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Orozco, Felipe; Redondo-Gomez, Carlos; Roberto Vega-Baudrit, Jose; Moreno-Villoslada, Ignacio

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Short Communication: Material Properties

On the comparison between diafiltration and isothermal titration calorimetry: Determination of the amount of analytes bound to water-soluble polymers

Felipe Orozco^{a,b}, Carlos Redondo-Gómez^{a,c}, José Roberto Vega-Baudrit^{a,d},
Ignacio Moreno-Villoslada^{e,*}

^a Laboratorio Nacional de Nanotecnología, Centro Nacional de Alta Tecnología, 1174-1200, Pavas, San José, Costa Rica

^b Department of Chemical Engineering, Product Technology, University of Groningen, Nijenborgh 4, 9747, AG, Groningen, the Netherlands

^c School of Engineering and Materials Science, Queen Mary University of London, Mile End Road, E1 4N, London, United Kingdom

^d Laboratorio de Polímeros, Universidad Nacional, 86-3000, Heredia, Costa Rica

^e Laboratorio de Polímeros, Instituto de Ciencias Químicas, Facultad de Ciencias, Universidad Austral de Chile, Casilla 567, Valdivia, Chile

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ABSTRACT

Aqueous systems composed of low molecular-weight species (LMWS) and water-soluble polymers (WSP) present important technological applications. Therefore, continuous advances on characterization techniques are crucial to gain a better understanding on how these systems interact and subsequently further modulate their properties by tuning their preparation process. For that matter, this work aims at comparing a developing characterization tool, diafiltration (DF), with a well-established method for studying LMWS/macromolecule interactions, isothermal titration calorimetry (ITC). Chlorpheniramine maleate (CPM) and poly(sodium 4-styrenesulfonate) (PSS) are used as a model aqueous LMWS/WSP system. The comparison is based on the CPM fraction bound to PSS measured by both methods. The values obtained are (0.89 ± 0.00) and (0.88 ± 0.05) for DF and ITC, respectively. The similarity between these values is noteworthy considering the dissimilarity of the process on which both techniques are based (filtration and heat exchange). This work constitutes a first step on setting DF and ITC as complementary and supporting techniques. Additional implications from these results are further discussed.

1. Introduction

A molecular understanding is crucial when it comes to achieve desired properties of a solution system. A number of characterization techniques have been developed in order to study the molecular behavior of systems composed of low molecular-weight species (LMWS) and macromolecules [1–8], since they present important applications in water treatment, delivery systems (of drugs, agrochemicals), sensorial materials, among others [9–13].

Diafiltration (DF) has been developed as a useful characterization tool for studying the interaction between LMWS and macromolecules such as water-soluble polymers (WSP) [7–11,14–22]. The technique (Fig. 1a) is based on the continuous filtration of a LMWS/WSP aqueous system through a membrane only permeable to the LMWS. By quantifying the eluted LMWS, a DF profile can be obtained as the DF carries

out while keeping constant the volume, pressure and temperature in the DF cell containing both interacting species. The resulting profiles can be used to estimate the apparent dissociation constant, the maximum retention capacities and the initial fraction of LMWS bound to the WSP [11,13,17]. These parameters have been determined for a wide variety of LMWS/WSP systems, allowing the study of long-range electrostatic interactions, short-range aromatic-aromatic interactions, non-chelating and chelating metal/WSP complexes, substrate retention, and oppositely charged polyelectrolytes complexes [7–11,14–22]. Although DF has proven to be a useful characterization tool, so far DF parameters have not yet been compared and corroborated with a widely accepted characterization technique.

Isothermal titration calorimetry (ITC) has been established as a universal detection and quantification technique for studying macromolecular interactions [23–25], but its fundamentals are quite distinct

Abbreviations: DF, diafiltration; ITC, isothermal titration calorimetry; LMWS, low molecular-weight species; WSP, water-soluble polymers; CPM, chlorpheniramine maleate; PSS, poly(sodium 4-styrenesulfonate)

* Corresponding author.

E-mail address: imorenovilloslada@uach.cl (I. Moreno-Villoslada).

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Fig. 1. (a) DF and (b) ITC scheme.

from those of DF. ITC (Fig. 1b) has received substantial attention compared to DF. It is based on the titration of a substrate A with a substrate B in an adiabatic chamber, so that any heat transfer events can be detected and counteracted by the equipment in such a way that isothermal conditions are maintained along the experiments. For that matter, being a LMWS titrated over a macromolecule, the interaction features of the system can be followed by the heat transfer involved in the process. ITC has found spread use on systems containing WSP such as proteins, DNA, and biological and non-biological polyelectrolytes [25–32]. Depending on the complexity of the LMWS/WSP system ITC experiments usually provide fundamental thermodynamic parameters such as binding constants, enthalpy, entropy and free energy changes, binding stoichiometry, onset point of phase separation, saturation point, as well as information about kinetics in some cases [23–32].

DF and ITC are based on very different events: one is a separation process, while the other is a titration one. Nonetheless, both techniques provide means to determine, under certain assumptions, the fraction of LMWS that is bound to the WSP; the former technique based on the observation of filtration profiles, and the latter based on measurements of heat exchange upon binding. Therefore, herein we present the first attempt to compare these two techniques by measuring the fraction of chlorpheniramine maleate (CPM, as LMWS) bound to poly(sodium 4-styrenesulfonate) (PSS, acting as WSP) in aqueous media (Fig. 2). Additional implications from these results are further discussed.

2. Experimental

2.1. Reagents

PSS (Aldrich; Mw 70 000 g mol⁻¹) was purified by diafiltration over a regenerated cellulose membrane of a nominal molecular weight limit (NMWL) of 10 000 Da (Millipore). After the polymer solution was washed at least eight times their initial volume, the solvent was removed by freeze-drying. CPM (Munnich, racemic mixture), NaOH (Merck), HCl (Merck) and NaCl (Merck) were used as received. For all experiments and purification procedures deionized water was used. NaOH and HCl

were used to adjust the pH, likewise, NaCl was used to adjust the ionic strength in ITC blank experiments. For ITC experiments, all solutions were degassed at 30 °C and 347 mmHg for 30 min using the equipment degasifier.

2.2. Equipment

The pH was controlled with a Thermo Fisher Scientific pHmeter (Oakton pH700). A diafiltration cell (Amicon 8010, 10 mL capacity) with a regenerated cellulose diafiltration membrane (Millipore) of a 5000 Da NMWL was used for diafiltration experiments. CPM concentration in the filtration fractions was quantified using Heλios γ UV-vis spectrophotometer (Thermo Electron Corporation, USA). Isothermal titration calorimetry experiments were performed with a NanoITC2G (T.A. Instruments, USA).

2.3. DF procedure

An aqueous mixture (10 mL) of CPM (0.750 mmol L⁻¹) and fractionated PSS (1.50 mmol L⁻¹) was placed in a 10 mL diafiltration cell bearing a 5000 Da NMWL membrane. The pH value of the solution was adjusted to 7.5 (including the water in the reservoir). The experiments were performed at room temperature. During the experiment the volume of the solution (10 mL) and pressure (3 bar) in the cell were kept constant. Fractions of 5 mL of the filtered solution were collected and the CPM concentration was quantified by UV-vis spectroscopy. Diafiltration control experiments were done in the absence of PSS to analyze the interaction of CPM with the cell components. The CPM/PSS experiments were carried out twice. The final result is expressed as a mean value, and the uncertainty as the standard deviation.

2.4. Determination of the LMWS bound fraction by DF

The LMWS is exponentially washed out from the DF cell. Thus, the DF profile can be described linearly by plotting the natural logarithm of the concentration of the LMWS eluted fractions ($\langle c_{LMWS}^{filtrate} \rangle$) along the diafiltration process. The extend of the process is described by the filtration factor (F), which is defined as the volumetric ratio between the solution that has been filtrated and the one that is kept constant on the DF cell. Such linear relation is noted in Eq. (1), where j is the absolute value of the slope, related with the rate at which the LMWS is eluted form the diafiltration cell, and $\langle c_{LMWS}^{filtrate-init} \rangle$, the concentration of the LMWS eluted fraction extrapolated to $F \rightarrow 0$ (the very beginning of the filtration) [13].

$$\ln(\langle c_{LMWS}^{filtrate} \rangle) = -j \cdot F + \ln(\langle c_{LMWS}^{filtrate-init} \rangle) \quad (1)$$

Fig. 2. Molecular structure of CPM and PSS.

Since LMWS filtrate is collected in fractions, both concentration terms in Eq. (1) are average values of the instantaneous LMWS concentration in the filtrate corresponding to each filtration fraction. The instantaneous LMWS concentration in the filtrate at the very beginning of the DF process is determined as [17]:

$$c_{LMWS}^{filtrate-init} = \frac{-j \cdot \Delta F \cdot c_{LMWS}^{filtrate-init}}{1 - e^{j \cdot \Delta F}} \quad (2)$$

Where ΔF is the volume of each collected fraction divided by the constant sample volume in the cell. In every instant, the LMWS concentration that is being filtered is equal to the concentration of free aqueous LMWS in the DF cell. As a result the total concentration of LMWS that are bound in the DF cell at the beginning of the DF process $c_{LMWS}^{bound-init}$ is expressed as Eq. (3), where $c_{LMWS}^{total-init}$ is the initial total concentration of LMWS in the DF system.

$$c_{LMWS}^{bound-init} = c_{LMWS}^{total-init} - c_{LMWS}^{filtrate-init} \quad (3)$$

In order to determine specifically the initial concentration of LMWS that are bound to the WSP, $c_{LMWS}^{bound-init-WSP}$, the initial value of bound molecules obtained in blank experiments made in the absence of the WSP (where the substrate could only bind to the diafiltration system) ($c_{LMWS}^{bound-init-DS}$) is subtracted from the $c_{LMWS}^{bound-init}$ value obtained in the LMWS/WSP experiments.

$$c_{LMWS}^{bound-init-WSP} = c_{LMWS}^{bound-init} - c_{LMWS}^{bound-init-DS} \quad (4)$$

Finally, the initial fraction of LMWS bound to WSP (f_{DF}^{bound}) is calculated as the ratio between $c_{LMWS}^{bound-init-WSP}$ and the concentration of LMWS present in the aqueous polymer system (i.e. subtracting what is initially bound to the diafiltration system from the total amount of LMWS) (Eq. (5)).

$$f_{DF}^{bound} = \frac{c_{LMWS}^{bound-init-WSP}}{c_{LMWS}^{total} - c_{LMWS}^{bound-init-DS}} \quad (5)$$

This subtraction is justified by the fact that a considerable fraction of LMWS may bind any of the diafiltration cell components, in particular to the high surface membrane.

The above mathematical treatment can be followed on the data sheets accessed through <https://data.mendeley.com/datasets/pwx9hvpqgz/1>.

2.5. ITC procedure

A CPM solution (7.10 mmol L^{-1} , pH 7.5) was titrated over aqueous PSS (1.50 mmol L^{-1} , pH 7.5). A volume of $950 \mu\text{L}$ of the PSS solution was loaded in the cell. 48 titrant solution aliquots of $5.14 \mu\text{L}$ were added every 180 s. During the experiment, the temperature was kept constant at 25°C , the system was continuously stirred (300 rpm) with the syringe, and data points were collected every second. Blank experiments, where CPM (7.10 mmol L^{-1} , pH 7.5) was titrated over NaCl (1.50 mmol L^{-1} , pH 7.5), were carried out. Aqueous NaCl was used instead of deionized water in order to account the heat exchange due to dilution of electrolytes through-out the titration. All experiments were carried out in duplicate. The resulting experimental values are presented as the mean value between replicates, and their uncertainties, as standard deviation.

2.6. Determination of the LMWS bound fraction by ITC

The calorimetric titration process is analyzed by plotting the molar heat produced after adding each aliquot (ΔQ) vs. the respective molar ratio ($\text{mol}_{LMWS}/\text{mol}_{WSP}$), where ΔQ is obtained upon dividing the difference between heat on experiments with and without WSP by the amount of LMWS that are being titrated (Δmol_{LMWS}). From this plot, an overall enthalpy change of the interacting LMWS/WSP system (ΔH) is defined as ΔQ when the molar ratio tends to zero. Once knowing ΔH it

is possible to estimate the amount of LMWS that is bound to the WSP after each aliquot is added since every binding event is associated with ΔQ as [2]:

$$\Delta Q \cdot \Delta \text{mol}_{LMWS} = \Delta H \cdot \Delta c_{LMWS}^{bound-WSP} \cdot V_{cell} \quad (6)$$

where $\Delta c_{LMWS}^{bound-WSP}$ is the difference on bound LMWS concentration, and V_{cell} is the volume of the LMWS/WSP solution.

The sum of all $\Delta c_{LMWS}^{bound-WSP}$ from the beginning of the titration until the desired molar ratio (in this case the molar ratio at which the DF experiments were set up, 0.5) grants the total concentration of LMWS bound to WSP at that specific molar ratio (target ratio) $c_{LMWS}^{bound-target-WSP}$. The summation must include a correction on the $\Delta c_{LMWS}^{bound-WSP}$ value of the first injection, since this injection generally presents a misleading ΔQ . For that matter, the $\Delta c_{LMWS}^{bound-WSP}$ value of the first aliquot is assumed to be equal to the one of the second aliquot. The fraction of LMWS bound to WSP is then calculated by ITC from Eq. (7), where $c_{LMWS}^{total-target}$ is the total concentration of LMWS at the target molar ratio.

$$f_{ITC}^{bound} = \frac{c_{LMWS}^{bound-target-WSP}}{c_{LMWS}^{total-target}} \quad (7)$$

The above determination is explained on a practical manner on the data sheets reached through <https://data.mendeley.com/datasets/pwx9hvpqgz/1>.

3. Results

3.1. Diafiltration

Fig. 3 depicts the DF profiles of CPM in the absence and in the presence of PSS. There is an evident affinity between CPM and PSS, evidenced by the difference in both slopes. Table 1 presents the DF parameters obtained experimentally.

3.2. Isothermal titration calorimetry

CPM/PSS isotherm is shown in Fig. 4 as a typical sigmoid curve. Table 2 presents ΔH and f_{ITC}^{bound} obtained experimentally, and includes the CPM/PSS stoichiometry (or saturation point).

4. Discussion

An outstanding similarity was found between f_{DF}^{bound} and f_{ITC}^{bound} (0.89 and 0.88, respectively), suggesting comparability between the two methods. The similitude is noteworthy considering that the processes on which these techniques are based are markedly different.

From the ITC isotherm it is worth noting the inflection point of the sigmoid isotherm at a molar ratio ~ 0.5 . This point traditionally

Fig. 3. DF profiles of CPM (0.75 mmol L^{-1}) in the absence (grey empty circles) ($y = -0.79x - 7.1$, $R^2 = 1.00$) and in the presence of PSS (1.5 mmol L^{-1}) (black filled circles) ($y = -0.33x - 9.5$, $R^2 = 0.99$) (for simplicity only one experiment is shown).

Table 1
DF experimental results.

Experiment	<i>j</i>	$\ln(\langle c_{LMWS}^{filtrate-init} \rangle)$	R^2	f_{DF}^{bound}
CPM (blank)	0.79	−7.1	1.00	–
CPM/PSS	0.35 ± 0.02	-9.5 ± 0.0	0.99 ± 0.00	0.89 ± 0.00

Fig. 4. (a) Raw data output of the titration of CPM over PSS (higher plot in black) and the blank experiment (lower plot in red). (b) Plot of the total heat furnished by mol of LMWS added per injection throughout the titration (data with blank experiments already subtracted).

Table 2
ITC experimental results.

Molar enthalpy $\Delta H / (kJ/L^{-1})$	Stoichiometry	f_{ITC}^{bound}
-14 ± 0	~ 0.5	0.88 ± 0.05

indicates the stoichiometry of the system. Therefore, even though both CPM and PSS (monomeric unit) have only one charged moiety at pH 7.5, the system gets saturated at a ratio of one CPM molecule per two PSS monomeric units. Moreno-Villoslada et al. have previously demonstrated the presence of aromatic-aromatic interaction by DF [18] and 2D NMR experiments [33]. In the latter, it was shown that both CPM pyridyl and chlorophenyl moieties interact with PSS styrene-sulfonate aromatic rings. This may have implications on the stoichiometry found, since the amount of aromatic rings per molecule is coincident at the CPM/PSS 1 : 2 ratio.

The results herein presented allow aiming to set DF and ITC as supporting and complementary techniques for the characterization of LMWS/WSP aqueous systems. The complementarity is based on the fact that both techniques can estimate different useful parameters that describe the molecular interactions between LMWS and WSP. For instance, from DF data, a mathematical treatment can be applied in order

to determine: the fraction of total LMWS that are irreversibly bound to the WSP, and the apparent dissociation constant of the remaining LMWS that are reversibly interacting with the polymer. The description of such mathematical treatment is out of the scope of this report (model described in Refs. [11,13,17]). On the other hand, ITC experiments enlarge the molecular scenario by granting different valuable parameters. In principle, and depending on the complexity of the system under study, ITC enables to look for phase separation endothermic peaks, ΔH , binding stoichiometry, study features of the system kinetics, and evaluate whether the polymer/substrate complex presents co-operative or non-cooperative binding (whether binding affinity increases or not along with site coverage) [31,32]. Such complementary set of data would be of high value, e.g. when designing a polymer carrier for controlled release of a given drug. The supporting and complementary nature of these techniques should help studying interactions involving macromolecules other than synthetic polyelectrolytes, such as natural proteins, and for nano/micrometric supramolecular arrangements such as micelles and oil-in-water nanoemulsions, providing supporting tools for studying molecular interactions in a broad range of research. Therefore, future perspectives should focus on exploring the universality of these findings through different substrate/macromolecular systems.

5. Conclusions

Remarkable alike values of the CPM fraction bound to PSS were found by DF and ITC: (0.89 ± 0.00) and (0.88 ± 0.05) , respectively. The similarity between these two values is noteworthy given the marked difference between the processes on which both techniques are based (filtration and heat exchange). This finding constitutes the first step on setting DF and ITC as supporting and complementary techniques for studying molecular interactions in LMWS/WSP aqueous systems. This complementarity should be highly valuable when developing substrate/macromolecule systems for applications such as controlled drug release.

Notes

Conflicts of interest

None.

Data availability

The raw data required to reproduce these findings are available to download from <https://data.mendeley.com/datasets/pwx9hvpqgz/1>. The processed data required to reproduce these findings are available to download from <https://data.mendeley.com/datasets/pwx9hvpqgz/1>.

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