Methods 76 (2015) 162–170

Contents lists available at ScienceDirect

Methods

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

Coupled equilibria of a self-associating drug loaded into polymeric nanoparticles

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ARTICLE INFO

Article history:
Received 24 September 2014
Received in revised form 13 December 2014
Accepted 15 December 2014
Available online 6 January 2015

Keywords:
ITC
Isothermal titration calorimetry
Stacking
Polymeric nanoparticle
Stepwise aggregation
Buffer protonation
Zeta potential
Doxorubicin
Dimer
DNA intercalators

ABSTRACT

Doxorubicin (DOX) and other anti-cancer drugs are often formulated using nanoparticles for passive or active targeting and reducing detrimental side effects. Anionic polymers have been shown to effectively facilitate loading of cationic DOX hydrochloride into nanoparticles with high efficiency. One powerful method to study DOX loading into anionic polymeric nanoparticles has been isothermal titration calorimetry (ITC), but the curves are complex and were previously interpreted in a largely qualitative manner only. Here we present detailed quantitative modelling of such ITC data, corroborated by zeta potential measurements and dynamic light scattering. The model takes into account 3 coupled equilibria. First, DOX self-associates in solution to dimers and larger aggregates. This effect is modelled in terms of the stepwise aggregation model. Second, DOX binds with a 1:1 stoichiometry to the carboxylic acids in the polymer at low salt. At about 33% saturation, the nanoparticles collapse in size and the enthalpy of further binding becomes less exothermic. Third, free DOX also stacks onto polymer-bound DOX. This stacking effect is very weak and hardly detected by ITC. It is, however, revealed by a positive zeta potential. The present work demonstrates the power of combining ITC with light scattering and zeta potential measurements for studying the thermodynamics of drug loading into polymeric nanoparticles.
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1. Introduction

The loading of the anti-cancer drug doxorubicin (DOX) into polymer-based nanoparticles (NP) is but one, particularly prominent example of the trend to enhance the efficacy of drugs and reduce their adverse side effects by utilizing passive or active targeting of these nanocarriers in the body [1–5]. The design of such formulations requires a detailed knowledge and understanding of drug–nanoparticle interactions. Pioneering studies of such interactions using isothermal titration calorimetry had aimed primarily at measuring the binding capacity of the polymer [6,7]. Govender et al. studied the binding of procaine to polyacrylic acid [8]. Previous works from Tam and co-workers [9,10] and our group [3] used ITC to characterize the loading of doxorubicin (DOX) into polymers. Binding was observed to be exothermic and depended mainly but not exclusively on electrostatic interactions. While the binding capacity of the polymer particles approached one DOX per carboxylic acid in the absence of extra salt, low pH and high salt conditions reduced the degree of ionization of the polymer and hence limited its DOX-loading capacity. These effects were straightforward to read from the ITC traces. However, a more comprehensive thermodynamic evaluation has been prevented so far by the self-association of DOX as well as DOX-induced changes in the cross-linked polymers. Note that, for example, dimerization effects cannot be corrected for by a simple blank subtraction but must be integrated into a comprehensive binding model.

Over the years, more and more complex equilibria have successfully been characterized by ITC [11,12]. A particularly important lead for our attempt to model ITC data of DOX–NP interactions presented here was the seminal work of Buurma and Haq [13] describing the binding of Hoechst 33258, a self-associating dye, to DNA. We largely followed their strategy in our effort to pursue a detailed fit of ITC data for DOX binding to polymethacrylate–starch nanoparticles [3,14]. The approach is based on calculating the amount of DOX in all states (aggregated in solution, bound to nanoparticle at low and high occupation, bound to nanoparticle as dimers) for a selected aqueous monomer concentration. The aqueous concentration is then numerically optimized so that the sums of all partial concentrations of DOX and polymer binding sites match the known, total concentrations. The parameters describing the self-association of DOX in solution are obtained by

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http://dx.doi.org/10.1016/j.ymeth.2014.12.015
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separate ITC dilution experiments [15–17] in the absence of nanoparticles.

While this approach was effective in general, there are also some fundamental differences in the thermodynamic properties of drug binding to DNA and polymeric nanoparticles. These differences also influenced the choice of the methods used to support and validate the ITC results. In particular, zeta potential measurements proved to be of major importance for interpreting the drug-loading data.

It should be noted that the results for the self-association of DOX are not only a prerequisite for modelling polymer binding but are of major interest in their own right. It is undoubtedly important for the handling of DOX to be familiar with its dimerization equilibrium but vastly different dimerization constants have been published (see Section 4).

2. Materials and methods

2.1. Materials

Doxorubicin (DOX) was obtained in its salt form as Doxorubicin Hydrochloride (DOX-HCl) in order to increase its solubility in water. DOX-HCl was purchased from Polymed Therapeutics Inc. (Houston, USA) with a purity of >98% (HPLC) and was used without further treatment. Nanoparticles (NPs) of poly(methacrylic acid)–polysorbate 80-grafted starch (PMAA–PS 80-g-St) were synthesized as previously described [3,14] by using a one-pot method that enabled simultaneous grafting of PMAA and PS 80 onto starch and nanoparticle formation in aqueous solution.

The buffers used in ITC experiments of DOX dissociation consisted of a 10 mM sodium phosphate buffer at pH 5.95 with 100 mM NaCl, a 10 mM Tris buffer at pH 7.40 with 140 mM NaCl, a 10 mM Tris buffer at pH 8.40 with 147 mM NaCl, and a 10 mM HEPES buffer at pH 7.41 with 145 mM NaCl. The concentrations of NaCl were selected to achieve similar ionic strengths (except the phosphate buffer). All ITC experiments with buffer were conducted at 25 °C, and those with water were conducted at 10 °C, 25 °C, and 40 °C.

ITC experiments with NPs in water were conducted at 25 °C to match the conditions for NP production and loading as described [3,14]. The concentration of the NPs in suspension was specified in terms of the effective concentration of carboxylic acid groups as determined by a potentiometric titration [14]. A typical value was 4.6 mmol COOH per gram of NP. The same experiment was also carried out in 10 mM Tris, 140 mM NaCl at pH 7.40.

2.2. Measurements

Isothermal titration calorimetry (ITC) was done using a MicroCal VP system (Northampton, MA) [18]. For each experiment, the 300 µL injection syringe was loaded with an aqueous solution of doxorubicin at a desired concentration. For dilution experiments, the 1.44 mL sample cell was filled with water or the matching buffer. For binding experiments, the cell contained a 0.1 mg/mL aqueous suspension of NPs (corresponding to an effective COOH concentration of 4.6 mM). The samples were degassed by stirring at reduced pressure prior to loading to avoid air bubbles.

The instrument records the differential heat power (DP) required to match the sample cell temperature with that of the reference cell as a function of time. Injections cause peaks of the DP signal which were integrated from a baseline adjusted manually or using NITPIC [19] and the resulting heat values were normalized with respect to the mole number of DOX injected to yield the normalized differential heat (NDH).

Dynamic light scattering and zeta potential measurements were performed on a Nano-ZS from Malvern Instruments (Malvern, UK) with the autotitrator accessory. The system uses a laser at 633 nm and a detection angle of 173° (backscattering), and automatically optimizes the effective path length within the cuvette.

3. Results

3.1. Self-association of DOX in water

Fig. 1A shows results of ITC experiments with the cell filled with water and the syringe loaded with 8.5 mM DOX. Each injection causes a positive power peak of the feedback heater (ordinate) to compensate for the endothermic effect accompanying the dilution of DOX into water. Integration of the peaks and normalization with respect to the injected mole number of DOX yields the normalized differential heat, NDH, as plotted in Fig. 1B (open circles). The panel combines a number of data sets recorded at different temperatures. The heats arise from the dissociation of DOX aggregates present at the relatively high concentration in the syringe upon

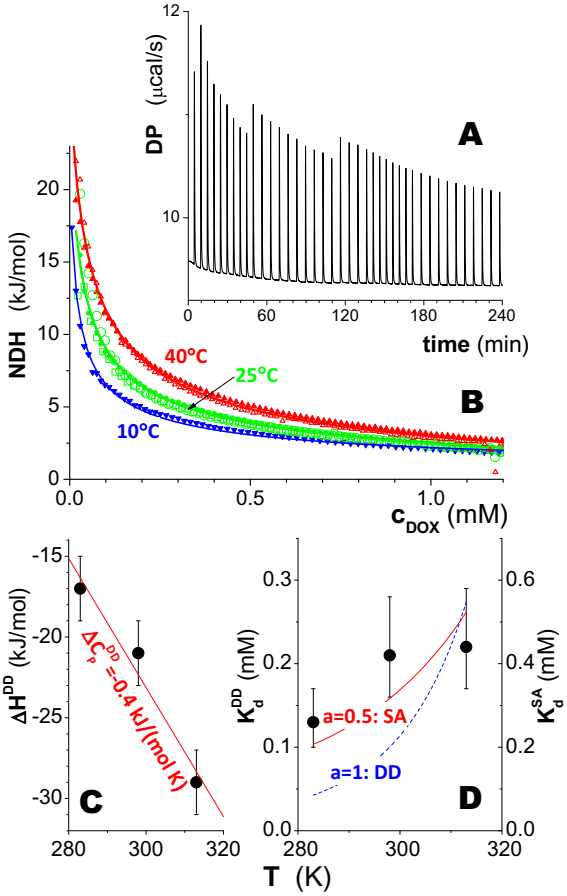

Fig. 1. Results of ITC dilution experiments of 8.5 mM DOX titrated into water. Panel A shows the beginning of a raw data set measured at 25 °C. Injection volumes and waiting times have been altered during the run to minimize evaporation. Panel B compiles integrated, normalized differential heats (NDH) versus the average, total DOX concentration in the cell, c_{DOX}, during each injection. Data of several runs (including duplicates) at 10 °C (down triangles), 25 °C (circles), and 40 °C (up triangles), are presented along with fit curves according to Eq. (13). The results of the fits, the enthalpy of association per monomer (ΔH^{DD}, $=\Delta H^{SA}$) and the dissociation constant assuming dimerization (K_d^{DD}, left ordinate) and stepwise aggregation (K_d^{SA}, right ordinate) are presented as a function of temperature in panels C and D, respectively. The fit line in C represents Eq. (5) and the curves in D are calculated using Eq. (7) for dimerization (dash-dot, $a = 1$) and stepwise aggregation (solid, $a = 0.5$).

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