PAPER

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IOP Publishing Journal of Physics: Condensed Matter
J. Phys.: Condens. Matter **34** (2022) 264001 (10pp) https://doi.org/10.1088/1361-648X/ac6559

The development of new oral vaccines using porous silica

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Received 25 October 2021, revised 22 March 2022
Accepted for publication 7 April 2022
Published 25 April 2022

Abstract

Ordered mesoporous silica (OMS) was proved to be an efficient oral adjuvant capable to deliver a wide in size variety of different antigens, promoting efficient immunogenicity. This material can be used in single or polivalent vaccines, which have been developed by a group of Brazilian scientists. The experiments performed with the model protein Bovine Serum Albumin (BSA) gave the first promissing results, that were also achieved by testing the virus like particle surface antigen of hepatitis B (HBsAg) and diphtheria anatoxin (dANA). Nanostructured OMS, SBA-15 type, with bi-dimensional hexagonal porous symmetry was used to encapsulate the antigens either in the mesoporous (pore diameter $\sim$ 10 nm) or macroporous (pore diameter $>$ 50 nm) regions. This silica vehicle proved to be capable to create an inflammatory response, did not exhibit toxicity, being effective to induce immunity in high and low responder mice towards antibody production. The silica particles are in the range of micrometer size, leaving no trace in mice organs due to its easy expulsion by faeces. The methods of physics, usually employed to characterize the structure, composition and morphology of materials are of fundamental importance to develop proper oral vaccines in order to state the ideal antigen load to avoid clustering and to determine the rate of antigen release in different media mimicking body fluids.

Keywords: oral vaccine, silica, SAXS, x-ray tomography, hepatitis B, diphtheria anatoxin

S Supplementary material for this article is available online

(Some figures may appear in colour only in the online journal)

1. Introduction

The worldwide health crisis triggered off by the Covid-19 virus turned thousands of scientists in a frenetic search for an efficient vaccine. Despite the strategy to produce antibodies [1], all certified immunizers used to combat the epidemic are administered by injection. Among the available immunization methods, the intramuscular inoculation is the more efficient one compared to the more friendly nasal and oral routes. Despite this advantage, the logistic and cost of this vaccination process justifies the search of easier and cheap administration routes. Therefore, even the most advanced clinical trials were focussed on injectable vaccines, in March of 2021, initiatives on clinical trials using nasal and oral immunizers against Covid-19 virus appeared in the news. The nasal immunization is strongly recommended since the lung infection is a serious and deadly consequence of this disease. On the other hand, the oral immunization against this virus can be argued looking at the protection through the lymphatic system.

Our research group, by more than fifteen years, is developing an oral route of vaccination using ordered mesoporous silica (OMS), with structurally organized mean pore size around

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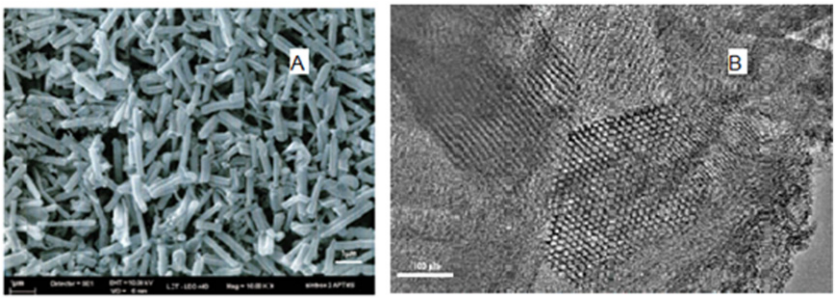

Figure 1. (A) SEM of SBA-15 shows the macroporosity (pores $>$ 50 nm) and (B) TEM image of SBA-15 shows the ordered mesoporous structure (mean pore size of $\sim$10 nm). The micropores ($<$2 nm) are present in the amorphous silica walls. (https://teses.usp.br/teses/disponiveis/43/43134/tde-17122008-091941/pt-br.php).

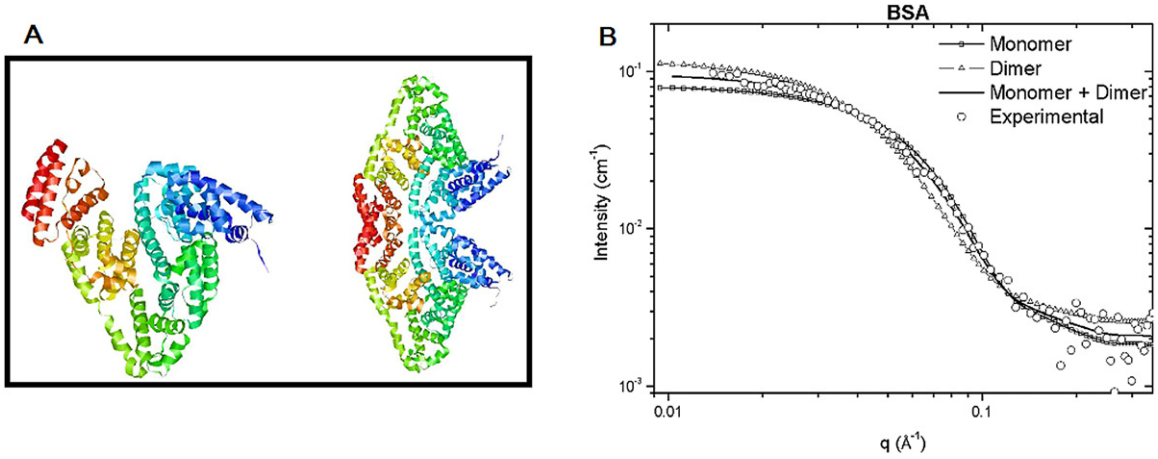

Figure 2. (A) Monomer and dimer representation of BSA. (B) BSA SAXS data, simulated as a monodisperse system of monomers, monodisperse system of dimers and polydisperse system of monomers and dimmers. Reprinted from [7], Copyright (2016), with permission from Elsevier.

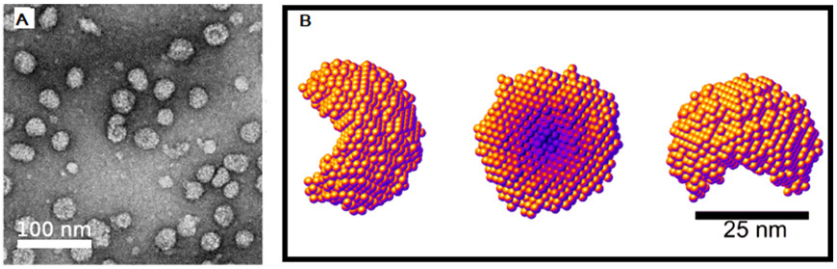

Figure 3. (A) TEM image of HBsAg virus like particles and (B) SAXS simulation of HBsAg in PBS solution. Reproduced from [11]. CC BY 4.0

10 nm, as the antigen vehicle, which is a Brazilian patent [2]. Besides the organized porous structure, the material also presents non-organized macropores (larger than 50 nm) inside the silica particles [2], whose size are in the micrometer scale. This team of researchers is formed by scientists with expertise in immunology and physics. Why this eclectic team can improve the efficacy of oral immunizers? The answer to this question relies on the tools available in usual material studies in physics, together with the biological tests in the field of immunology. The complementary information allows determining the best immunogenic complexes to be proposed as oral vaccines, regarding efficacy and practical storage plus delivery.

In 2013, a review pointed out the existence of few works on the use of mesoporous silica as a vehicle of vaccines [3]. Our research focussed on the optimization of immunogenic complexes (OMS plus antigen), regarding their physical properties, their antigen proper load in the silica matrix and antibody titers were checked for many different cases, including Intimin β protein of *Escherichia coli*, venom proteins from the *Micrurus ibiboboca* snake, model proteins such as Bovine Serum Albumin (BSA) and Human Gammaglobulin G (HGG), virus like particles of Hepatitis B surface antigen (HBsAg) and diphtheria anatoxin (dANA), comprising different sizes of antigens to fit inside the protective silica meso and macroporosity [4–13].

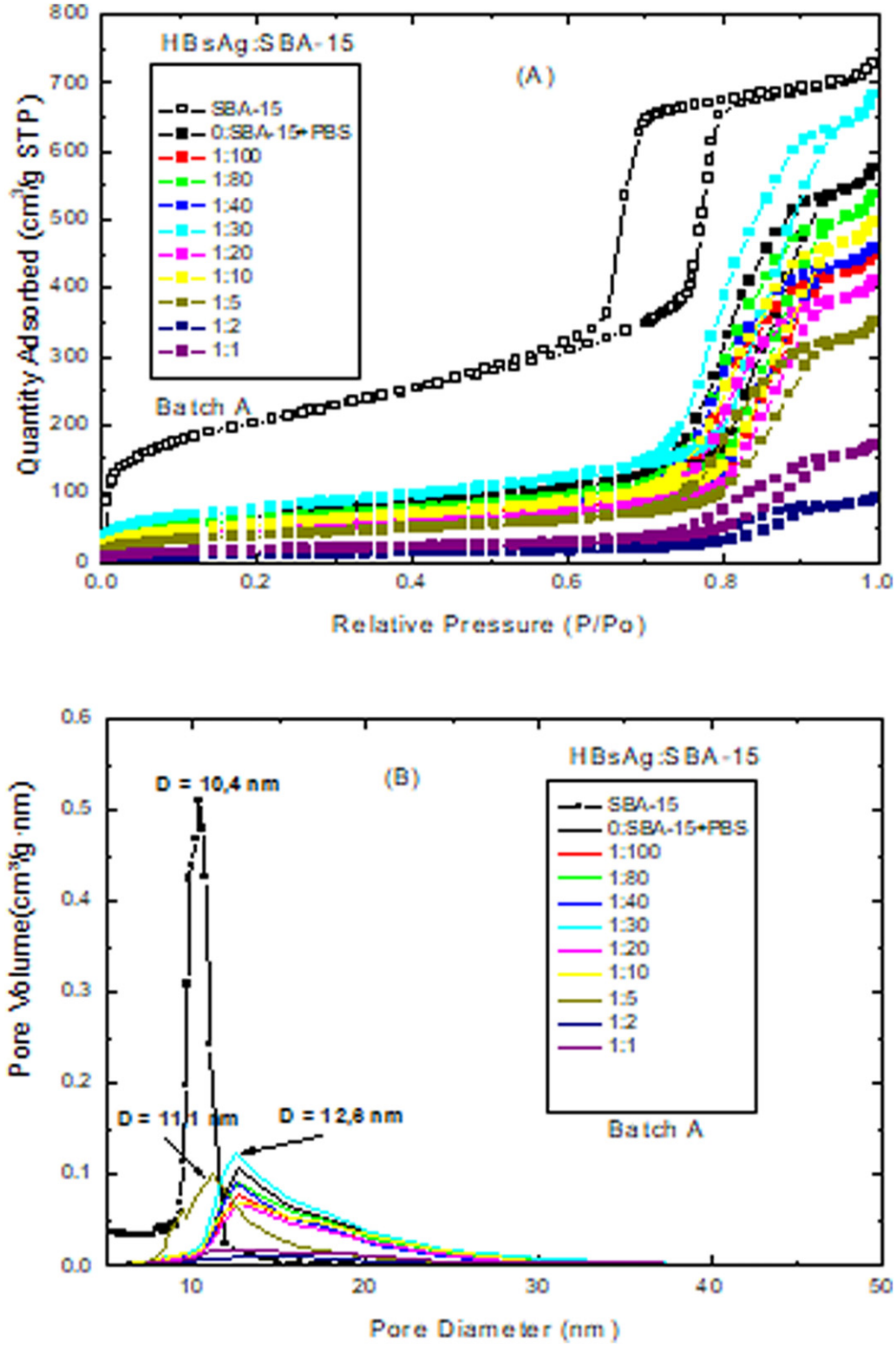

Figure 4. (A) Isotherms and (B) pore size distribution of the SBA-15 samples encapsulated with HBsAg and PBS.

Our challenges included the optimization of the encapsulation (imbibition process and loading degree), such that higher antibody titers could be reached even after the vaccine passed through the harsh gastric medium. Strategies to block protein denaturation can be achieved by using polymeric coatings insoluble in acidic medium and soluble in basic one, such that the oral immunizer could reach the intestine Peyer's patches.

The interface of this work with the research area of soft matter is related to the investigation of antigens in solution and their encapsulation inside the OMS amorphous silica matrix.

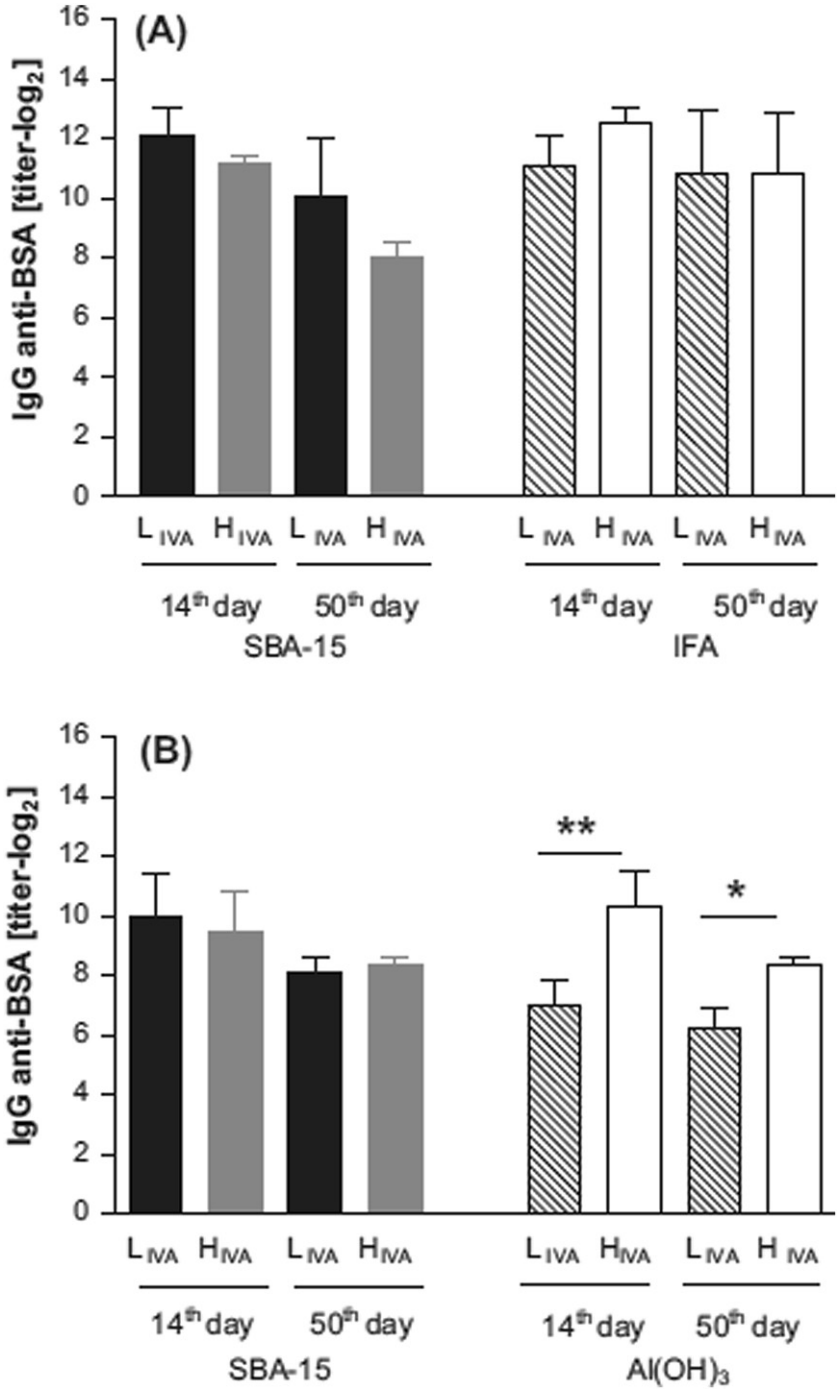

Figure 5. Anti-BSA IgG antibody production produced by selected mice for high (HIVA) and low (LIVA), $n = 4–5$ groups, antibody response of selection IVA. (A) Intramuscular route with SBA-15 and IFA and (B) Oral route with SBA-15 and $Al(OH)_3$. Reprinted from [5], Copyright (2010), with permission from Elsevier.

2. Experimental

The large part of bench top experimental tools used in this research is available in Brazil. The ones that require synchrotron and neutron sources were available through collaboration with a Brazilian scientist, working in Denmark. This fruitful collaboration improved especially the visualization of the antigens inside the OMS. This knowledge is important to determine the encapsulation process that results in homogeneous distribution of antigens without clustering, which is a condition that maximizes the production of antibodies, since the immune system cells have to reach as much as possible the antigen's surface.

The silica matrix was prepared according to the published recipe to obtain the SBA-15 structure [9, 14]. Synthesis processes that allow swelling of the mesopores to entrap larger antigens (>10 nm) were also investigated [7].

The detailed description of the characterization experimental apparatus was described in our previous papers, but it is important to identify the capabilities of each technique [4–13].

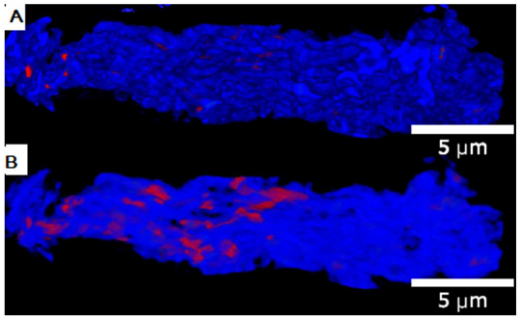

Figure 6. X-ray tomograms of the (A) surface and (B) interior of a SBA-15 particle encapsulated with HBsAg. Aggregates in the macroporous region are visualized in red. Reproduced from [11]. CC BY 4.0

Our most used experimental method is small angle x-ray scattering (SAXS), that, together with nitrogen adsorption isotherm (NAI), provide important structural and morphological parameters such as the pore mean size (∼10 nm), ordered porous structure lattice constant (∼12.5 nm), surface area (∼670 $m^2\ g^{-1}$), mesopore's volume without (∼1 $cm^3\ g^{-1}$) and with antigen load [6, 7]. By analysis of four different loading process of BSA in SBA-15 [6], using SAXS (not shown) and NAI results (figure S1 (https://stacks.iop.org/JPCM/34/264001/mmedia), table S1), indicated that the larger imbibition of BSA occurred when the mixture was kept at rest during evaporation of the liquid content (RE nomenclature in figure S1). Also, in order to evaluate the incorporation yield of proteins with different sizes, Human Gammaglobulin G (HGG) and Bovine Serum Albumin (BSA) proteins were incorporated into the mesoporous silica with expanded pores, as well as the phosphorous buffer saline solution (PBS), used in the encapsulation process. Again, the combination of SAXS (not shown) and NAI (figure S2, table S2) provided information about the incorporation of PBS inside the microporosity (pore diameter less than 2 nm), competing with the antigens for free space inside the mesopores (2 < pore diameter < 50 nm) and in the macro porous region (pore diameter > 50 nm). [7]. First of all, all these analysis have to be followed by SAXS data of the pristine antigens in buffered solutions, which give information on the antigen size, which is an important parameter to determine the possibility of incorporation inside the silica mesopores [11, 13].

These results are also cross-checked by dynamic light scattering, whose results must be consistent and complementary to SAXS, since aggregation process can be detected. Also, *in situ* SAXS experiments in different conditions, determine the time scale in a continuous liberation process of the antigens from the OMS [6–13].

The SAXS results are theoretically simulated by using a modified model [15], allowing information on variations in the electronic density contrast that indicates the encapsulation site (meso or macro), clustering in the OMS matrix or even in solution after release. The model presented in reference [15] was developed to simulate the formation of the silica network in solution, including the polymeric directing structure agent. Our model has to describe the powder OMS material after the removal of the surfactant by calcination, leaving open pores. Also, instead of loose micelles in solution, we had to include micropores in the dried silica wall [7]. SAXS experiments allow to verify the incorporation of antigens in dried powders, used in NAI measurements, but also can be utilized in *in situ* release of antigens from the silica matrix. These structural and morphological studies are complemented by direct visualization using scanning and transmission electron microscopies (SEM and TEM) [5, 11, 13], besides neutron and x-ray tomographies [11, 12].

The distribution of the antigen inside the silica matrix can be scrutinized by absorption spectroscopy at the carbon *K*-edge, together with scanning microscopy, because carbon is present only in the antigen formula [11].

Complementary techniques were utilized to better describe a specific property of the studied system, as Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry [9, 12, 13].

The structural integrity of the antigens must be checked in different pHs and temperatures in order to plan its distribution as a real vaccine. For this purpose a series of experiments were carried out using synchrotron radiation circular dichroism (SRCD) and intrinsic tryptophan (Trp) fluorescence spectroscopies [10, 13]. Finally, an important question is the toxicity of silica if retained inside the body.

In order to quantify the presence of silica in calcined mice organs (lungs, spleen, kidneys, liver) and faeces, proton induced x-ray emission (PIXE) was used to analyse the amount of silica retained after oral and injectable administration for a period of time that extended up to 70 days [6, 8]. All in all, the combined use of the several experimental techniques provides a detailed set of information on the studied system.

3. Results and discussion

The OMS material is formed by an amorphous silica matrix having various mesoscopic porosities in micro (<2 nm), meso (2 nm < pore dimension < 50 nm) and macro (>50 nm) scales, that allows entrapment of many different sizes of molecules and large aggregates, as illustrated in figures 1 (A) and (B).

Our first attempts to use OMS as an adjuvant proved its exceptional performance when compared to incomplete Freund adjuvant (IFA), because low responder mice line produced titers of antibodies as good as high responders, when inoculated by injection with Intimin β protein of *Escherichia coli* and venom proteins from the *Micrurus ibiboboca* snake [4].

The same type of remarkable result was obtained with the model protein BSA in PBS. The encapsulation site inside the silica matrix depends strongly on the antigen size and eventual clustering. Therefore, it is important to determine the size of the antigens in solution by SAXS (figure 2), or, when possible, by electron microscopy. In this case, it was concluded that the PBS solution containing BSA is formed by a mixture of monomers (66%) and dimmers (34%), having dimensions of 2.8 nm × 7.0 nm × 4.1 nm and 3.0 nm × 14.0 nm × 4.0 nm, respectively, proving that part of this protein solution can enter

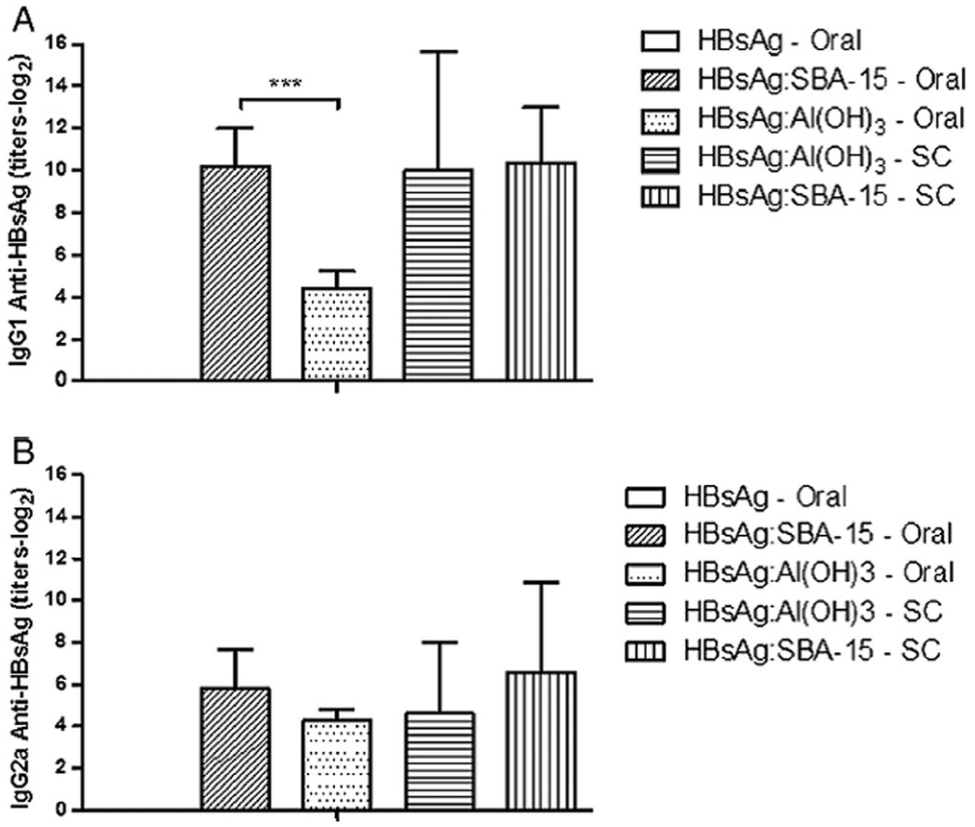

Figure 7. Immunization results of Balb C mice by means of subcutaneous (SC) and oral routes, using HBsAg antigen in SBA-15 and $Al(OH)_3$ vehicles. Reprinted from [8], Copyright (2016), with permission from Elsevier.

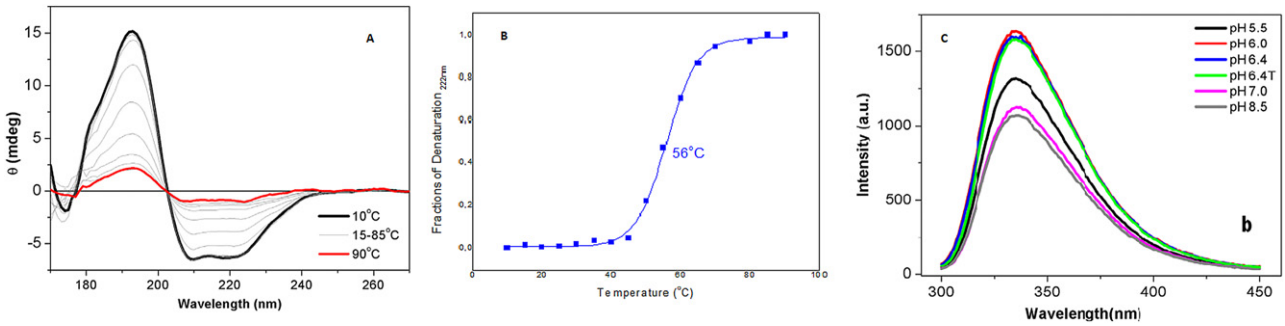

Figure 8. (A) Effect of temperature on the SRCD spectra of HBsAg antigen, and (B) its respective thermal denaturation curve. (C) Trp emission spectra of HBsAg at different pHs (from 5.5 to 8.5). Reprinted from [10], Copyright (2019), with permission from Elsevier.

the mesopore' cavities. The encapsulation of the protein was made by wet contact of SBA-15 with the BSA in the PBS aqueous solution.

Our first investigation with true antigens used in commercial vaccines focussed on the Hepatitis B surface antigen (HBSAg) virus like particle [8, 10–12]. More recently, the diphtheria anatoxin (d-ANA) was analysed [13].

Both antigens were encapsulated by wet imbibition in PBS. In the case of BSA, it was demonstrated that it fits inside the mesopores, once discarding the formation of clusters in an optimized protein concentration in PBS [5–7]. SAXS simulation of the experimental data confirms the presence of BSA inside the mesopores [7]. On the other hand, the size of the HBsAg virus like particles of ∼22 nm exceeds the SBA-15 mean mesopore diameter of ∼10 nm, as shown in figure 3. Therefore, this antigen is entrapped in the SBA-15 macroporosity.

The SAXS and NAI basic characterization techniques of the dried silica powder plus antigens were carried out. NAI results for different HBsAg:SBA-15 mass ratio (%) are shown in figure 4. These results were summarized in reference [8].

Regarding the efficiency of SBA-15 as an adjuvant, the results can be visualized in figure 5. In this study the presence of SBA-15 resulted in excellent antibody titers and it was compared with IFA and with the human certified aluminium hydroxide adjuvant, $Al(OH)_3$ [5–7]. Intramuscular and oral

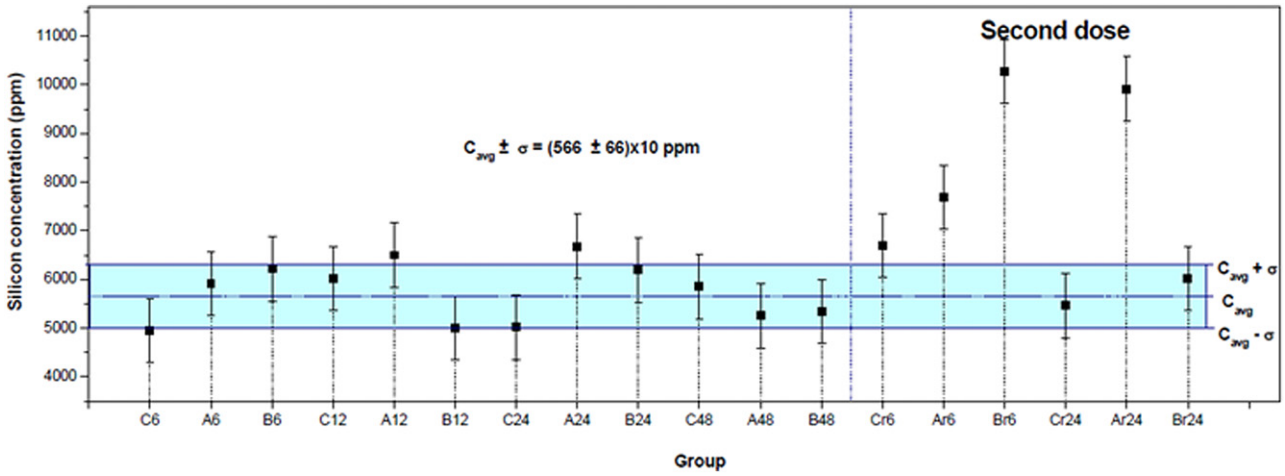

Figure 9. PIXE of silicon of mice faeces. Silicon concentration versus time of oral inoculation. Groups A and B received SBA-15 and the control group C received PBS. The symbol r stands for extra silica administration. The coloured area corresponds to the concentration detected for the control group. Reprinted from [8], Copyright (2016), with permission from Elsevier.

administrations were tested, opening up the possibility of using SBA-15 as an oral vehicle for real vaccines.

In order optimize the HBsAg concentration in the immunogenic complex and also to avoid aggregation, which decreases the immune cells contact with the antigen, a series of imaging techniques and absorption spectroscopy were used to visualize the HBsAg inside the macroporosity of SBA-15. Figure 6 depicts x-ray tomograms of a silica particle in which is evidenced the presence of the HBsAg inside the macroporosity. Large clusters of HBsAg are avoided by increasing the HBsAg:SBA-15 mass ratio up to 1:40.

Even at this less protected part of the OMS, in terms of contact with body fluids, the immunogenic complex achieves high titers of antibody [8] when administered in mice, as shown in figure 7.

Scanning transmission x-ray microscopy and near edge x-ray absorption fine structure were performed at the C *K*-edge (290 eV), and images with spatial resolution of 80 nm and photon energy of 390 eV, sensitive to the presence of carbon, were obtained. The results showed the presence of clusters but also of isolated HBsAg particles, that are responsible for better immunological response [11]. Finally, the release of HBsAg from the silica matrix was investigated by thermogravimetric analysis (TGA), FTIR and incoherent inelastic neutron scattering showing that there are different interactions of HBsAg with the silica matrix [12], providing it slow release from it along more than 6 h, detected by SAXS, which reinforces the efficiency of this adjuvant to induce high titers of antibodies.

The analysis about the viability to use HBsAg plus SBA-15 in an oral vaccine formulation was performed by SRCD and intrinsic tryptophan (Trp) fluorescence spectroscopies [10]. The SRCD method was used to check the secondary structure of the antigen protein in solution, whilst Trp fluorescence spectroscopy evaluated the aromatic residues exposure to aqueous solvent. A series of assays with these techniques examined the stability of the pristine antigen and inside SBA-15 at different temperatures and pHs [10], as shown in figure 8. The stability of the formulation is maintained for a 6 months storage period under refrigerated conditions (4 °C) or at room temperature (20 °C) and at extreme values of pH, from 5.5 up to 8.5. Also, the thermal melting point of HBsAg protein is close to 56 °C, but loss of vaccine potency and antigenic properties only occurs at 100 °C. These findings reinforced the use of this immunogenic complex as an oral vaccine. In conclusion, these techniques proved to be efficient for antigen characterization. For vaccine producers, this information is useful because it allows them to verify changes in the product over time, as well as provide subsidies for development and post licensure process changes.

Oral vaccines even though have advantages as 'safety and compliance, and easier manufacturing and administration', still presents important challenges, such as the bypass of the gastro acidic medium preserving protein integrity and the tolerance limit, that can be avoided [16].

A strategy that can be used in the case of oral administration is the introduction of an extra cap on top of the silica particles made of a polymer that has different solubility in acid and basic media, such that it can maintain the integrity of the antigen through the gastric fluid [6].

An important aspect of SBA-15 particles is that they preserve the phagocytic activity of macrophages, in a certain level of concentration, much lower than those needed for vaccination. The silica is eliminated through faeces, a remarkable aspect, preventing toxicity, as evidenced in figure 9, where the silicon concentration in mice faeces was determined by PIXE after 6, 12, 24 and 48 of oral administration.

The results clearly show that excess of silica is delivered probably with dead cells, and complete elimination is attained after 48 h.

The presence of silicon in mice organs, obtained by PIXE revealed that after 70 days, the silicon levels in inoculated mice, presented in figure 10, were similar to the control ones, remembering that animal feed contains silica, as well as many human provisions, even at negligible amounts.

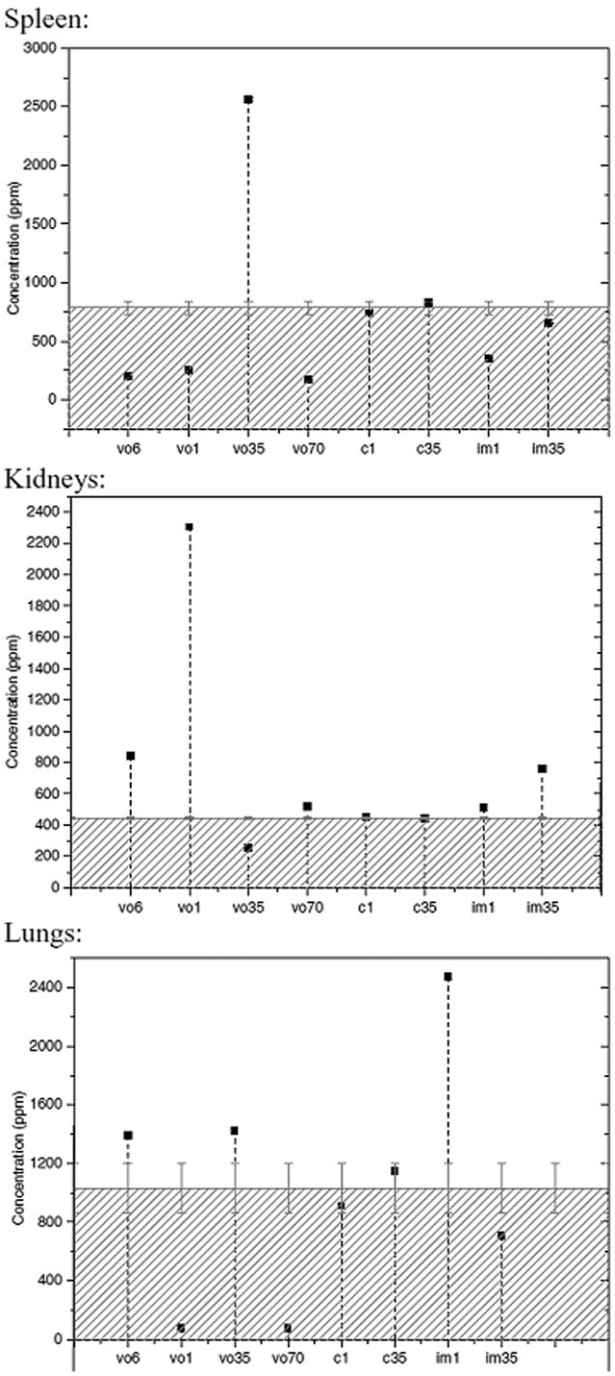

Figure 10. Pixe results of silicon concentration in spleen, kidneys and lungs for oral (vo) and intramuscular (im) administration as a function of time in days. Reproduced from [6]. © IOP Publishing Ltd. All rights reserved.

The silica particles are micrometric in size (mean values $\sim$20 μm), as depicted in figure 6, which is an important parameter to disregard nano safety issues [6, 8]. This characteristic together with the PIXE results prove that the proposed adjuvant is promising for future commercial oral vaccines.

The research on the incorporation of diphtheria anatoxin (dANA) [13], a much small antigen (maximum size of $\sim$9.2 nm), used the already described experimental methods to characterize the encapsulation yield and antigen release from the SBA-15 matrix. The NAI results evidenced dANA inside SBA-15 (figure S3). The TEM images showed the presence of the diphtheria inside the silica mesopores, giving to this complex an extra protection from any external liquid medium. This result corroborates the NAI data, that showed an almost complete filling of the mesopores for a dANA:SBA-15 mass ratio of 1:10.

The presence of the antigen inside the silica matrix was confirmed by SAXS of powder samples, as depicted in figure 11.

The simulation of the scattering curve is given by the general expression of the convolution between form and structure factors [15]:

$$I(q) = \mathrm{Sc}_1 P_{\mathrm{rod}} \langle F^2{}_{\mathrm{CS}}(q)\rangle (1 + \beta(q)[\langle Z(q)\rangle - 1]G(q)) + \mathrm{Sc}_2 I(q)_{\mathrm{chain}} + \mathrm{Sc}_{\mathrm{const}}. \quad (1)$$

With a resolution function $R(q)$, such that the experimental curve is given by:

$$\tilde{I}(q) = \int R(\langle q\rangle, q) I(q) \mathrm{d}q \quad (2)$$

where Sc_1 is the scale of the mesopores, P_{rod} is the form factor of the silica cylinder, F_{CS} is the cross sectional amplitude of the mesopores, $Z(q)$ is the lattice factor describing the spatial distribution of the mesopores, $G(q)$ is a Gaussian distribution related to distortion of the periodic lattice. Sc_2 is scale factor of the micropores and $I(q)_{\mathrm{chain}}$ is the scattering intensity due to micropores. The $\beta(q)$ factor is related to the polydispersity in pore diameter. The $\mathrm{Sc}_{\mathrm{const}}$ parameter represents a background. A nonlinear least-square method was used to fit the curves given by equation (1) to the experimental data. Detailed mathematical functions of this model are described in reference [7].

Successful SC and oral immunization using SBA-15 as the vehicle for dANA vaccine exhibited higher antibody titers compared with the classical aluminum hydroxide adjuvant. High thermal stability at pH 7 was observed for dANA secondary structure in PBS solution upon heating from 20 °C to 60 °C.

Again, like the results obtained with hepatitis B, the diphtheria oral vaccine using SBA-15 gave excellent figures, regarding stability, safety and antibody production (figure 12).

More recently this strategy of OMS adjuvant to produce oral immunizers was tested in a vaccine for respiratory disease of piglets, with promising results [17].

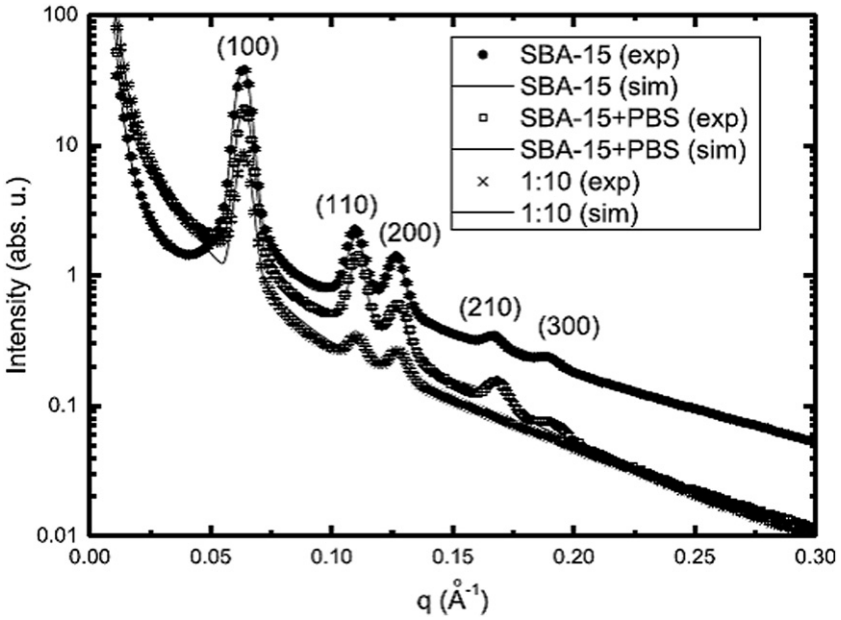

Figure 11. SAXS experimental results and simulated curves of SBA-15, SBA-15 + PBS and 1:10 powder samples.

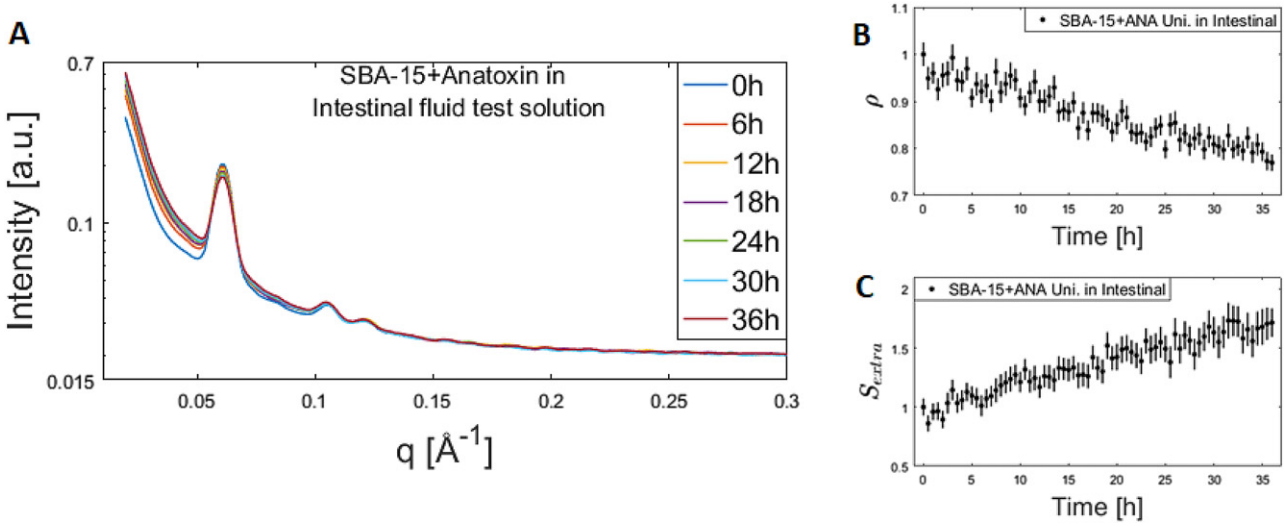

Figure 12. (A) SAXS data of dANA release from SBA-15 matrix in a intestinal test solution as a function of time, (B) electronic density contrast (ρ) and (C) extra scattering factor (S_{extra}) vs time from fitting of the experimental data.

4. Conclusions

Our results obtained during more than 15 years of research provided a robust database to conduct the evaluation of OMS as a vehicle for future oral vaccines. Recent encouring results were obtained with a diphtheria and tetanus polyvaccine, tested by intramuscular injection using SBA-15 as the antigen's vehicle. Also the influence of morphological characteristics of this silica, such as mesopore mean diameter, macroporous region dimension, silica cylinder and particle sizes are being under study to determine the influence such parameters on antigen load and release. Besides hepatitis B and diphtheria, our studies include tetanus, aiming to produce multivalent vaccines. Clinical trials have to be performed and we expect that the unusual and important experience obtained with clinical trials of Covid 19 vaccines could be a source of other protocols in our area of research. Not only more complicated human vaccines but also animal ones [17] can be explored in near future, relying in easier, friendly and low cost platform of oral immunization.

Acknowledgments

Authors are grateful to the Danish financial support (project 5132-00054B given to Dr Martin K Rasmussen). Thanks are due to CNPq, Brazil (CLPO, JLSL and MCAF are fellows) and FAPESP, Brazil (Grants 2017/17844-80 and 2018/19546-7). HNB acknowledge the support by the Danish Agency for Science, Technology and Innovation, European Synchrotron Radiation Facility, Paul Scherrer Institut, Villigen, Switzerland, for beamtime at PolLux of SLS (German Minister für Bildung und Forschung, contracts 05KS4WE1/6 and 05KS7WE1). We would like to thank the assistance during the

experiments of Alfredo Duarte, Julie Villanova and Benjamin Watts.

Data availability statement

The data that support the findings of this study are available upon reasonable request from authors.

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