Enzyme-Labeled Phages Detected by Amperometry: A New Method to Study Inline Virus Retention in Membrane Processes

L. Soussan, C. Guigui, C. Mathe, S. Alfenore, and C. Cabassud
Université de Toulouse; INSA, LISBP, 135 Avenue de Rangueil, F-31077 Toulouse, France
INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, F-31400 Toulouse, France
CNRS UMR5504, F-31400 Toulouse, France

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A new method is presented to characterize the retention dynamics of membrane processes with a new virus surrogate used as a tracer and developed in our laboratory. This virus surrogate is an enzyme-labeled MS2 bacteriophage whose activity can be directly and rapidly detected and quantified by amperometry, which is a sensitive electrochemical technique. In the first step, the amperometric measurement was developed and validated. Microfiltration and ultrafiltration experiments consisting of injecting tracers into the feed and monitoring the tracer presence in permeate (or in retentate) by amperometry, and then validating the use of the tracer and its detection technique in the field of membrane filtration. In particular, global retention experiments demonstrated the ability of this method to differentiate among membrane behaviors and dynamic experiments showed the ability of the method to characterize dynamics of retention in a reproducible way.
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Introduction

Membrane processes (in particular ultrafiltration) are widely used for their ability to retain particles and notably viruses. However, membrane history (aging, fouling), defects and operating parameter changes can lead to possible modifications of the virus retention where the virus retention is defined as the ratio of the virus concentration in the feed to that in permeate, usually expressed in log removal value (LRV), i.e., the logarithm in base 10 of the virus retention. Some studies have presented, for example, the influence of the transmembrane pressure[1] or the membrane fouling state[2] on the virus retention. However, these parameters are likely to vary during filtration. A tool able to characterize the dynamics of retention of virus species directly in membrane systems is, thus, interesting. Such characterizations can hardly be performed with native pathogenic viruses. A nonpathogenic virus surrogate is, therefore, necessary. This virus surrogate should notably be detected/quantified by a method that is fast, easy, sensitive and applicable *in situ* (directly in the membrane systems), and inline (during filtration). The global aim of such a method would be, thus, to characterize quickly the retention variations of a suited virus surrogate as a function of operating conditions, and not to access to the absolute virus retention of the membrane process, nor to find the "ideal" virus surrogate that could be the extrapolation model to anticipate the intrinsic retention of the membrane systems.

Methods based on different virus surrogates were proposed in the literature. Bacteriophages (especially MS2 bacteriophages), which are nonpathogenic bacteria viruses, are commonly used as reference microorganisms to test the retention performances of membrane systems,[3–5] because they are very similar to pathogenic viruses carried by water (e.g., poliovirus or hepatitis virus) in terms of size and shape. Current methods to quantify bacteriophages are those used to quantify viruses: direct enumeration by plate counting, flow cytometry, quantitative PCR and biosensors. Direct enumeration by plate counting[6] is widely used because of its low cost, ease of use and high sensitivity (up to 0.01 pfu mL^{-1}). This method requires, however, putting the bacteriophages in contact with host bacteria for at least 24 h, which is too long for an inline application. Flow cytometry[7,8] and quantitative PCR[9–11] are faster techniques. Flow cytometers are for the moment very expensive and has low mobility. They also need low-flow rates (ca. 100 μL min^{-1}) to avoid coincindence of viral particles that make this technique currently unsuitable for the filtration application. Quantitative PCR that is more sensitive than flow cytometry (with a current detection threshold of 10^2 pfu mL^{-1}) is still not used for inline studies as it requires at least 1 h 30 min for the amplification step of the labeled genetic support. This promising method is, however, under development. Biosensors (mechanical,[12] electrical[13,14] or optical[15]) have also been

Correspondence concerning this article should be addressed to L. Soussan at laurence.soussan@enscm.fr.

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developed, but the establishment of the immunochemical bonding between the capture molecules and the viruses currently imposes two flow rates for the aimed application.

This explains why modified bacteriophages were also considered in the literature. Bacteriophages with their surface modified by the grafting of different probes allowing their direct detection have been notably tested (1) MS2 bacteriophages labeled with fluorescent dyes detected by fluorimetry,[16] (2) MS2 bacteriophages labeled with enzymes developed in a previous study[17,18] and detected by spectrophotometry, and (3) T4 bacteriophages conjugated with enzymes detected by ECL (Electro-ChemiLuminescence).[19] No quantification of the enzymes grafted on the T4 phage enzymes has, however, yet been reported in the literature.[19] Similarly to the enzyme-labeled T4 phage, no quantification of the grafted dyes has been performed at present for the fluorescent-dye-labeled MS2 phage. Such quantification of the grafted probes (either enzymes or dyes) is nevertheless necessary to conclude on the proper purification of these modified bacteriophages from the excess dyes or enzymes in order to use these modified phages in filtration and to assess their lower detection threshold. In the case of the MS2 bacteriophage labeled with enzymes, the average number of enzymatic probes grafted per phage was determined,[17] and the lower detection threshold of this virus surrogate by classical spectrophotometry (with pyrogallol and hydrogen peroxide) was found to be 4 10^7 eq. pfu mL^{-1}, which underlines a lack of sensitivity of this method.

Recently, norovirus like particles (NVLPs)[20] morphomogically and antigenically similar to native norovirus were proposed in the literature as another kind of virus surrogate. These NVLPs are for the moment detected and quantified by a static immunosensor (ELISA), which cannot allow an inline detection of these particles.

Methods based on nonbiological virus surrogates were also considered, such as (1) gold nanoparticles detected by potentiometry,[21] (2) fluorescent microspheres detected by fluorimetry,[22] and (3) iron oxide nanoparticles detected magnetically.[23–26] Although these methods allow the respective surrogates to be detected/quantified directly and quickly in permeate, these nonbiological virus surrogates are still not truly representative of actual viruses (deformability, density, surface charge density,…).

This work focuses on the development of a new method based on an enzyme-labeled MS2 bacteriophage as a virus surrogate. In a previous article,[17,18] the principle and detailed procedure for producing this MS2 virus surrogate were presented, together with its characterizations. The activity of this enzyme-labeled MS2 bacteriophage (induced by its grafted enzymes) has been measured by spectrophotometry up to now.[18] In order to use this virus surrogate (or tracer) in filtration; the tracer detection threshold has to be low in comparison with spectrophotometric detection. In this work, amperometric detection was chosen and developed for its higher sensitivity, its specificity, and its ability to be applied inline. This article focuses on the development of the amperometric detection of this new virus surrogate, and its use in following membrane retention dynamics.

The amperometric measurement was first developed in perspective of an inline application, which required study of the whole chemistry of the substrate of catalyzed reaction, and to use this substrate in an original way. The specific activities of three different HRP enzymatic species (including the tracer) were then measured by amperometry, and compared to spectrophotometry (considered as the reference method) on high-concentration area in order to validate the amperometric measurement. The tracer threshold of this method was thereafter investigated. This new method was finally applied in filtration experiments, consisting of injecting tracers into the feed and monitoring the tracer presence in permeate by the amperometric detection method developed.

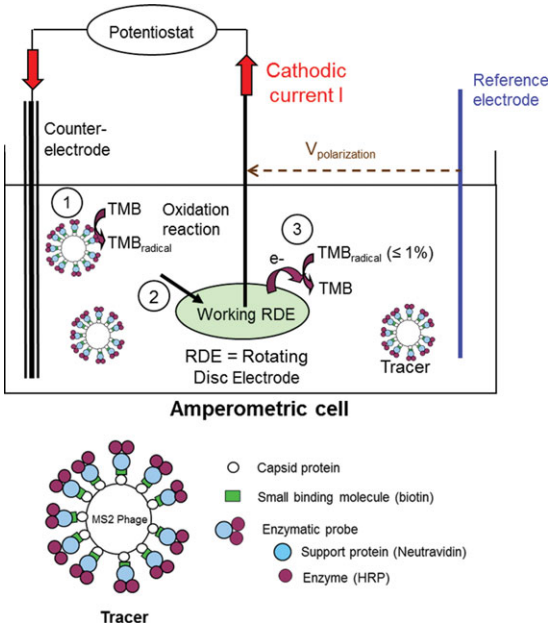

Figure 1. Scheme of enzyme-labeled MS2 bacteriophage, amperometric cell and measuring principle.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The enzyme-labeled MS2 bacteriophage and the principle of its amperometric detection are first detailed in the following section.

Principle of Amperometric Detection of an Enzyme-Labeled MS2 Bacteriophage used as a Virus Surrogate

Virus surrogate

HRP enzymatic probes used for the grafting on the MS2 bacteriophage are neutravidin-HRP conjugates composed, as shown in Figure 1, of a support protein (neutravidin) covalently linked to HRP enzymes. In this study, two HRP enzymes on average were bound per neutravidin protein (provider data). The HRP enzyme was chosen because of its small size and its high-catalytic activity.[27] These enzymatic probes were grafted on the capsid phage via small binding molecules (i.e., biotin) covalently bonded to the capsid proteins. The interaction between biotin and neutravidin is very specific and very strong, which makes this interaction comparable to a covalent bond.[18,28] In particular, the size characterization of the enzyme-labeled MS2 bacteriophage at neutral pH and at 20°C showed a spherical monomodal population centered on an average diameter of 64 nm, which is within the size range of pathogenic viruses (from 10 to 110 nm).[29] At 64 nm, our surrogate is within the range of

sizes of mammalian viruses evaluated in filtration studies, larger than parvoviruses (20–28 nm), but smaller than retroviruses (80–110 nm). It is also a similar size to PR772, the phage used to standardize the nomenclature of large virus retentive filter. The average molecular weight of the tracers was also quantified and found to be between 12,000 and 22,000 kDa (depending on the average number of probes grafted per phage), which is within the molecular weight range of pathogenic viruses (from 1,000 to 30,000 kDa).[29] Details on the tracer synthesis protocol and properties are presented in Soussan et al.[17,18]

The enzymatic entity of the enzymatic probes grafted on the MS2 bacteriophage (i.e., the HRP enzyme) catalyzes the oxidation reaction of an electron donor R into its oxidized form O, and the direct detection/quantification of this virus surrogate, thus, results from its induced enzymatic entity.[30]

Amperometric detection

The enzymatic entity (HRP) of the chosen enzymatic probes thus catalyzes the oxidation of an electron donor in the presence of hydrogen peroxide as enzyme substrate. The HRP-catalyzed reaction allows a number of electron donors to be used, notably TMB (or 3,3′,5,5′-tetramethylbenzidine), which permits high sensitivity to electrochemical detection[31,32] and was, thus, chosen as the electron donor for the study. Iodide-ion and a commercial stabilized mixture of H_2O_2/TMB (Ultra TMB, from Perbio, France) were also tested as electron donors but appeared to be less efficient (data not shown). The irreversible HRP-catalyzed reaction is, thus, written as described in Eq. 1, where TMB_{radical} is the TMB oxidized form which corresponds to the complex resulting from the rapid equilibrium with the native radical form.[33]

$$\text{TMB} + {}^1\!/_2\text{H}_2\text{O}_2 \rightarrow {}^1\!/_2\text{TMB}_{\text{radical}} + \text{H}_2\text{O} \quad (1)$$

Figure 1 shows the amperometric cell used for the amperometric measurements together with the measuring principle.

The amperometric cell is commonly composed of a working electrode (that is a rotating disc electrode, RDE), a reference electrode and a counterelectrode.[34] The principle of the amperometric measurement can be described in three steps. In step 1, when the new virus surrogate is present in solution and in the presence of TMB and hydrogen peroxide, the oxidation reaction catalyzed by the grafted enzymes occurs, producing TMB_{radical} that diffuses in step 2 toward the working electrode where a small quantity of TMB_{radical} ($\leq 1\%$) is reduced in step 3 (due to the imposed polarization potential $V_{\text{polarization}}$ corresponding to a diffusion limitation of TMB_{radical} to the electrode), thus, generating a cathodic current I. These three steps occur simultaneously during the measurement. Under given conditions, this current decreases linearly with time and the slope at the origin of the regression line obtained is proportional to the production rate of TMB_{radical}, thus, to the total concentration of enzymes grafted on the phages, thus, to the total concentration of enzymatic probes grafted on the phages and, thus, to the virus surrogate concentration.

Equation 2 describes the fact that the slope $V0_A$ (A min^{-1}) of the tangent at the origin of the regression line giving the current vs. time is proportional to the maximal initial production rate Vi (L mol min^{-1}) of TMB_{radical} in solution

$$|\text{V0}_\text{A}| = \text{K.Vi} \quad (2)$$

where $(\text{dI}(t)/\text{dt})_{t=0}$ (A min^{-1}) is the slope $V0_A$ of the tangent at the origin of the regression line giving the current I vs. time (and cutting the time axis at zero), Vi (L^{-1} mol min^{-1}) is the maximal initial production rate of TMB_{radical} in solution due to the catalyzed reaction, K (A L mol^{-1}) is the constant of the diffusion law linking the cathodic current produced to the concentration of TMB_{radical} formed over time in solution by the enzymatic reaction.

Equation 3 describes the proportionality of the slope $V0_A$ with the concentration of grafted enzymes and, thus, the tracer concentration

$$|\text{V0}_\text{A}| = \text{K.k}_{\text{cat}}.[\text{G}_{\text{enzyme}}] \quad (3)$$

where $[G_{\text{enzyme}}]$ (mol L^{-1}) is the concentration of grafted enzymes in the amperometric cell, k_{cat} (mol mol^{-1} min^{-1}, i.e., min^{-1}) is the specific activity of the grafted enzymes. The specific activity k_{cat} (min^{-1}) is defined as the number of moles of TMB_{radical} produced per mole of grafted enzyme in the conditions of the amperometric measurement. If *TMB* and hydrogen peroxide are in large excess with respect to the quantity of grafted enzymes in solution during the amperometric measurement, the specific activity k_{cat} of the grafted enzymes is optimal and the production rate of TMB_{radical} due to the grafted enzymes is maximum and constant over time.

It should be noted that the oxidation reaction of *TMB* into TMB_{radical} in the presence of hydrogen peroxide (Eq. 1) can also occur spontaneously in solution (in parallel with the catalyzed reaction), and give rise to an amperometric slope. Volpe et al.[31] have shown that the spontaneous reaction is sufficiently slower than the catalyzed reaction to permit a good detection level of HRP enzymes with *TMB*. Experimentally, global amperometric slopes due to the catalyzed reaction and to the spontaneous reaction are measured. The amperometric slope $V0_A$ due to the catalyzed reaction only (Eq. 3) is then obtained by subtracting the slope corresponding to the spontaneous reaction from the global measured slope.

Tracer use and tracer quantification principle

The developed virus surrogate is used as a tracer that is monitored in permeates during filtration by the amperometric detection. In this context, the amperometric detection needs less than 10 min for analysis.[30] This detection method can be performed inline by using an amperometric flow cell. However, a classic amperometric cell requiring batch sampling was preferred in this work focusing on the method development, so as to decrease the number of experimental parameters of the measuring system and to obtain more precise measurements with the amperometric slopes than with instantaneous single current measurements. Volumes from 1 to 30 mL can be analyzed at the laboratory scale. Figure 2 summarizes the different steps of tracer use and quantification.

First of all, a blank is performed (with the reactants alone: *TMB* and H_2O_2 in the electrolyte solution) to determine the amperometric slope due to the spontaneous reaction. The tracers are injected into the feed (either by step or by peak), and permeate samples (collected during filtration) are analyzed. Each permeate sample collected is introduced into the amperometric cell then the reactants are added and the measurement of the current I is started. It continues for a time that gives access to the global amperometric slope due to the catalyzed reaction and to the spontaneous reaction. The amperometric slope $V0_A^{\text{unknown sample}}$ of the unknown

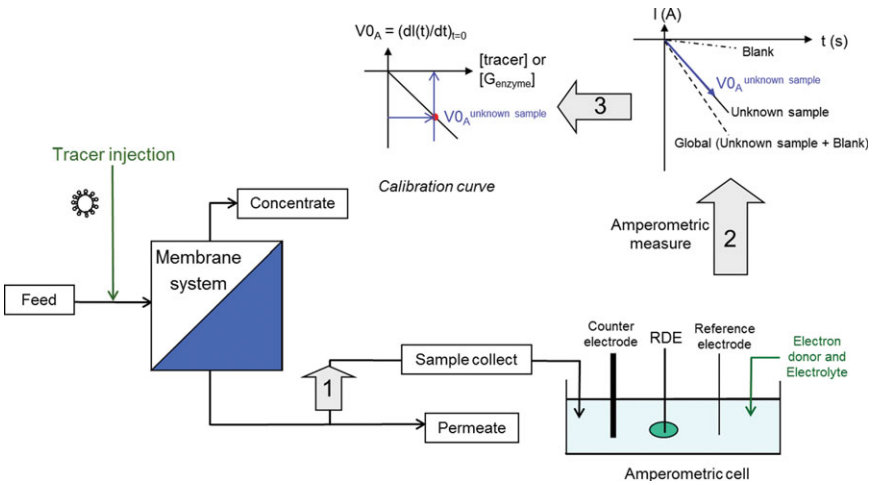

Figure 2. Principle of use and quantification of the virus surrogate.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sample is then obtained by subtracting the slope of the blank from the global measured slope.

The unknown tracer concentrations of the samples in the amperometric cell are then deduced from a calibration curve plotted just before the measurements. This curve is obtained by plotting the $V0_A$ values measured for known tracer concentrations vs. these concentrations. Tracer concentrations can be expressed either in pfu equivalent (as a tracer is a modified bacteriophage), or in equivalent of grafted enzymatic probes. A protocol developed by Soussan et al.[17,18] quantifies the mass of enzymatic probes grafted on the phages, and, thus, the concentration of grafted probes in the tracer suspensions produced.

It is worth noting that such an analysis over time can also be performed on the feed and on the concentrate.

Consumables and Materials

Consumables

Tracers were prepared according to the protocol described in a previous work.[17] Tracer suspensions used for the filtration experiments were prepared in neutral PBS buffer solution, i.e., in the same matrix in which tracers were synthesized. Concentrations of the prepared tracer suspensions were between 5.0 10^8 pfu mL^{-1} and 1.0 10^9 pfu mL^{-1}.

Chemicals involved in the enzymatic quantification by amperometry and spectrophotometry were 3,3′,5,5′-tetramethylbenzidine (from Sigma Aldrich, France), and Hydrogen peroxide H_2O_2 (30% (w/w) in aqueous solution) provided by Roth, France. Citrate phosphate buffer, 0.1 mol L^{-1} phosphate plus 0.1 mol L^{-1} NaCl, pH = 5.20 ± 0.04, was made using disodium phosphate Na_2HPO_4, $2H_2O$ (from Roth, France), citric acid (from Sigma Aldrich, France) and sodium chloride (from Roth, France). Neutral phosphate buffer solution (PBS) 0.1 M, pH = 7.0 ± 0.1 was provided by Perbio Science, France. All the chemicals used in this study were of the highest grade of purity.

Materials for enzymatic activity measurements

Amperometric device. Enzymatic activity measurements by amperometry were performed using a μ-Autolab III potentiostat-galvanostat (from Metrohm, France) with a 30 fA resolution, and the acquisition software was GPES (version $4.9_{.007}$). A Karl Fisher cell made of glass was used as the amperometric cell. Its maximal volume was 130 mL. Its flat lid made it easy to set up and wash the electrodes and also to introduce the reactive agents. A small opening for air was provided at the top of this air-sealed cell in order to avoid any excess pressure in the amperometric cell during measurement. The working electrode was a rotating disk electrode (RDE) with a 5-mm-dia. platinum disc as the active area (from Metrohm, France). The RDE control system allowed the rotation rate to be fixed between 100 and 10,000 rpm. The maximal rotation velocity was fixed at 1,130 rpm in this study. The counterelectrode (manufactured by Heraeus, France) was a platinum grid with an active area 6.5 times the area of the RDE. The reference electrode was a double junction Ag/AgCl electrode (from Metrohm, France).

Plate Reader Spectrophotometer for the Validation of the Amperometric Measurement. Enzymatic activity measurements by spectrophotometry were performed using a Multiskan Ascent, 100–120 V plate reader (Thermo Scientific, France). These experiments were carried out using disposable transparent 96-well plates (Greiner Bio-One, France) at a wavelength of 620 nm and at room temperature.

Materials for the application of the developed method in filtration

Membranes. Two flat sheet membranes were used for the filtration tests: an ultrafiltration UF membrane (Novasep Process, Orelis, France) made of poly(ether sulfone) (PES) with a mean nominal cut-off of 100 kDa, and a microfiltration MF membrane (Alfa Laval, France) made of polysulfone (PS) with a mean pore size of 0.1 μm. In particular, the permeability to ultrapure water of the UF membrane was Lpo_{UF} = 57 ± 4 L h^{-1} m^{-2} bar^{-1} at 20°C, and the permeability Lpo_{MF} to ultrapure water of the MF membrane ranged from 362 ± 46 to 469 ± 58 L h^{-1} m^{-2} bar^{-1} at 20°C.

Filtration System. A filtration system for flat sheet membranes was used for the filtration experiments, which were performed in a dead-end mode and at a constant transmembrane pressure (TMP). Figure 3 illustrates the membrane system used. A filtration cell (Millipore, France, Model 8050) without stirring and with a membrane surface area of about

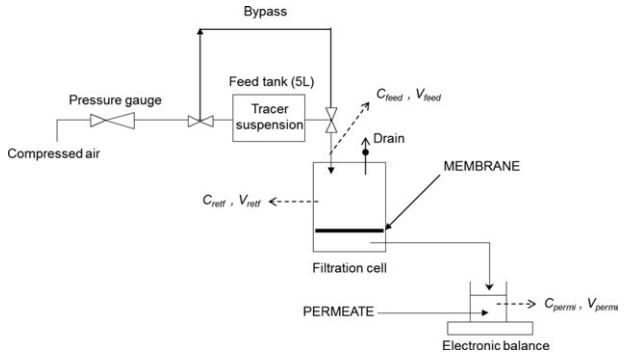

Figure 3. Membrane system used for dead-end filtration experiments at constant TMP.

C_{feed} is the tracer feed concentration measured at the inlet of the filtration cell. C_{permi} is the tracer permeate concentration at time i and V_{permi} is the volume of permeate collected at time i. C_{retf} is the tracer concentration in the retentate at the end of the filtration, and V_{retf} is the volume of retentate collected at the end of the filtration.

12.5 cm^2 was employed for these experiments. This filtration cell was connected to a large stainless steel vessel (5 L) containing the tracer suspension, to allow longer filtration times. Permeate volumes were monitored with an electronic balance. A bypass also allowed the contents of the filtration cell alone to be filtered and retentate to be collected. Before each experiment, the whole filtration system without membrane was disinfected in order to avoid any microbial contamination of the membranes or the suspension to be filtered, and was then washed with ultrapure water.

Validation of the amperometric measurement

The amperometric measurement was first developed (see the Appendix): the parameters of the measuring system were notably determined and the whole chemistry of the TMB as an electron donor was characterized in view of an inline application. The amperometric measurement was then compared to a reference measurement in order to assess the validity of the amperometric measurement. Spectrophotometric measurement was, thus, chosen as a reference as TMB_{radical} is a blue compound that absorbs between 600 and 670 nm. The comparison was made by measuring the specific activities of three HRP enzymatic entities (i.e., HRP enzyme, neutravidin-HRP conjugate and tracer) by amperometry, and by spectrophotometry on high-concentration area as the spectrophotometric measurement is less sensitive.

Methods for the validation of the amperometric measurement

The specific activity, k_{cat} (min^{-1}), of any HRP enzymatic entity was defined as the number of moles of TMB_{radical} produced per mole of enzymatic entity in 1 min at room temperature, and at pH = 5.20 in the following solution: *TMB* 2.00 10^{-4} mol L^{-1}, and H_2O_2 1.00 10^{-3} mol L^{-1}. The same HRP enzymatic entities were analyzed by amperometry and spectrophotometry on the same day in the same conditions (see following sections). Specific enzymatic activities measured by amperometry were noted as k_{catA} (min^{-1}), and specific enzymatic activities measured by spectrophotometry were quoted as k_{catS} (min^{-1}). Each enzymatic entity was tested at high concentrations to compare the amperometry and the spectrometry methods with accuracy. Dilutions of the HRP enzymatic entities were made in ultrapure water just before measurements; a new dilution was made for each characterization. Lowest concentrations were always analyzed first.

Amperometric Measurement of Specific Activities f k_{catA} or HRP Enzymatic Entities (Including the Tracer): Method and Experimental Protocol Method for the Measurement of k_{catA}. Amperometric measurements of specific enzymatic activities were performed with maximal initial rate experiments,[36] based on Eq. 3, that were generalized to any HRP enzymatic entity

$$|\text{V0}_\text{A}| = |(\text{dI}/\text{dt})_{\text{t}=0}| = \text{K}.\text{k}_{\text{catA}}.\alpha.[\text{EnzEntity}] \quad (4)$$

$$\text{Vi}_\text{A} = |\text{V0}_\text{A}|/(\text{K}.\alpha) = \text{k}_{\text{catA}}.[\text{EnzEntity}] \quad (5)$$

where $V0_A$ (A min^{-1}) is the tangent slope at the origin of the curve giving the current I vs. time, Vi_A (mol L^{-1} min^{-1}) is defined as the maximal initial production rate Vi of TMB_{radical} measured by amperometry, [EnzEntity] (mol L^{-1}) is a known concentration of the enzymatic entity studied, K (A L mol^{-1}) is the constant of the diffusion law, and α (−) is the average number of HRP molecules per molecule of HRP enzymatic entity. In particular, $\alpha = 1$ for free HRP enzymes and, according to provider data, $\alpha = 2$ for neutravidin-HRP probes either free or grafted on the phages for tracers.

It appears, notably, that the K constant allows $V0_A$ values (A min^{-1}) to be expressed in Vi_A values (mol L^{-1} min^{-1}) in order to determine k_{catA}.

Equation 4 shows the proportionality of the $V0_A$ (A min^{-1}) slope to the concentration of the studied enzymatic entity. $V0_A$ values were, thus, measured for different known concentrations of the studied HRP enzymatic entity. Vi_A (mol L^{-1} min^{-1}) values were calculated from the measured $V0_A$ values according to Eq. 5. Then the Vi_A values were plotted vs. the corresponding enzymatic entity concentrations [EnzEntity] (mol L^{-1}), and the slope of this regression line (cutting the x-axis at zero) was k_{catA} (according to Eq. 5). For each quantification of the specific activity k_{catA}, four concentrations of the HRP enzymatic entity under study were tested.

Experimental protocol for the measurement of k_{catA}

Whatever the HRP enzymatic entity tested (i.e., free HRP enzymes, free neutravidin-HRP probes or tracers), the kinetic curves giving current vs. time, and the $V0_A$ slopes of the tangents at the origin to these curves were obtained according to the following protocol.

A fresh TMB solution and a fresh hydrogen peroxide solution were prepared, respectively, at 0.01 mol L^{-1} and 0.88 mol L^{-1} in ultrapure water. The total analysis volume in the amperometric cell was fixed at 78.0 ± 0.5 mL. TMB solution and hydrogen peroxide solution volumes were set, respectively, to 1.56 ± 0.01 mL and 88.60 ± 0.18 μL so that respective concentrations of TMB and H_2O_2 in the cell were 2.00 ± 0.11 10^{-4} mol L^{-1} and 1.00 ± 0.01 10^{-3} mol L^{-1} at $t = 0$. In particular, the TMB and H_2O_2 concentrations were chosen so as to maintain a large excess of electron donor and substrate during the amperometric measurements. The TMB concentration was lower than that of hydrogen peroxide in order to limit the spontaneous oxidation reaction of *TMB* into TMB_{radical} in the presence of hydrogen peroxide.[31] The rest of the cell volume was filled with a citrate phosphate buffer solution (pH = 5.20 ± 0.04)

and the sample of HRP enzymatic entity to be analyzed. Analysis volumes ranged from 1.0 ± 0.3% to 30.0 ± 1.2%. The rotation velocity of the working electrode was fixed at 1,130 tr min^{-1} and the polarization potential was fixed at 0.240 ± 0.001 V relative to an Ag/AgCl reference electrode.

First of all, a blank characterizing the spontaneous oxidation reaction of the TMB in the presence of hydrogen peroxide was performed over 15 min with the operating conditions given perviously.

For a sample to be analyzed, the amperometric assay consisted of filling the cell with the citrate phosphate buffer then the sample to be analyzed. Then the working RDE was put in rotation in order to mix the compounds of the cell and to fix the hydrodynamics in the cell. Afterwards, the TMB solution was added and then the H_2O_2 was also introduced. Hydrogen peroxide could indistinctly be introduced before launching the acquisition of the current vs. time or a short time (up to 60 s) after the start as the polarization potential was stabilized quasi-instantaneously. The duration of the analysis was chosen so that the tangent at the origin of the curve giving the current vs. time was obtained with a high number of points; 7.5 min were sufficient for the lowest detection. Amperometric analyses were performed at room temperature (without temperature variation during measurements), and no deoxygenating of the cell contain was carried out.

Spectrophotometric Measurement of Specific Activities k_{catS} for HRP Enzymatic Entities (Including the Tracer) Method for the Measurement of k_{catS}. Spectrophotometric measurement was chosen as a reference as $TMB_{radical}$ is a blue compound that absorbs between 600 and 670 nm. As for the amperometry, measurements of specific enzymatic activities were performed with maximal initial rate experiments; the only difference lay in the fact that, in this case, absorbances were measured instead of currents. Substrate and electron donor concentrations were identical to those used for the amperometric measurements, and, thus, we have

$$V0_S = (dOD(t)/dt)_{t=0} = I.\varepsilon_{620}.\alpha.k_{catS}.[EnzEntity] \quad (6)$$

$$Vi_S = V0_S/(I.\varepsilon_{620}.\alpha) = k_{catS}.[EnzEntity] \quad (7)$$

where OD(t) (−) is the absorbance due to the catalyzed reaction measured at 620 nm at time t, $(dOD(t)/dt)_{t=0}$, also named $V0_S$ (min^{-1}), is the slope of the tangent at the origin to the kinetic curve giving the absorbance OD as a function of time, Vi_S (mol L^{-1} min^{-1}) is defined as the maximal initial production rate Vi of $TMB_{radical}$ measured by spectrophotometry, [EnzEntity] (mol L^{-1}) is a known concentration of the HRP enzymatic entity studied, α (−) is the average number of HRP molecules per molecule of HRP enzymatic entity, and $l.\epsilon_{620}$ (L mol^{-1}) is the Beer-Lambert constant of $TMB_{radical}$ at 620 nm.

The Beer-Lambert constant $l.\epsilon_{620}$ was deduced from the Beer-Lambert constant $l.\epsilon_{652}$ at 652 nm in the same operating conditions ($l.\epsilon_{652}$ = 3.90 10^4 L mol^{-1})[33]. The $l.\epsilon_{620}$ constant was found to be $l.\epsilon_{620} = 3.58 \pm 0.10\ 10^4$ L mol^{-1}.

It appears, thus, that the knowledge of the constant ($l.\epsilon_{620}$) allows $V0_S$ values (min^{-1}) to be expressed in Vi_S values (mol L^{-1} min^{-1}) in order to determine k_{catS}.

Equation 6 shows the proportionality of the $V0_S$ (min^{-1}) slope to the concentration of the studied enzymatic entity. $V0_S$ values were, thus, measured for different known concentrations of the studied enzymatic entity. Vi_S (mol L^{-1} min^{-1}) values were calculated from the measured $V0_S$ values according to Eq. 7. Then the Vi_S values were plotted vs. the corresponding enzymatic entity concentrations (mol L^{-1}), and the slope of this regression line (cutting the x-axis at zero) was k_{catS} (according to Eq. 7). Four concentrations of the HRP enzymatic entity studied (similar to those tested by amperometry) were tested for each determination of specific activity k_{catS}.

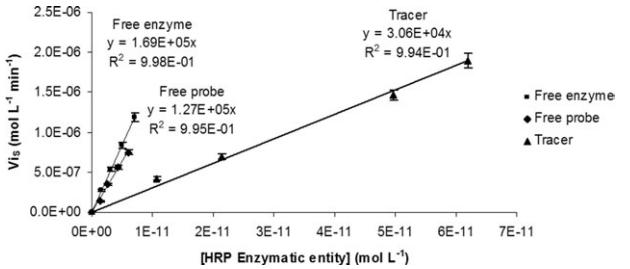

Figure 4. Initial production rates of $TMB_{radical}$ measured by spectrophotometry (Vi_S) vs. the corresponding HRP enzymatic entity concentrations.

k_{catS} values are the slopes of the regression lines corresponding to each enzymatic entity. In that case, $[tracer]_{eq.\ pfu/mL} = 9.90 \times 10^{18}\ [tracer]_{eq.\ mol/L}$, where [tracer] is the tracer concentration expressed either in pfu equivalent ($[tracer]_{eq.\ pfu/mL}$) or in equivalent of grafted enzymatic probes ($[tracer]_{eq.\ mol/L}$).

Experimental protocol for the measurement of k_{catS}

Initial concentrations of TMB and H_2O_2 in the wells, and the analysis temperature, were the same as for the amperometric measurements. A fresh TMB solution and a fresh hydrogen peroxide solution were prepared at 0.01 mol L^{-1} and 0.088 mol L⁻1, respectively in ultrapure water. The total analysis volume of wells was fixed at 320.00 ± 2.88 μL and reactive volumes were set to 6.40 ± 0.02 μL of TMB and 3.64 ± 0.01 μL of H_2O_2. Wells were filled first with a citrate phosphate buffer (pH = 5.20 ± 0.04) then with the TMB (first) and the H_2O_2. Wells were shaken for few seconds and then the sample was introduced. Wells were quickly shaken and the kinetics determination was started immediately. Kinetics were studied for 175 s (one acquisition every 7 s). A conformity control of the wells was carried out before adding the sample and the wells were shaken for 5 s between each acquisition. Blank assays were performed for each analysis by replacing the sample volume with citrate phosphate buffer and the blank assay results were subtracted from the enzymatic assay results for exploitation in order to obtain OD.

Validation of the Amperometric Measurement—Results

Comparison and validation

Figures 4 and 5 plot the initial production rates of $TMB_{radical}$ measured by spectrophotometry (Vi_S), and by amperometry (Vi_A) vs. the corresponding concentrations.

Table 1 gives the specific activities of the different HRP enzymatic entities tested, measured by spectrophotometry (k_{catS}), and amperometry (k_{catA}). Uncertainty on the k_{cat} values includes uncertainty on $V0$ measurements and on the dilutions, and also on the constants K and $l.\epsilon_{620}$. Tracer concentrations are given in equivalent of grafted neutravidin-HRP probes (refer to section Tracer use and tracer quantification principle) for the comparison.

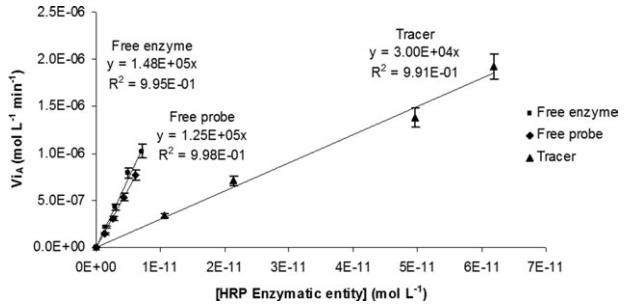

Figure 5. Initial production rates of $TMB_{radical}$ measured by amperometry (Vi_A) vs. the corresponding HRP enzymatic entity concentrations.

$kcat_A$ values are the slopes of the regression lines corresponding to each enzymatic entity. In that case, $[tracer]_{eq.\ pfu/mL} = 9.90 \times 10^{18}\ [tracer]_{eq.\ mol/L}$, where [tracer] is the tracer concentration expressed either in pfu equivalent ($[tracer]_{eq.\ pfu/mL}$) or in equivalent of grafted enzymatic probes ($[tracer]_{eq.\ mol/L}$).

Results demonstrated a good concordance between the two methods (with a shift between the k_{cat} values of 6% on average, which is within the experimental error). These results, thus, validated the amperometric measurement relative to the spectrophotometric measurement.

Current Tracer Threshold of the Method Developed. The lower current threshold for tracer detection of the amperometric method developed was determined experimentally with a minimal measurable amperometric slope fixed at two times the slope of the blank. In the experiments, the mean slope value of the blank was equal to $1.01 \pm 0.07\ 10^{-10}$ A s^{-1}. The lower tracer detection threshold was obtained in the maximum analysis time (7.5 min) for the maximum analyzed volume (30 mL), with tracers synthesized on the same day as the characterization and diluted in neutral PBS, with an average number of probes grafted per phage of 126 ± 38, and a specific enzymatic activity of the grafted probes of $8.47 \pm 0.65\ 10^4$ min^{-1}. This threshold was found to be $2.9 \pm 0.5\ 10^6$ eq. pfu mL^{-1} in the sample studied. A comparative study made in the same conditions between the amperometric measurement, and the spectrophotometric measurement showed that the spectrophotometric measurement is 6.4 less sensitive than the amperometric one, with a lower detection threshold of $1.9 \pm 0.3\ 10^7$ eq. pfu mL^{-1}.

For the moment, the lower threshold of the amperometric detection remains quite high in comparison with the quantification techniques for native bacteriophages. However, this threshold could be improved by using new enzymatic probes (now available on the market from Perbio Science France, Ref. 21130) that are nearly 10 times more sensitive than the probes used for the phage labeling in this study (according to provider data). Complementary ways to improve this threshold are also available, notably concerning the working electrode material.

It should be noted that the maximal analyzable tracer concentration in the cell was also determined ($7.0 \pm 1.1\ 10^8$ eq. pfu mL^{-1} at a maximum) so that only $TMB_{radical}$ was formed during the current measurement (see Appendix).

The amperometric measurement was, thus, validated by comparison with a reference measurement which then allowed the developed tracer and its amperometric detection to be tested in filtration experiments.

Application of the New Method in Filtration and Validation for Some Examples

The method developed, based on the coupling of the new tracer and its amperometric detection was used in filtration experiments. Global filtration experiments were first performed to compare the global retention of different membranes (microfiltration and ultrafiltration) in order to assess the ability of the method to characterize different types of membrane behavior then dynamic experiments were carried out to study the ability of the method to characterize retention dynamics.

For each filtration experiment, a fresh tracer suspension was prepared and a calibration curve, giving the $V0_A$ amperometric slopes as a function of the tracer concentrations expressed in pfu equivalent, was made from the dilution of the prepared tracer suspension (refer to section Tracer use and tracer quantification principle and section Experimental protocol for the measurement of k_{catA}). A fraction of the prepared tracer suspension was then sampled at the beginning of the filtration experiment and, like the suspension to be filtered, kept at room temperature without protection from light in order to verify the stability of the enzymatic activity of the tracers during the experiment.

All samples collected during the filtration experiments were taken in glass containers to prevent tracer adsorption phenomena. The protocol used for all the amperometric characterizations is described in section Experimental protocol for the measurement of k_{catA}. All the amperometric analyses were performed on the same day as the filtration experiments.

Global Experiments. Global filtration experiments consisted in analyzing the permeates collected at the end of filtrations performed with different membranes (ultrafiltration and microfiltration) for the same filtration conditions.

Protocol for the global experiments

These experiments were performed at a constant TMP (56 ± 3 kPa) by filtrating the same tracer feed at a concentration of $2.0 \pm 0.4\ 10^8$ eq. pfu mL^{-1}. The same specific volume (55.5 ± 0.3 L m^{-2} corresponding to a volume of 69.7 mL) of the tracer suspension was filtered for each membrane tested.

Global experiments— Results

Figure 6 shows the amperometric responses obtained by analyzing the tracer feed and permeates collected at the end

Table 1. Specific Enzymatic Activities of the Different Enzymatic Entities Tested, Measured by Spectrophotometry (k_{catS}) and Amperometry (k_{catA}) at a 21°C Temperature

Enzymatic entity	Concentration ranges (mol L^{-1})	$kcat_S$ (min^{-1})	$kcat_A$ (min^{-1})
Tracer	$1.07 \pm 0.01\ 10^{-11} - 6.19 \pm 0.06\ 10^{-11}$	$3.06 \pm 0.17\ 10^4$	$3.00 \pm 0.23\ 10^4$
Free neutravidin-HRP probe	$1.30 \pm 0.01\ 10^{-12} - 6.04 \pm 0.05\ 10^{-12}$	$1.27 \pm 0.07\ 10^5$	$1.25 \pm 0.10\ 10^5$
Free HRP enzyme	$1.51 \pm 0.01\ 10^{-12} - 7.05 \pm 0.06\ 10^{-12}$	$1.69 \pm 0.09\ 10^5$	$1.48 \pm 0.12\ 10^5$

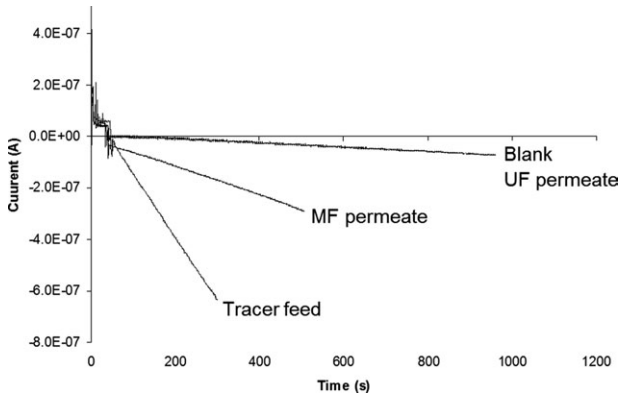

Figure 6. Amperometric responses obtained by analyzing the tracer feed and permeates collected at the end of the filtration for the microfiltration membrane (MF) and the ultrafiltration membrane (UF).

(Lpo_{UF} = 57 ± 4 L h^{-1} m^{-2} bar^{-1} at 20°C; Lpo_{MF} = 362 ± 46 L h^{-1} m^{-2} bar^{-1} at 20°C; TMP = 0.56 ± 0.03 bar and C_{feed} = 2.0 ± 0.4 10^8 eq. pfu mL^{-1}).

of the filtration for the microfiltration membrane (MF), and the ultrafiltration membrane (UF).

The amperometric response obtained for the MF permeate demonstrated that tracers passed through this membrane and the concentration of tracers in permeate (5.2 ± 0.8 10^7 eq. pfu mL^{-1}) was lower than in the tracer feed (2.0 ± 0.4 10^8 eq. pfu mL^{-1}) as the slope of the MF permeate was lower than the slope related to the tracer feed. The associated LRV value was 0.6; such a modest LRV value can notably be explained by the fact that the nominal mean pore size of the MF membrane used (0.1 μm) is the same order of magnitude as the average tracer diameter (64 nm).

In contrast, the response relative to the UF permeate was similar to that for the blank, which means that there was no detection of tracers in the UF permeate.

Dynamic experiments

From the previous results, it was known that tracers passed through the microfiltration membrane. This membrane was, therefore, used in the dynamic experiments. Two types of dynamic experiments were carried out (1) to test the ability of the method to monitor a dynamics of retention, and (2) to assess the method reproducibility. Dynamic experiments consisted in collecting permeate regularly during filtration and analyzing permeate samples.

Protocols for the dynamic experiments. All these experiments were performed with a tracer feed at 2.0 ± 0.4 10^8 pfu mL^{-1} and at a constant TMP of 56 ± 3 kPa. Samples were stored at 4°C and equilibrated at room temperature before analysis. Analyzed volumes ranged from 22 to 25 mL, which means that nearly all permeate collected during each dynamic experiment was analyzed.

Assessment of the method's ability to characterize retention dynamics

Ultrapure water was left in the filtration cell (ca. 70% of the cell volume) to study the effect of the tracer feed dilution. The drain of the filtration cell (Figure 3) was then opened in order to entirely complete the cell volume with the tracer feed, and the cell drain was then closed to filter the tracer suspension. The permeate was collected regularly

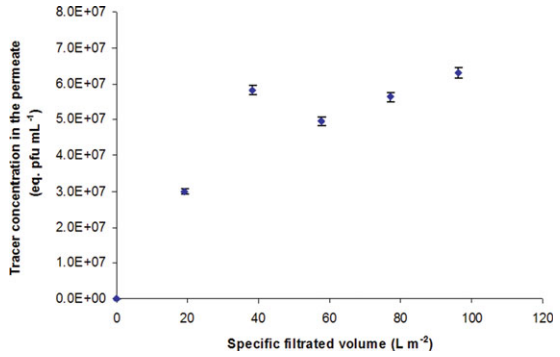

Figure 7. Tracer concentrations in permeate vs. the specific filtered volume for a dynamic experiment with the microfiltration membrane.

The filtration cell volume corresponds to a specific volume of 55.6 L m^{-2}. (Lpo_{MF} = 413 ± 51 L h^{-1} m^{-2} bar^{-1} at 20°C; TMP = 0.56 ± 0.03 bar and C_{feed} = 2.0 ± 0.4 10^8 eq. pfu mL^{-1}).

during filtration (every 19.3 L m^{-2} on average corresponding to a volume of 24.3 mL), and retentate was collected at the end of the filtration.

Assessment of the method reproducibility

In order to assess reproducibility of the method, two dynamic experiments were performed in the same operating conditions (TMP = 56 ± 3 kPa) with two distinct tracer feeds at the same concentration (2.0 ± 0.4 10^8 eq. pfu mL^{-1}). Tracer feeds were directly filtered and permeate was collected regularly during filtration (every 19.4 L m^{-2} on average corresponding to a volume of 24.4 mL).

Dynamic Experiments—Results

Assessment of the method's ability to characterize retention dynamics

Figure 7 shows the tracer concentrations in permeate (eq. pfu mL^{-1}) vs. the specific filtered volume (L m^{-2}).

The results shown in Figure 7 show that tracers passed through the membrane as soon as the tracer suspension was filtered (i.e., for positive specific filtered volumes), due to the mixing of the tracer feed with the water left in the cell. The tracer concentration in permeate increased with the filtered volume

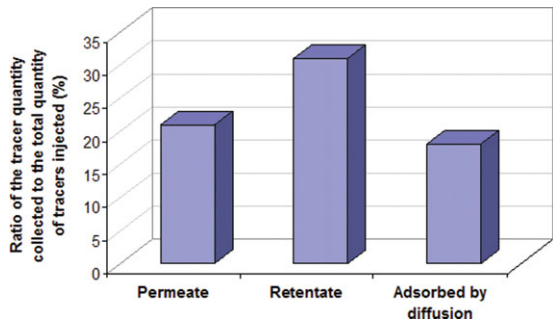

Figure 8. Ratios of the quantities of tracers recovered in permeate and retentate, and adsorbed by diffusion to the total tracer quantity injected for the dynamic experiment that demonstrated the ability of the new method to characterize dynamics of tracer retention.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

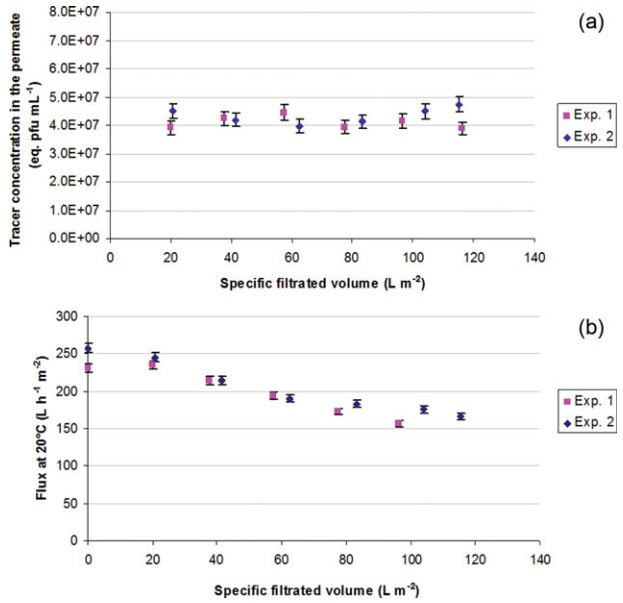

Figure 9. Tracer concentrations in permeate (a), and permeate flux at 20°C (b) vs. the specific filtered volume for two dynamic experiments performed in the same operating conditions (TMP = 0.56 ± 0.03 bar; C_{feed} = 2.0 ± 0.4 10^8 eq. pfu mL^{-1}) with the microfiltration membrane.

(For experiment 1, Lpo_{MF} = 420 ± 52 L h^{-1} m^{-2} bar^{-1} at 20°C and for experiment 2, Lpo_{MF} = 469 ± 58 L h^{-1} m^{-2} bar^{-1} at 20°C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

until a pseudo-stabilization was reached. These results, thus, show the ability of the method developed to characterize the dynamics of tracer retention (within the detection limits).

It can be noted that the error bar on the tracer concentrations in Figure 7 corresponds to the experimental error on the amperometric measurements (i.e., the error on the current: ± 0.2% cumulated with the sampling errors).

A mass balance on the tracer illustrated in Figure 8 showed that 70% of the tracers injected for this filtration was recovered, with 21% in permeate, 31% in retentate, and 18% adsorbed by diffusion on the membrane system (filtration cell walls and membrane). The quantity of tracers adsorbed by diffusion on the membrane system was determined in a preliminary study by putting a tracer suspension fivefold more concentrated than the tracer feed in contact with the membrane system over the same period as the period of filtration (see Ref. [37], chapter III p 160-162, and Appendix III.4). The rest of the tracers (30%) was probably adsorbed onto the membrane during filtration as a loss of permeability of 35% was observed during filtration.

Assessment of the method reproducibility

Figure 8 shows, respectively the tracer concentrations (eq. pfu mL^{-1}) in permeate (Figure 9a) and permeate flux (L h^{-1} m^{-2}) at 20°C (Figure 9b) vs. the specific filtered volume (L m^{-2}) for two dynamic experiments carried out in the same operating conditions with the microfiltration membrane.

Figure 9a shows similar retention dynamics in the two experiments and, thus, the reproducibility of the method. In fact, the instantaneous tracer concentrations in permeate were quite stable throughout the filtration in each experiment. Flux variations (Figure 9b) were probably at the origin of the slight differences observed on the tracer concentrations in permeate from approximately 80 L m^{-2}, where the flux measured in experiment 2 began to be significantly higher than that measured in experiment 1.

In conclusion, these first dynamic experiments demonstrated that the method developed allows characterizing dynamics of retention in a reproducible way. The method, thus, constitutes a new tool for the characterization of the dynamics of virus retention and opens ways for the inline study of the filtration behavior and the risk of virus passage for different membranes and operating conditions.

Conclusion

A new method based on the use of a new virus surrogate that can be directly quantified by amperometry, was developed to characterize the dynamics of retention of membrane systems. This new tracer is an enzyme-labeled MS2 bacteriophage. This article has focused on the development and the validation of the amperometric detection. In particular, this detection is fast as it requires less than 10 min for analysis and the mean reagent cost per analysis is low (ca. 2 euros in batch conditions with the current reagent prices). Besides, this detection method will be able to be applied inline by using an amperometric flow cell. However, a classic amperometric cell requiring batch sampling was preferred for the method development in order to decrease the parameters of the measurement system.

The operating parameters of the measurement (polarization potential fixed at 240 mV vs. Ag/AgCl, active area and rotation velocity of the working electrode imposed, respectively, to 0.196 cm^2 and to 1130 tr min^{-1}) were, thus, optimized first. The overall chemistry of the TMB (3,3′,5,5′-tetramethybenzidine) as an electron donor was also studied. In particular, catalytic conditions were found such that the catalytic oxidation of TMB by the enzymes grafted on the tracers was limited to the first step only resulting in the sole formation of $TMB_{radical}$ in solution over at least 15 min, which constitutes an original result.

Then, the comparison of the specific activities of three different enzymatic species (free HorseRadish Peroxidase, free neutravidin-HRP probes and tracers) measured with TMB by spectrophotometry (considered as the reference method for enzymatic activity measurement), and by amperometry allowed the new amperometric measurement to be validated. The difference between these two methods was found to be 6% on average (which is within the experimental error). This comparison was performed on high-concentration area of the tested enzymatic entities, but the amperometric measurement system appeared to be 6.4 times more sensitive than the spectrophotometric one. The amperometric measurement was then used in lower tracer concentration area, and the lower detection threshold was experimentally found to be 2.9 ± 0.5 10^6 eq. pfu mL^{-1} in the sample for the moment with the laboratory equipment. Some ways to further improve this current detection threshold are already available.

Filtration experiments consisting of injecting tracers stepwise in the feed and monitoring the tracer presence in permeate by the amperometric detection developed validated the method in a third stage by showing its ability to differentiate among membrane behaviors and to characterize dynamics of retention in a reproducible way. This method clearly distinguished an ultrafiltration membrane from a microfiltration one in terms of tracer retention. This method also

allowed the transient state of the tracer passage through a microfiltration membrane letting the tracers pass to be followed until the tracer concentration in permeate was practically stable. It is finally worth noting that this new method was first performed in neutral phosphate buffer and ultrapure water but can also be applied to drinking water,[27] which makes this method possibly transferable to drinking water filtration setups. Similarly, the developed method could also be used to study real large virus retentive filters like Viresolve NFR or Pall Ulitpor DV50.

In conclusion, this method meets many of the required criteria for the target application (i.e., the characterization of the dynamics of retention of membrane systems) and the filtration results are promising. These filtration experiments allowed to validate the operating conditions for a dynamic monitoring of the tracers in permeate, and, constitute a basis for a broader study of the retention dynamics of different membrane systems. This new tool for the characterization of the dynamics of virus retention opens ways to study the filtration behavior and the risk of virus passage for a variety of membranes, operating conditions and working histories.

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Appendix

In a preliminary study detailed in Ref. [37] (chapter II p 107–116), the amperometric measurement was developed. The overall chemistry of the *TMB* as an electron donor was first characterized by spectrophotometry as *TMB* is also a chromogenic substance. This characterization showed up the two steps of the oxidative mechanism of *TMB* that have been described by Josephy et al.[32]

Step 1: $TMB + ½\ H_2O_2 \rightarrow ½\ TMB_{radical} + H_2O$ (1)
Step 2: $½\ TMB_{radical} + ½\ H_2O_2 \rightarrow TMB_{ox} + H_2O$ (8)

Two reductive species ($TMB_{radical}$ and TMB_{ox}), can, thus, be produced and generate a current of reduction. The characterization of the TMB chemistry also showed that the realization of these two steps is linked to the catalytic activity of the enzyme sample (depending on the concentration of the enzyme sample and on its specific activity), which is an original result. In particular, catalytic conditions were found with HRP enzyme (with a specific activity of $1.59 \pm 0.11\ 10^5\ min^{-1}$, and at concentrations $\leq 7.05 \pm 0.06\ 10^{-12}$ mol L^{-1}), so the oxidative mechanism of *TMB* is limited to the first step only, i.e., limited to the only formation of $TMB_{radical}$ over at least 15 min. These catalytic conditions allows to quantify all the $TMB_{radical}$ produced in solution as the $TMB_{radical}$ is not consumed in the second step of the mechanism, which was a necessity if *TMB* was to be used as an electron donor. These catalytic conditions were then generalized to all HRP enzymatic entities (i.e., neutravidin-HRP conjugates and tracers) by using the notion of catalytic unit, where a catalytic unit (U) is defined as the amount of HRP enzyme (in the HRP enzymatic entity) required to form 1.0 μmol of $TMB_{radical}$ in 20 s at room temperature and at pH = 5.20 in the following solution: *TMB* $2.00 \pm 0.11\ 10^{-4}$ mol L^{-1} and H_2O_2 $1.00 \pm 0.01\ 10^{-3}$ mol L^{-1}.

In a second stage, the parameters of the amperometric measurement were optimized in a usual way. The polarization potential (0.240 ± 0.001 V relative to an Ag/AgCl reference electrode) was determined by voltamperometry so as to impose the $TMB_{radical}$ reduction reaction at the working electrode and a diffusion state. The active area and the rotation velocity of the working electrode (a platinum disc of 5 mm dia. with a rotation velocity of 1130 tr min^{-1}) were defined so that the consumption of $TMB_{radical}$ at the working electrode is minimal (<1%) compared to the quantity of $TMB_{radical}$ produced in solution over the maximal analysis time (fixed at 7.5 min) in order not to modify the concentration of $TMB_{radical}$ in solution.

In these conditions, the constant K of the diffusion law was experimentally determined and found to be $0.173 \pm 0.009\ A$ L mol^{-1}. According to Eq. 2, the constant K was obtained by measuring the amperometric slope $V0_A$ and the maximal initial production rate Vi of $TMB_{radical}$ for the same enzymatic entity analyzed on the same day in the same conditions. The maximal initial production rate Vi was measured by spectrophotometry. It is noteworthy that K is independent of the nature of the enzymatic entity used for its determination and free HRP was chosen. Each measurement was made in triplicate. A high concentration of free HRP enzyme was chosen ($7.05 \pm 0.06\ 10^{-12}$ mol L^{-1}) as the spectrophotometric measurement is less sensitive than the amperometric measurement.

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