a dilution in PBS (e.g. 1000-fold).[3]

TRPS was successfully applied to proteinaceous samples, for which an increase in submicron particle counts was detected after a short duration of stirring stress. Data analysis for proteinaceous samples is proposed to be conducted by integrating the obtained histograms from the maximum towards the upper limit of the measurement range. An underestimation of the particle count at particle sizes lower than the peak maximum was proven, adversely affecting the data quality, if the particle concentration across the whole size range is reported. A similar approach as suggested in this study for the data evaluation of TRPS results was performed during data analysis of

MRPS (nCS1, Spectradyne LLC.) measurements of proteinaceous samples.[12,18] Particles below the detection threshold were similarly excluded from the reported particle concentration resulting in similar particle size distributions compared to TRPS (when using the proposed cutoff).[12,18] Therefore, both resistive pulse sensing technologies are capable of quantifying the submicron particle concentration in proteinaceous samples and report particle concentration of heterogeneous samples (e.g. stressed protein samples) in a comparable way.

This study presents TRPS as a promising technology for submicron particle analysis in biopharmaceuticals. Further studies will investigate the comparability of TRPS to other submicron particle counting techniques used for SMP analysis in biopharmaceutical formulations and investigate the impact of the sample handling procedure on submicron particle populations.

Acknowledgements

The support from the Formulation Development Parenterals group within the Bayer AG is kindly acknowledged.

Sources of funding: Andreas Stelzl is a PhD student at the LMU, financially supported by Bayer AG, Pharmaceuticals, Formulation Development Parenterals.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.xphs.2021.07.012.

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