Analytica Chimica Acta 690 (2011) 190–198

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

A new biosynthetic tracer for the inline measurement of virus retention in membrane processes: Part I – Synthesis protocol

L. Soussan*, C. Guigui, S. Alfenore, S. Mathe, C. Cabassud

Université de Toulouse, INSA, LISBP, 135 Avenue de Rangueil, INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, CNRS, UMR5504, F-31400 Toulouse, France

ARTICLE INFO

Article history:
Received 9 July 2010
Received in revised form 28 January 2011
Accepted 8 February 2011
Available online 18 February 2011

Keywords:
Virus retention
Biosynthetic tracer
MS2 bacteriophage
Probes
Enzymatic assay
Chromatography

ABSTRACT

In this study, a new biosynthetic tracer was developed to characterize the virus retention dynamics of membrane systems. This new tracer is a modified bacteriophage obtained by the grafting of enzymatic probes to an MS2 bacteriophage, one of the smallest non-pathogenic bacteria viruses, with an average diameter of about 30 nm. A protocol for the synthesis and purification of this new tracer was developed in this work. The production of this biosynthetic tracer was first qualitatively shown by a chromatographic characterization and an enzymatic test. The average number of probes grafted per phage was then quantified for three batches of tracers made from the same native phage suspension and the same batch of enzymatic probes. This quantification demonstrated the reproducibility of the synthesis protocol developed.

1. Introduction

Membrane molecular weight cut-offs are currently assessed by small size tracers, which are polymers or proteins (such as PEG, i.e. polyethylene glycol, or dextran) and are determined for 90% retention of these compounds. The properties of these tracers differ from those of viruses in terms of size, shape or density. They are thus not suitable to mimic the filtration behaviour of viruses and consequently they cannot satisfactorily characterize the retention dynamics of filtration systems during filtration. It was therefore necessary to develop a non-pathogenic tracer as representative as possible of viruses, allowing detection/quantification that is fast, continuous, applicable inline, compatible with large volumes of water produced and not too expensive.

Bacteriophages (also known as phages) are non-pathogenic viruses of bacteria. They are commonly used as reference microorganisms to quantify the virus retention of membrane systems [1–3] as they are very similar to the viral pathogens carried by water from the viewpoint of size, shape and surface properties. However, existing methods for bacteriophage quantification (such as direct enumeration by plate counting, epifluorescence microscopy on filtering membrane [4,5], flow cytometry [6], quantitative PCR [7], or biosensors [8–12]) are not sufficiently rapid to characterize retention during filtration and/or not applicable inline and/or analyse volumes that are too small for the sample to be representative. In addition, their to cost is sometimes high.

In the literature, other surrogates for viral pathogens have also been tested as tracers, such as gold nanoparticles detected by potentiometry [13] or iron oxide nanoparticles detected magnetically [14–16]. Although gold and iron oxide nanoparticles have similar sizes to native viruses, they are denser and have significantly smoother surfaces than viruses, which makes these nanoparticles inappropriate to mimic viruses in terms of their behaviour during filtration. Fluorescent microspheres detected by fluorimetry have also been considered [17] but appear to be poor virus surrogates, probably due to differences in surface charge density that could result in electrostatic interactions, adsorption or adhesion with the membranes.

In the same way as biosensors (suggesting the surface modification of germs so that they can be collected and detected), modified bacteriophages have also been considered as tracers [18,19]. One of the proposed modifications was notably the grafting of fluorochromes on to the surface of MS2 bacteriophages to make the modified bacteriophages directly detectable by fluorimetry. However, this relevant approach remains limited by low analysis volumes (about 1 mL), which could lead to problems of statistical analysis. Moreover, no quantification of the average number of fluorescent dyes grafted per phage has been performed yet, although it is necessary to check that these modified bacteriophages have

* Corresponding author at: Université de Toulouse, INSA, LISBP, 135 Avenue de Rangueil, F-31077 Toulouse, France. Tel.: +33 5 61 55 97 90.
E-mail address: Laurence.Soussan@hotmail.fr (L. Soussan).

0003-2670/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2011.02.022

been purified of the excess dyes if the tracers are to be used in filtration. The labelling of enzymes on the surface of T4 bacteriophages has also been considered. In this particular case, the major drawbacks of the approach lie, first, in the large size of the T4 phage (about 200 nm long and 80 nm wide, being one of the largest bacteriophages [20]), which limits the monitoring of the retention dynamics of ultrafiltration systems; secondly, in the associated detection method (ECL: Electro-ChimiLuminescence) which allows only very small analysable volumes (about 50 μL) and, finally, in a lack of tracer characterization (the average number of enzymes grafted per phage and the enzymatic activity of the grafted enzymes have not been determined yet).

Considering the literature and the objectives, the bio-artificial tracer that appeared the most relevant was an MS2 bacteriophage modified on its surface by the grafting of enzymes. The MS2 phage was chosen as it is non-pathogenic, has a spherical capsid structure and, above all, is small (among the smallest viruses [21]). HRP (HorseRadish Peroxidase) enzymes were chosen for the grafting because of their high enzymatic activity (that generates greater signals than conventional chromogenic or fluorigenic probes) and their small size. The HRP enzymes catalyze a highly specific and rapid reaction, permitting its detection, and could be operated in drinking water without inhibition by the molecules present in solution [22,23]. This tracer was thus built to enable direct detection of its induced enzymatic activity, notably by an amperometric method that is fast, continuous, applicable inline and compatible with large volumes of samples [24].

In this study, a method for tracer synthesis and purification was developed (Part I). The grafting protocol and chromatographic purification steps are described in this work, together with the tracer chromatographic characterization. A quantitative characterization protocol, based on enzymatic assays, was also developed and used to investigate the average number of enzymatic probes grafted per phage for three batches of tracers made from the same native phage suspension and the same batch of enzymatic probes.

2. Materials and methods

2.1. Bacteriophages, strains and culture media

MS2 bacteriophages (15597-B1) were obtained from the American Type Culture Collection (ATCC, Molsheim, France), received as freeze-dried pellets. Non-pathogenic *Escherichia coli* K12 (*E. coli* K12) were obtained from the LISBP Laboratory (Toulouse, France). Different media were used for phage cultivation and enumeration. Hard and soft agar media and broth medium were prepared with trypticase 10.0 g L^{-1} (from BD, 211921), Bacto agar 15.0 g L^{-1} for hard agar and 5.0 g L^{-1} for soft agar (from Biokar, A1010 HA), yeast extract 1.0 g L^{-1} (from Biokar, A1202HA), glucose 1.0 g L^{-1} (from Prolabo, 06C170020), calcium chloride dihydrate $CaCl_2, 2H_2O$ 0.3 g L^{-1} (from Prolabo, 23317.297) and sodium chloride 8.0 g L^{-1} (from Riedel, 31414). Neutral phosphate buffer solution (PBS) 0.1 M, pH = 7.0 ± 0.1 was provided by Perbio Science France.

2.2. Preparation of a native MS2 phage suspension

A pure, concentrated and enumerated suspension of phages was required for tracer synthesis. Phage cultivation was achieved by a double-layer plaque assay method provided by the ATCC [25,26] using *E. coli* bacteria as hosts. This reference method was modified slightly to obtain a more highly concentrated phage suspension. In particular, a 1:10 ratio of phages to fresh host bacteria cultivation (reseeded just before phage cultivation) was applied in a non-nutrient soft agar, which resulted in total lysis, to produce large quantities of viruses. After 8 h of incubation at 37 °C, the overlayers containing the infected bacterial cells were scraped with an inoculation loop and collected into centrifuge glass tubes with a minimum of neutral PBS buffer. The amplified phage suspension was then mixed with chloroform to remove bacterial fragments and/or to release phages contained in host bacteria. Chloroform was thus added to each tube at 10% v/v, without damage to the phages [25]. The tubes were then centrifuged at 9000 rpm for 20 min at 4 °C in a Sorvall RC-5B refrigerated superspeed centrifuge. Subsequently, supernatants containing phages were filtered through a sterile 0.2-μm filter and collected in a sterile glass container. Chloroform extraction and filtration were repeated twice. The phage suspension was then stored in its neutral PBS solution, in the dark, at 4 °C in a sterile glass container to limit protein adsorption. These storage conditions were chosen according to the literature [27] to minimize capsid deterioration in the long term.

The enumeration of the phage suspension obtained was carried out by a double-layer plaque assay method proposed by ISO standard reference 10 705-1 [28], using *E. coli* K12 bacteria as hosts. Samples of the phage suspension obtained were diluted by decades until lysis plaques were countable, each lysis plaque corresponding to one phage. Once the dilution range was determined (minimum 5-log), plates were replicated at least five times for each chosen dilution and negative control plates were made. The concentration of the phage suspension was calculated as the average of the lysis plaque number divided by the spotted volumes, taking the corresponding dilution factors into account.

2.3. Chemicals for labelling

The chemicals involved in the tracer preparation (EZ-Link Sulfo-NHS-LC-Biotin and ImmunoPure Neutravidin–HRP Conjugated) were supplied by Perbio Science France. Neutravidin–HRP conjugates will also be referred to indifferently as HRP enzymatic probes or enzymatic probes since these conjugates are composed of HRP enzymes covalently linked to a support protein (neutravidin). Neutral phosphate buffer solution (PBS) 0.1 M, pH = 7.0 ± 0.1 was also provided by Perbio Science France. All the chemicals used in this study were of the highest grade of purity.

2.4. Tracer purification and chromatographic characterization

Tracer purification and chromatographic characterization were performed by HPLC-SEC (High Performance Liquid Chromatography by Size Exclusion). HPLC-SEC was achieved using a Superose6 size exclusion column (GE Healthcare, France) integrated in an analysing chromatographic system, ÄKTApurifier UPC 100 (GE Healthcare, France). The Superose6 column allows compounds from 5 to 5000 kDa to be fractionated and tolerates a 40 000 kDa maximal load. Its bed volume is 24 mL. The ÄKTApurifier system was equipped with a 1.00-mL injection loop, a detection at three UV wavelengths (210, 254 and 280 nm), a pH/conductivity monitor (Monitor pH/C-900, GE Healthcare) and a fraction collector (Fraction Collector Frac-950, GE Healthcare). Neutral PBS was used as the eluant. This solution was first filtrated on a 0.2-μm filter and then sonicated for 200 s. Before column equilibration, pumps were rinsed with eluant and then the column was equilibrated with 50 mL of eluant. Analyses were performed by eluting samples with 35.00 mL eluant at a 0.40 mL min^{-1} flow rate (except for the biotinylated phages at 0.50 mL min^{-1}). Phage suspensions were analysed in a concentration range higher than 10^{10} pfu mL^{-1} in order to obtain a significant and stable signal.

Tracer concentrations were determined from peak area measurements [29]. For quantitative characterization, peak areas were determined using the UNICORN software version 5.01 (Amersham Biosciences, Sweden) allowing direct integration and triangulation (via a tangent method). Partial peak areas were measured by a