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Regular Article

Dynamics of encapsulated hepatitis B surface antigen*

A combined neutron spectroscopy and thermo-analysis study

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Abstract. As a consequence of its ordered pore architecture, mesoporous SBA-15 offers new possibilities for incorporating biological agents. Considering its applicability in oral vaccination, which shows more beneficial features when compared with parenteral vaccines, SBA-15 is also seen as a very promising adjuvant to carry, protect, and deliver entrapped antigens. Recent studies have shown several remarkable features in the immunization of hepatitis B, a viral disease transmitted mainly through blood or serum transfer. However, the surface antigen of the hepatitis B virus, HBsAg, is too large to fit inside the SBA-15 matrix with mean pore diameter around 10 nm, thus raising the question of how SBA-15 can protect the antigen. In this work, thermal analysis combined with neutron spectroscopy allowed us to shed light on the interactions between HBsAg and SBA-15 as well as on the role that these interactions play in the efficiency of this promising oral vaccination method. This information was obtained by verifying how the dynamic behaviour of the antigen is modified under confinement in SBA-15, thus also establishing an experimental method for verifying molecular dynamics simulations.

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1 Introduction

Vaccination is a powerful and cost-effective form of preventing the spread of infectious diseases. However, most vaccines are delivered by injection, which makes mass immunisation costly and less safe, particularly in resource-poor developing countries. Oral delivery of vaccines has many benefits, and is becoming a preferred approach to effective vaccination for many diseases. Unfortunately, often the vaccine-active proteins are unable to produce an immune response when encountering the digestive tract. There is clear evidences that oral immunisation is a feasible alternative for preventing infections transmitted through non-mucosal routes [1]. For these reasons, and as a result of its structural stability and low toxicity, mesoporous silica SBA-15 has been shown to be a promising adjuvant for the oral delivery of vaccines for hepatitis B [2], a viral disease which attacks the liver and has infected about 2 billion people.[1]

The pore structure of SBA-15 is bi-modal, having hexagonal ordered 10 nm mesopores, together with disordered macropores larger than 50 nm. This bimodal porosity is therefore expected to protect the vaccines from the gastric acid of the stomach before initiating their release in the intestine. However, the release behaviour will depend on both the physical properties of the carried vaccine (antigen) and the morphology of the carrier SBA-15 (adjuvant). In this work, loaded SBA-15 was characterised using thermogravimetric Fourier Transform Infrared spectroscopy (TGA-FTIR) and inelastic neutron scattering (INS) to obtain a better understanding of the dynamics of the Hepatitis B surface antigen (HBsAg) encapsulated in SBA-15. Neutron scattering is a well suited method for probing the dynamics of HBsAg inside SBA-15 due to the penetrating power of neutrons and their strong interaction with hydrogen atoms [3].

2 Experimental details

2.1 Sample preparation

To encapsulate the antigen in SBA-15, HBsAg was prepared in a phosphate buffered solution (PBS, 10 mM Na_2HPO_4) with a concentration of 0.09 mg/mL and added to commercially produced SBA-15 [4], to obtain an HBsAg to SBA-15 mass ratio of 1:5. The material (SBA-15+PBS+HBsAg) was then dried at 35 °C, following the most optimal encapsulation method reported in [5], to obtain a powder. A sample containing the same amount of PBS (SBA-15+PBS) was also prepared to be used as a reference. The only difference between the two samples was the presence of encapsulated antigen.

2.2 Thermogravimetric analysis and Fourier-transform infrared spectroscopy

To determine and characterise the decomposing substances upon heating of the samples, the mass loss and the chemical composition of the released gases from SBA-15+PBS and SBA-15+PBS+HBsAg were measured by thermogravimetric analysis (TGA) and Fourier-transform infrared spectroscopy (FTIR) using a Perseus TG 209 F1 Libra (Netzsch, Germany) with an attached ALPHA FTIR spectrometer (Bruker Optics Inc., Germany). About 10 mg of each sample was placed in an aluminum oxide crucible and the temperature was varied from 30 °C to 1050 °C with a heating rate of 10 °C/min under

[1] World Health Organization, http://www.who.int/mediacentre/factsheets/fs204/en/

nitrogen gas flow. An empty crucible was measured under identical conditions and used for background correction, i.e. instrument and buoyancy influences. A new FTIR spectrum of the evolved gases was recorded for every 3 degrees of data collection.

2.3 Neutron spectroscopy

Incoherent inelastic neutron scattering (IINS) along with molecular dynamics (MD) simulations offers real possibilities of investigating the dynamics associated with a molecule's biological function(s). Using the large incoherent scattering cross section intrinsic to naturally abundant hydrogen atoms, information on the elastic (ENS), quasielastic (QENS), and inelastic (IINS) neutron scattering response of a molecule can be obtained. This experimental information when combined to MD simulations offers unique information on the dynamics of biological molecules in confinement, deepening our understanding of the relationship between a molecule's dynamics and its function.

In this work, quasi elastic neutron scattering (QENS) combined with the elastic fixed window (EFW) method were performed on both samples in order to disentangle the dynamics of the protein confined within the different pore sizes of SBA-15. This approach was used because the degree of confinement shifts the onset of the local protein dynamics to different time-scales and/or temperatures [8]. The EFW method gives information on the evolution of the elastic scattering intensity as a function of temperature, thus allowing us to observe the onset of proton mobility by points of inflexion [7,9]. Also, by analysing the variation of the line width of the QENS signal, changes in the diffusion coefficient (mainly related to the residual water in the PBS or hydrating the protein) can be readily obtained [6]. This information is crucial in elucidating how the dynamical properties of the hydration water surrounding the salt and/or antigen are modified when confined in the carrier.

EFW scans were performed during heating from 20 K to 300 K using the EMU high-resolution backscattering spectrometer at the Australian Centre for Neutron Scattering [10]. EMU is a Si (111) crystal backscattering spectrometer characterised by an energy resolution in the order of 1.2 μeV (FWHM) and a wavelength of $\lambda = 6.27$ Å. To probe the confined proton dynamics, we performed QENS experiments on the same samples using the backscattering spectrometer BASIS with wavelength centered at $\lambda = 6.4$ Å and an elastic energy resolution of 3.5 μeV (FWHM) [11]. This corresponds to a time scale in the nanosecond (ns) range. During both experiments the samples were placed in annular cylindrical holders with a thickness of 0.4 mm, relevant to the minimization of effects like multiple scattering and absorption. The QENS data was collected at 310 K, corresponding to body temperature.

3 Results and discussion

Comparing the TGA curves presented in Figure 1a, no observable mass loss was detected for pure SBA-15, indicating that the percentage of polymeric template left in the structure after washing was negligible [12,13]. On the other hand, we observe a mass loss of ∼8% for SBA-15+PBS+HBsAg starting at 250 °C, which is not present in the SBA-15+PBS sample. As the peaks observed in the FTIR signal obtained from the gases released at 300 °C can be assigned to stretching of C–H, C–O and C=O bonds, Figure 1b, and considering that encapsulated HBsAg is a protein consisting of more than 389 amino acids, we can attribute this mass loss to initial stages of protein degradation [8,14]. On further heating the mass remains almost constant

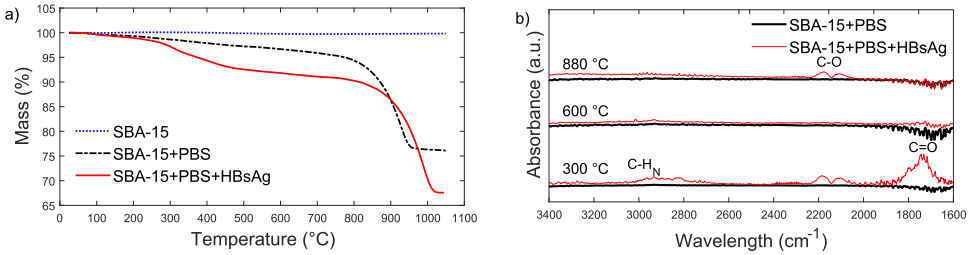

Fig. 1. Comparison of TGA curves (a) and FTIR spectra at temperatures of interest (b) for SBA-15+PBS containing only PBS salt and SBA-15+PBS+HBsAg with encapsulated antigen. Organic material is observed to be released at both 300 °C and 880 °C from SBA-15+PBS+HBsAg indicating two different configurations of HBsAg encapsulation. No mass loss was detected for pure SBA-15.

between 500 °C and 800 °C, while a second mass loss starts at 800 °C, corresponding to ∼25% and ∼20% for SBA-15+PBS+HBsAg and SBA-15+PBS, respectively. We attribute these to degradation of the strong hydrogen binding to the surface silanol groups formed during the incorporation of PBS salt [13]. Furthermore, from the FTIR spectra obtained at 880 °C, Figure 1b, we also observe a weak C–O signal for SBA-15+PBS+HBsAg, indicating final degradation of strongly bound antigen.

The combined TGA and FTIR results seem to indicate that the surface antigen HBsAg is confined in two different environments. The majority, which degrades at 300 °C, is present in the larger macropores of SBA-15, and a smaller amount, most likely attached to the entrance of mesopores, starts degrading at 800 °C.

We now turn to the analysis of the neutron spectroscopy results. By comparing the EFW scans of the two samples in Figure 2, we observe a more rapid decrease of the elastic scattering response for SBA-15+PBS+HBsAg. At 300 K an immobile hydrogen fraction of 50% is observed, corresponding to the hydrogen tightly bound in altered PBS or PBS+HBsAg structures. This population has a lower mobility than the observation time provided by the instrument. Additionally, the slightly higher fraction of immobile hydrogens observed for the confined PBS+HBsAg (50%) when compared to the sample containing only salt (45%), indicates stronger confinement in the former in the observable time window of EMU. Also, the lower temperature of the inflexion point (120 K, marked with a red dashed line) in SBA-15+PBS+HBsAg, when compared to that for SBA-15+PBS (160 K, marked with a black dashed line) indicates that the presence of the antigen facilitates the activation of hydrogen dynamics [15]. The second inflection point, around 240 K is caused by thermal activation of the OH groups present in the salt and in the antigen, plus hydration water tightly bound to the protein and/or water confined in pores of tenths of nm [16].

By focusing on the analysis of the quasi-elastic (QE) region of the IINS spectrum obtained on BASIS, we accessed unique information on the dynamical process occurring in the two samples. To this end, we have used the simplest analytical model to describe the hydrogen mobility assuming decoupled translational and rotational motions. Details of the data analysis approach can be found in [17]. Following this method, the experimental data was analysed by fitting the QE signal using a delta function describing the particles seen as immobile in the instrumental observation time window, one Lorentzian function representing the broadened energy distribution resulting from distinct relaxation processes, and a background term which takes into account fast dynamical processes. For all samples, the instrument background was defined based on the signal recorded at 20 K, while its slope was free to vary.

By plotting the half-width at half-maximum (HWHM) as a function of the scattering vector squared, Q^2, Figure 3, one may check whether Fick's law applies, viz.

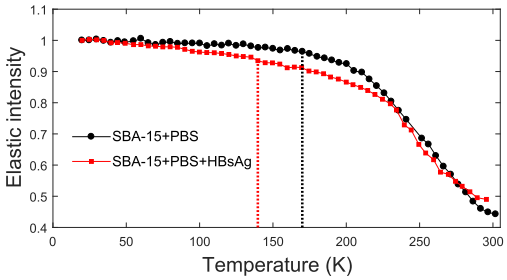

Fig. 2. EFW scans during heating obtained using the backscattering spectrometer EMU for SBA-15+PBS and SBA-15+PBS+HBsAg. The inflexion points, indicating the activation of diffusive motions, are marked by dashed lines.

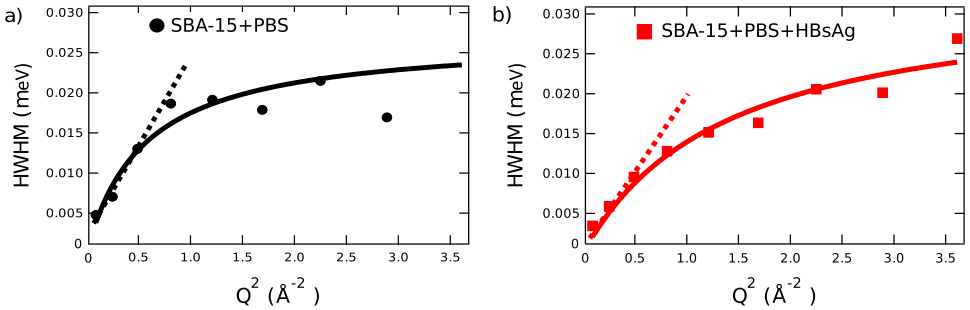

Fig. 3. Evolution of the half-width at half maximum of the quasi-elastic line calculated as a function of Q^2 modelled using the Singwi-Sjölander model from the data obtained using the backscattering spectrometer BASIS for SBA-15+PBS and SBA-15+PBS+HBsAg at 310 K. Dotted lines depict the straight line dependence expected for Fick's diffusion. The error bars are within the size of the markers.

Table 1. Average diffusion coefficient (D), residence time (τ_0) and mean jump length l_{AV}, obtained using the Singwi-Sjölander model for the confined water in SBA-15+PBS and SBA-15+PBS+HBsAg using an observation time of ns.

Sample	D (10^{-9} m^2/s)	τ_0 (ps)	l_{AV} (Å)
SBA-15+PBS	0.8 ± 0.3	24 ± 3	3.4 ± 0.7
SBA-15+PBS+HBsAg	0.37 ± 0.06	20 ± 2	2.1 ± 0.2

when a straight line is obtained, where the slope gives directly the self- diffusion coefficient. In the present case, while a linear variation of the broadening is found at small Q, the width deviates from a straight line at larger values. This implies that the continuous diffusion model is no longer valid at small distances and that the details of elementary diffusive steps have to be taken into account. Therefore, the interpretation of the QENS spectra requires a model containing the characteristic lengths and times of such mobility steps as free parameters. Considering the progressive convergence to an asymptotic value, the data were analysed using the Singwi-Sjöander model [18]. The results are given in Table 1.

Assuming that in the pure and almost dry HBsAg motions in the time window covered by BASIS are absent [6], and that the coherent scattering contribution from both PBS and PBS+HBsAg is negligible, we hypothesize that in a very simplistic approximation the QE broadening results from the hydration water, i.e. water surrounding the protein and/or tightly bound to the salt. It is well known that under

confinement water mobility will shift to longer relaxation times with respect to the bulk water processes ($D_t = 2.3 \times 10^{-9}\,\mathrm{m}^2/\mathrm{s}$). Thus our results indicate that while the diffusion of the hydration water in the PBS solution is reduced by a factor close to 3, when the antigen is introduced the diffusion coefficient is reduced by a factor 6, whilst the mean jump length, l_{AV} is comparable. This confirms that the length scale of localization in the inner structure of the SBA-15 is the same for both samples, while the hydration water becomes more hindered when the antigen is added to the solution, inducing a controlled release of the vaccine.

4 Conclusion

Molecular dynamics simulations have the potential for providing a powerful tool for understanding and predicting the release mechanics of HBsAg from SBA-15, both under relevant biological environments and for various loading degrees of vaccine in the porous silica. Such simulation methods would also be very useful for predicting the behaviour of antigens used in other types of vaccines, with different physical properties, including antigens small enough to enter the 10 nm mesopores. In order to develop such models, however, a variant of state of the art experimental methods is required to verify the simulated dynamics. In this work we have presented results obtained using thermal analysis and incoherent inelastic neutron scattering that reveal properties of the antigen HBsAg encapsulated in the mesoporous silica SBA-15. Through analysis of the neutron data we were able to probe the dynamics of the hydrogen atoms in the confined HBsAg. In the future we expect that comparison between experimental incoherent inelastic neutron scattering data and molecular dynamics simulation can be used for improving vaccine-active protein encapsulation in SBA-15.

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Author contribution statement

H.N.B. and M.C.A.F. designed and supervised the project. M.K.R., J.E.M.P., G.N.I. and H.N.B. performed the E.M.U. experiments and M.K.R. analysed the data with inputs from M.C.B. and H.N.B. M.K.R. performed and analysed the TGA-FTIR experiments. N.H.J. carried out the BASIS experiments and M.C.B. analysed the data with inputs from M.K.R. and H.N.B. V.F.B. and O.A.S.A. provided the samples and N.R.S. provided support. M.K.R. and H.N.B. wrote the paper with input from all authors, which was approved in the final version by all authors.

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