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Nanostructured SBA-15 silica: An effective protective vehicle to oral hepatitis B vaccine immunization

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Abstract

Due to its physicochemical properties, nanostructured mesoporous SBA-15 silica shows great potential as a vaccine adjuvant. This study evaluated the capacity of SBA-15 to encapsulate/adsorb the recombinant purified HBsAg from the Hepatitis B virus and the immunoresponsiveness of mice orally immunized with HBsAg inside SBA-15. A simulation of small angle X-ray scattering experimental results, together with the nitrogen adsorption isotherms data, allowed to determine the appropriate mass ratio of HBsAg:SBA-15, indicating antigen encapsulation into SBA-15 macroporosity. This was also evaluated by bicinchoninic acid assay and gel electrophoresis. The recruitment of inflammatory cells, an increase in production of specific antibodies, and the non-influence of silica on T_H1 or T_H2 polarization were observed after oral immunization. Besides, SBA-15 enhanced the phagocytosis of ovalbumin by dendritic cells, an important key to prove how this adjuvant works. Thus, it seems clear that the nanostructured SBA-15 is an effective and safe adjuvant for oral immunizations.

Key words: Nanostructured SBA-15 silica; Oral immunization; Adjuvant/vehicle; Vaccine

The SBA-15 silica is an inorganic material with ordered channels of uniform hexagonal nanostructured mesopores (pore diameter between 2 nm and 50 nm, *International Union of Pure and Applied Chemistry, IUPAC*) measuring approximately 10 nm in diameter. Described in 1998, these 20 μm particles of amorphous silicon oxide show great potential as an adjuvant. Despite several studies focused on the use of this silica as a vehicle for other substances, it has not been tested for its ability to efficiently activate antigen presenting cells (APC) and carry and release antigens.[1–5]

A large number of biological applications of ordered mesoporous silica (OMS) is found in the literature. The first study regarding the adjuvant effect of SBA-15 showed that the isogenic BALB/c and heterogeneous genetically selected Low responder mice[6] immunized with distinct immunogens encapsulated/adsorbed in SBA-15, such as the 16.5 kDa *Escherichia coli* Intimin 1β protein or *Micrurus ibiboboca* snake venom, presented an increase in the specific antibody titers, besides inducing equivalent or higher humoral response compared to immunizations using the traditional adjuvants.[7] Moreover, SBA-15 proved to be safe and non-toxic, and modulated

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positively the humoral immune response of two Low responder mouse lines, which after subcutaneous or intramuscular immunizations with bovine serum albumin (BSA), produced IgG titers as high as the High responder mouse lines.[7,8] Other researches also proved the applicability of OMS as vaccine adjuvant,[9–14] reinforcing the importance of our pioneer work for its use in oral vaccination. Other important aspects of OMS use for human and animal health are related to the silica toxicity[15] biocompatibility[16] and *in vivo* bio-distribution.[17]

Since the natural route of most infections is the intestinal mucosa, the administration of antigens through this can mimic the induction of natural protection. Oral immunizations can be particularly advantageous due to its easy administration and minimal side effects. Unfortunately, environmental factors, such as the harsh gastric environment, enzymatic and intestinal epithelium barriers, and pH variations, may lead to antigen denaturation with the destruction of epitopes in the intestinal lumen and decreased capture of antigens by *lamina propria* (LP) antigen presenting cells (APC) and M cells at the Peyer's patches (PP), therefore impairing significantly the immune response.[18–25] Thus, it's relevant to develop new mucosal adjuvants/vehicles that effectively stimulate the immune system and modulate the response of low responder individuals without inducing a bias towards T_H1 or T_H2, and that are safe, economically viable, and easily handled.[26–36]

Most infectious agents enter the body through mucosal surfaces and, therefore, the endothelial respiratory and alimentary tracts' immune responses act as a first-line of defense. Protective responsiveness is most effectively induced by mucosal immunization through the oral or nasal routes, but the vast majority of vaccines in use is administered by injection and can be associated with high cost and discomfort. Therefore, alternative routes for immunization, such as the natural oral one, should be investigated.

Successful oral immunization must overcome several barriers during its progression through the gastrointestinal tract.[37] Taking into account the gastrointestinal natural barriers among the vehicles, the nanostructured silica seems very attractive.

The properties of silica that qualify it as a proper system for oral vaccines are: its large porous surface with wide pores, which can be filled with antigen; its resistance to the effects of gastric juice, maintaining immunogenic properties; and finally an optimal nanoparticle size that enables phagocytosis in antigen presenting cells.

Herein, the applicability of SBA-15 as an adjuvant in oral immunizations to Hepatitis B antigens is presented. The high levels of specific antibodies and the increased numbers of innate immune cells found in the gut associated lymphoid tissue (GALT) after oral immunizations supported our hypothesis that the incorporation of antigens to this mesoporous silica protects the main epitopes. SBA-15 is captured by the APC, leading to the stimulation and the efficacious enhancement of immune responsiveness.

Methods

Animals

Female 8 to 12 week-old BALB/c mice, supplied by Butantan Institute animal facilities, were used to evaluate the adjuvant effect of SBA-15 silica toward oral immunizations. The experiments were conducted according to protocols approved by the Animal Use and Care Ethics Committee from the Butantan Institute (Protocol 672/09) and under ethical conditions according to the international rules of animal care by the International Animal Welfare Recommendations.[38]

SBA-15 silica synthesis and physical characterization

Mesoporous SBA-15 silica samples were synthesized as described by Matos et al.[3] The Small Angle X-ray Scattering (SAXS) of pristine and encapsulated SBA-15 samples was measured in a Bruker AXS Nanostar equipment, with a bi-dimensional HiStar detector or in a Xenocs Xeuss set-up with a Pilatus bidimensional detector, using the same wavelength (Cu Kα radiation, $\lambda = 0.1542$ nm). The samples with recombinant HBsAg protein diluted in PBS were also analyzed in a diamond anvil cell with a pressure of 0.45 GPa in order to promote a higher and more efficient encapsulation/adsorption of the molecules inside the SBA-15 matrix. The nitrogen adsorption isotherm (NAI) measurements were performed in a Micromeritics ASAP 2020. The BET (Brunauer-Emmett-Teller) method was used to calculate the pore surface area, the BJH (Barrett-Joyner-Halenda) method determined pore volume and pore size distribution, and t-plot methods calculated the micropore (<2 nm, *IUPAC*) volume.

Incorporation of HBsAg in SBA-15 silica

The encapsulation and/or adsorption of the purified HBsAg in SBA-15 were determined by *in vitro* tests. Briefly, the HBsAg (4.72 mg/10 mL) was added v/v in different proportions to SBA-15 (1:2 to 1:100) diluted in phosphate buffer saline (PBS) solution at a pH 7.4 in a final volume of 500 mL, and maintained at 4 °C for 24 h. The non-encapsulated/adsorbed protein was removed by decantation and the HBsAg concentrations in the supernatant were determined by bicinchoninic acid (BCA) kit (Thermo Scientific Pierce, Rockford, IL, USA) and spectrophotometric measurement at 562 nm. As control, a solution with HBsAg (5 mg/10 mL), SBA-15 and PBS was used. The samples of HBsAg (10 μg/well) with or without different proportions of SBA-15 (HBsAg:SBA-15 of 1:2 to 1:100 in PBS) were also analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) performed by using 12.5% polyacrylamide slab gel sand stained with Coomassie brilliant blue to visualize the protein encapsulation.

HBsAg protein on SBA-15 silica was fully analyzed using samples with the mass ratio 1:10 (0.5 μg HBsAg:5 μg SBA-15). Samples with HBsAg were prepared using the HBsAg received in a concentration of 200 UI/g of HBsAg to SBA-15. Other ratios (1:2 up to 1:100) were analyzed by SAXS, NAI, BCA method, and electrophoresis.

Immunizations with HBsAg

BALB/c mice (n = 5/group) received either by subcutaneous (s.c) injection or gavage (oral) 0.5 μg of the HBsAg adsorbed or not in SBA-15, or in $Al(OH)_3$ in a ratio of 1:20 in a final volume of 0.25 mL PBS. The immunogen was mixed in a ratio of 1:10 antigen:SBA-15, v/v (0.5 μg and 5 μg, respectively); the

mixture was kept at rest at 4 °C for 24 h before immunizations. As control, we evaluated specific antibody levels in non-immunized animals. The s.c. immunization with the HBsAg was diluted at the ratio of 1:20 in PBS for further comparative analysis. The second dose was given 30 days after the first immunization by the same route. After 14 days, individual blood samples were collected for anti-HBsAg antibodies titration by enzyme-linked immunosorbent assay (ELISA).

Fecal pellet extract collection

Fresh fecal pellets from orally immunized mice were collected and weighed. Five mL of 1 mM phenyl-methyl-sulphonyl fluoride (PMSF) (Sigma-Aldrich Biotechnology Co., St. Louis, MO, USA) enzyme inhibitory solution, BSA 1% in PBS *per* 1 g of fecal pellet was added. After 15 min, the material was vigorously vortexed and samples were centrifuged at 20,000 *g*/10 min at 4 °C.

Specific antibody titrations

To determine specific anti-HBsAg titers by ELISA,[39] 96-well microplates with high binding properties (Maxisorp Nunc International, Rochester, NY, USA) were coated with 1 μg/mL of the corresponding antigen diluted in carbonate-bicarbonate buffer pH 9.6 (0.05 M Na_2CO_3 and 0.05 M $NaHCO_3$) for 16 h at 4 °C. Titers were calculated neglecting 20% of saturation dilution absorbance and expressed as $\log_2$.

A double sandwich ELISA was done to measure the anti-HBsAg IgG1 and IgG2a antibodies. After incubation with the samples, anti IgG1 and IgG2a antibodies (Sigma®, St. Louis, USA) diluted at 1:1000 and 1:500, respectively, were added to the plate. The same washing procedure was repeated and peroxidase-labeled anti goat IgG (KPL, Maryland, USA) diluted 1:3000 was added.

Phagocytosis assay with labeled fluorescein isothiocyanate (FITC) ovalbumin

To evaluate if SBA-15 silica is able to enhance the phagocytosis of soluble antigens such as ovalbumin (OVA) by dendritic cells (DC), 5×10^5 BMDCs were incubated for 2 h at 37 °C, 5% CO_2 in 1 mL of complete RPMI media with labeled OVA-FITC (5 μg) (Invitrogen, Eugene, OR, USA) or OVA-FITC:SBA-15 (5 μg:125 μg). After incubation and consecutive washes, cells were stained with anti-CD11c (APC), anti-MHCII (V450), and the death cell marker 7-AAD (PerCP) to evaluate the amount of OVA inside the DC by flow cytometry and confocal microscopy.

In vitro generation of dendritic cells

Bone marrow cells from inbred BALB/c mice were collected from femurs and tibias through successive washes using syringe and hypodermic needles with complete RPMI media (10% fetal calf serum, 2 mM L-glutamine, 100 μg/mL penicillin, 100 μg/mL streptomycin, 1.25 μg/mL fungizone and 1 μL/mL β-mercaptoethanol at 50 μM (Gibco, Grand Island, NY)). After centrifugation at 400 *g* for 5 min, cells (5×10^6) were cultured in Petri dishes for seven days in 10 mL of complete media containing 1 μL of GM-CSF (100 μg/mL) (Peprotech, Rocky Hill, NJ) at 37 °C, 5% CO_2. After three days, cells were supplemented with more media and 1 μL of GM-CSF (100 μg/mL). After one week, the differentiated dendritic cells from bone marrow (BMDCs) were used in the tests.

Flow cytometry

The BMDCs treated with labeled OVA, were characterized using a FACScalibur and FACSCanto flow cytometer (Becton Dickinson, San Jose, CA, USA). Cell staining (2-5 × 10^5 cells/mL) was performed using the following monoclonal antibodies (BD PharMingen, San Diego, CA, USA) at appropriate concentrations as determined by titration: mouse $IgG_{2b,\kappa}$ anti-rat CD11b (clone M1/70), mouse $IgG_{1,\lambda 2}$ anti-Armenian hamster CD11c (clone HL3), mouse IgG_{2a} anti-rat MHCII (clone M%/114.15.2). To reduce nonspecific antibody binding, Fc block hamster anti-mouse CD16/CD32 (clone 2.4G2) was included in cell surface staining. The death cell marker 7-AAD (BD PharMingen, San Diego, CA, USA) was used to exclude dead cells from the analysis. Data were analyzed for at the least 10,000 events with BD Cell Quest® version 3.3 (Becton Dickinson, San Jose, CA, USA) or using the software FlowJo (Tree Star Inc., Ashland, OR, USA) and the results were expressed as mean values ± standard deviation of the numbers of positively stained cells in the population gated in at least two independent experiments.[40]

Confocal microscopy

Glass slides with 5×10^4 BMDCs from different treatments were obtained by cytocentrifugation (400 *g* for 5 min). After washes in cold acetone and PBS, a drop of Vectashield Mouting media with DAPI (Vector Laboratories, Burlingame, CA, USA) was added to each sample for nuclei staining. OVA-FITC phagocytosis by BMDC was visualized with Apotome Imager.ZI microscope and the images were acquired by the digital Carl Zeiss camera AxioCam MRM and the software Axiovision version 4.7.2.

Statistical analysis

Results were expressed as mean values ± standard deviation. Statistical significance was determined by unpaired *t* test or two-way ANOVA and post-test Bonferroni and set at $P < 0.05$ using GraphPad Prism 4.0 software (La Jolla, CA, USA).

Proton induced X-ray emission (PIXE) experiments to analyze the presence of SBA-15 in the feces after oral administration

The proton induced X-ray emission (PIXE) experiments were performed at LAMFI (Laboratory of Material Analysis by Ionic Beams; USP), and studied as described in Reference.[1] BALB/c mice excrements were collected from 9 mice and divided in three boxes, and 6 mice were inoculated with SBA-15. A second immunization with a time interval of 7 days was also performed and the PIXE results were analyzed. A control group (C) of 3 mice was used to determine the silicon standard level. Animals in the experimental Groups A (n = 3) and B (n = 3) received, by gavage, 1 mg of SBA-15 diluted in 200 μL of PBS or only 200 μL of PBS (control Group C, n = 3). Their excrements were collected after 6 h, 12 h, 24 h and 48 h. After 6 h of administration, each group was placed in clean boxes for a period of 30 min and all the excrements produced during this

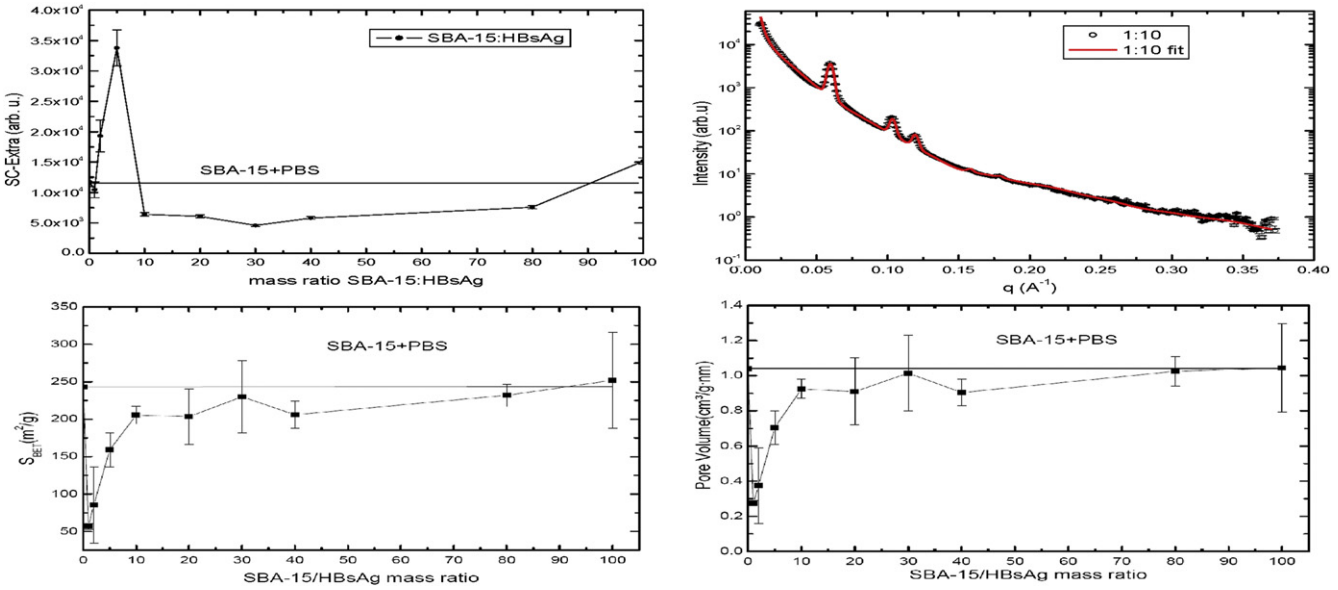

Figure 1. Small Angle X-ray Scattering (SAXS) and Nitrogen Adsorption Isotherm (NAI) results of HBsAg encapsulated in SBA-15. *SC-Extra* parameter obtained from simulation of SAXS curves (for example, right),[1] related with HBsAg particles. Surface area (S_{BET}) and pore volume versus the mass ratio SBA-15/HBsAg.

period of time were collected. Similar procedures were performed 12, 24, and 48 h following the SBA-15 administration. The excrements were inserted into *Falcon* tubes and kept at 4 °C until their use. Seven days after the first administration, the animals received again the same amount of SBA-15 and/or PBS and their excrements were collected after 6 h and 24 h. The PIXE analyses were performed in samples heat treated at 900 °C in air for 2 h, in order to remove the organic compounds.

Results

Incorporation analysis of HBsAg into nanostructured SBA-15 silica

Considering the dimension of HBsAg around 22 nm with more than 3000 kDa of molecular weight,[41, 42] it is reasonable to suppose that the proteins are encapsulated inside the silica macroporosity (macropores larger than 50 nm, *IUPAC*), obstructing the entrance of the 10 nm mesopores.

Figure 1 presents the SAXS results, in particular, including the extra scattering factors (*SC-Extra*) related to the HBsAg presence in the silica matrix, according to the SAXS model of Reference,[1] as well as the simulation of the experimental SAXS result for the sample having a 1:10 HBsAg:SBA-15 mass ratio, as an example of fit quality. All the results were compared to the parameters obtained from the sample embedded with PBS, which penetrates the micropores and mesopores. In the same figure, the NAI results are shown for all analyzed samples. As determined from the change in the *SC-Extra* parameter, for larger HBsAg:SBA-15 mass ratios (1:5, 1:2 and 1:1), there is an agglomeration of HBsAg particles, which are not protected by the silica macroporosity, confirmed also by the increase in the gyration radius (data not shown). The surface area and pore volume values for samples having larger HBsAg:SBA-15 mass ratios (1:5, 1:2 and 1:1) present an abrupt decrease, indicating that at least an HBsAg:SBA-15 mass ratio of 1:10 has to be used to guarantee that the antigen is encapsulated inside the material macroporosity. Also, powder samples prepared with an HBsAg:SBA-15 mass ratio equal to 1:10, were analyzed by SAXS in order to verify the influence of pressure (sample identification is p) on the encapsulation process.

Figure 2, *A-C* presents the results of different scattering vector ranges. Figure 2, *C* shows the diffraction peaks after a straight-line background removal in order to evidence the effect of molecule encapsulation. The diffraction peaks were fitted by a pseudo-Voigt function and their areas were computed and compared. The scattering of the original SBA-15 sample increased with pressure due to the densification of the material. The ordered mesoporous structure was not destroyed, evidencing the possibility to produce SBA-15 pills for oral vaccination. On the other hand this effect of scattering intensity increase was not observed in the sample containing HBsAg. This result is attributed to the size of these particles (~22 nm) being larger than the SBA-15 mesopores, which have a mean diameter around 10 nm.[1] HBsAg should be encapsulated in the macroporosity of the silica matrix, obstructing the entrance of the mesopores and decreasing the electronic density contrast under pressure.

Other results related to the encapsulation capacity of SBA-15 were obtained by BCA analysis and electrophoresis. Around 50% of HBsAg was incorporated inside the silica matrix for a 1:10 dilution of HBsAg:SBA-15. Our data suggest that a higher amount of SBA-15 is necessary to incorporate the total concentration of the antigen. Larger capacities were measured for higher silica content, as depicted in Figure 3, *A*. In Figure 3, *B* the electrophoresis analysis performed with the supernatant collected after the incubation of the HBsAg with different amounts of SBA-15 shows that the antigen is

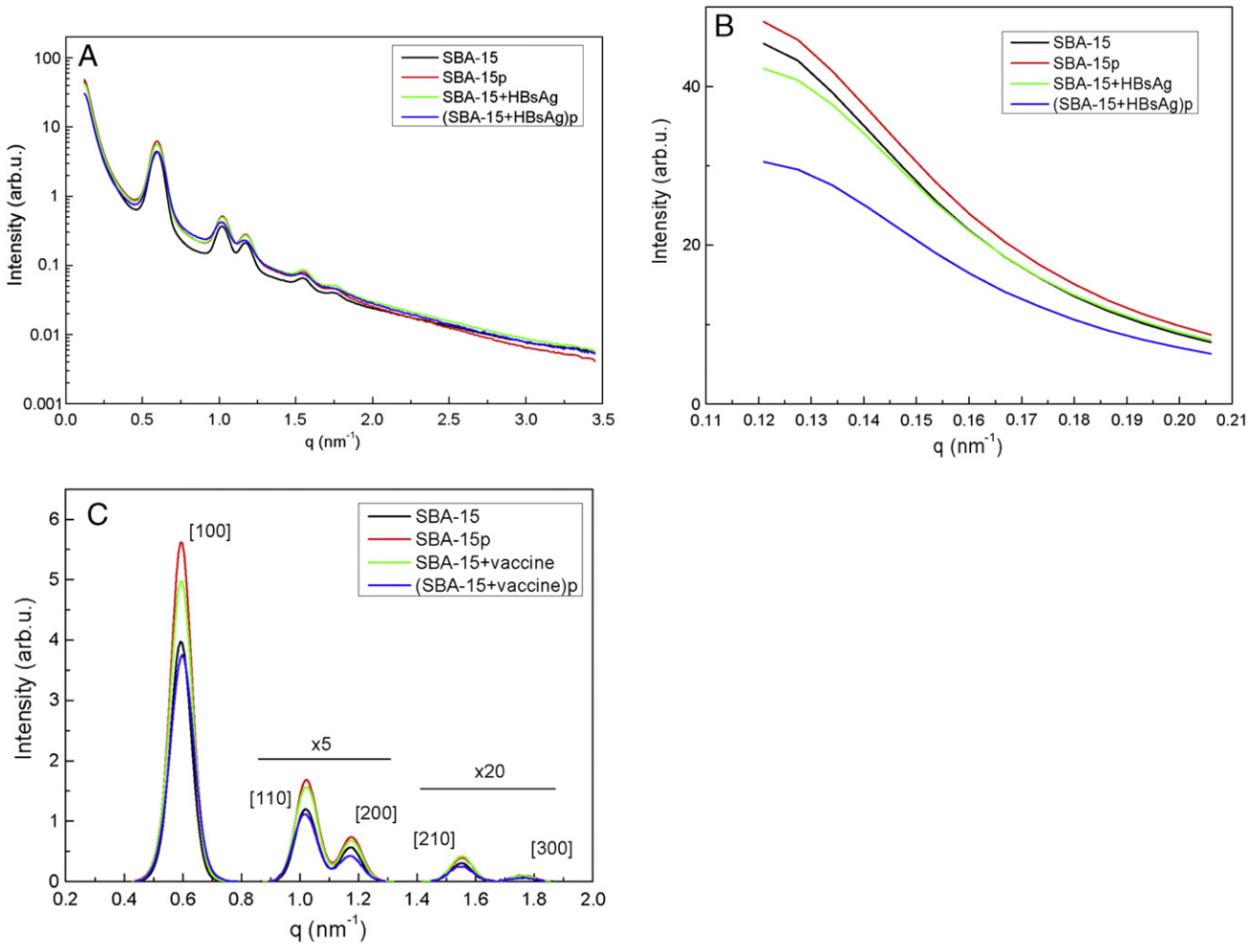

Figure 2. Effect of pressure on the structure of SBA-15 and SBA-15 plus HBsAg (1:10). (**A**) SAXS curves of SBA-15 and SBA-15 plus HBsAg in regular sample holder and under pressure (p). (**B**) SAXS curves of SBA-15 and SBA-15 plus HBsAg in regular sample holder and under pressure (p). (**C**) SAXS peaks after background removal of SBA-15 and SBA-15 plus HBsAg in regular sample holder and under pressure ("p" suffix).

completely encapsulated in the silica in the proportions higher than 1:40. Therefore, even though all the biological tests were successfully performed with a mass ratio of 1:10, the present results suggest, for future human use, an increase of the silica mass in order to promote total protein encapsulation.

Specific anti-HBsAg antibody production after oral immunizations

The immunization schedule with only subcutaneous or oral administrations presented no differences in IgG production 14 days after subcutaneous booster using Alum (>11 $\log_2$ in 4 mice while 1 mouse showed no response) or SBA-15 (>11 $\log_2$, 8 $\log_2$, 6 $\log_2$, 6 $\log_2$ and 11 $\log_2$) indicating that the two adjuvants were equally effective (data not shown). Oral immunizations with silica presented IgG1 (13 $\log_2$, 9 $\log_2$, 9 $\log_2$, 11 $\log_2$, 9 $\log_2$) and IgG2a (9 $\log_2$ 2 $\log_2$, 5 $\log_2$, 6 $\log_2$ and 7 $\log_2$) titers significantly high ($P < 0.001$, in relation to the group immunized with HBsAg in PBS where no specific antibodies were detected. Surprisingly, the antibody titers in mice immunized subcutaneously were similar to those of animals immunized orally with silica (Figure 4).

Phagocytosis assay

To verify whether SBA-15 silica enhances the phagocytosis of dendritic cells, OVA-FITC labeled pre-incubated or not with silica has been tested to quantify and visualize the phagocytosis. Data obtained by confocal microscopy and flow cytometry clearly show an increase in percentage and mean fluorescence intensity in OVA-FITC$^+$CD11c$^+$ BMDCs in the group with SBA-15 (Figure 5). It must be stressed that this SBA-15 silica is not cytotoxic.

Proton induced X-ray emission (PIXE)

Figure 6 depicts the silicon concentration in mice excrements. Groups A and B are identical and received silica. Group C is the control group and only received PBS. The number after the letters indicates the time of material uptake. The results clearly evidenced the silicon release in part per million (ppm) after the re-administration, since the amount of silicon is above the range detected for the control group. Also, it was verified that the silicon is constantly eliminated. The significant difference between groups A/B and C after re-administration is perhaps

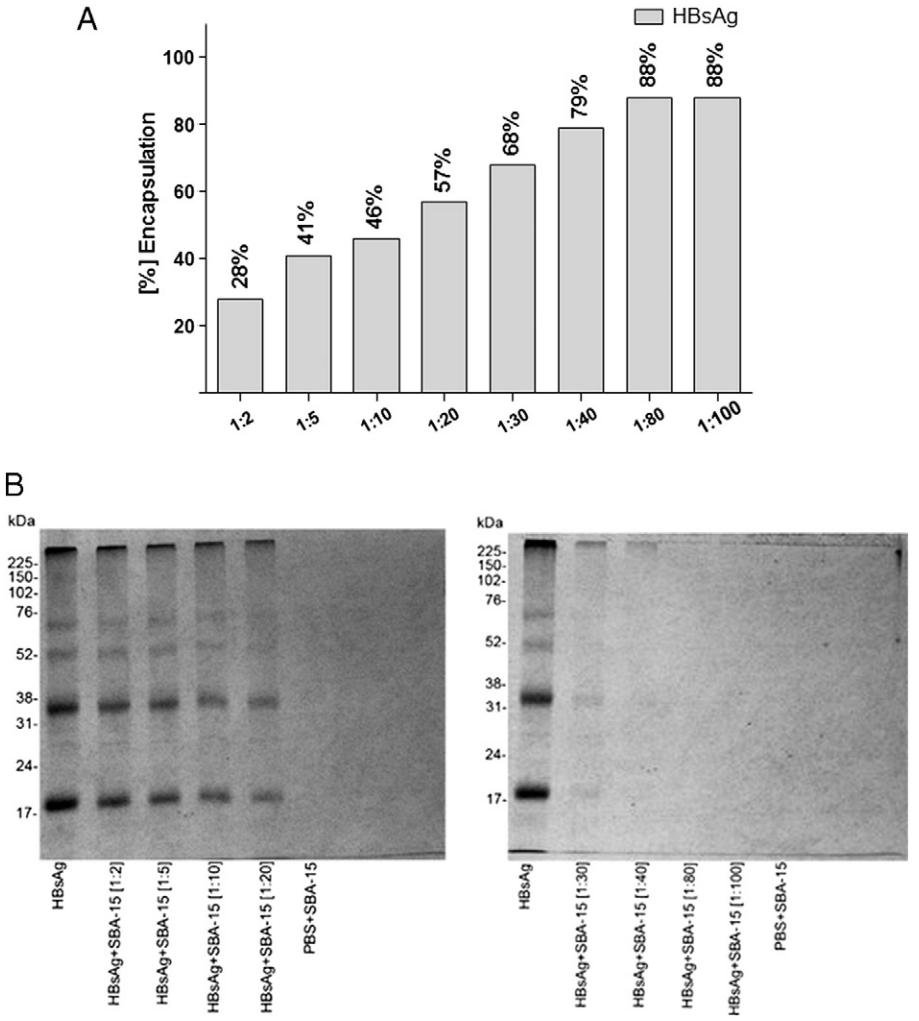

Figure 3. Analysis of the encapsulation of HBsAg in SBA-15. (**A**) The HBsAg samples with increasing dilution of SBA-15 and PBS used with negative control. The samples were centrifuged and the supernatant analyzed by BCA kit. (**B**) Samples (10 μg/mL) of HBsAg with increasing dilution of SBA-15 and/or PBS used with negative control. The samples were centrifuged and the supernatant solubilized in non-reducing sample buffer to analyze by SDS-PAGE of the 12.5%. The gels stained with Coomassie blue R250.

related to silicon accumulation in antigen presenting cells, and subsequent elimination.

Discussion

Nature itself is definitively the major agent of vaccination, and the most important and efficacious memory promoter. Silica is the second most common element on earth, present in all environments, and as ordered nanostructures, it constitutes a variety of diatomaceous algae species, abundant in aquatic environments. The physical chemical characterization of the SBA-15 particles loaded with HBsAg was important to show the presence of the proteins in the silica macroporosity, as well to point out that a mass proportion of Antigen/SBA-15 of 1:40 is recommended for eventual future preparations to be administered as an oral vaccine.

Experimental data suggest the participation of the APC in increasing the epitopic recognition, leading to an efficient memory induction of T and B lymphocytes and prevention of clonal deletion and anergy, which are regulated independently by polygenes. Ineffective vaccination schedules, from the qualitative and quantitative viewpoint would act as selective agents for many characteristics of a pathogen or analogous microorganisms; and the quantitative traits such as virulence, toxicity, infectivity degrees, and repertory of receptors, phenotypes that are under modulation by the immune system, could negatively modify the natural history of the relationships between microorganisms and host species.

Considering that the mucosa is the natural locus of sensitization to a massive variety of antigens, artificial oral vaccinations could reduce the impact of environmental factors, modulating the phenotypes of a given pathogen. Moreover, among these factors, the adjuvants play important roles on the capacity and intensity of immuneresponsiveness and memory induction to pathogens.

Until now, few orally administrated vaccines were licensed for human use, such as those against poliomyelitis and rotavirus,

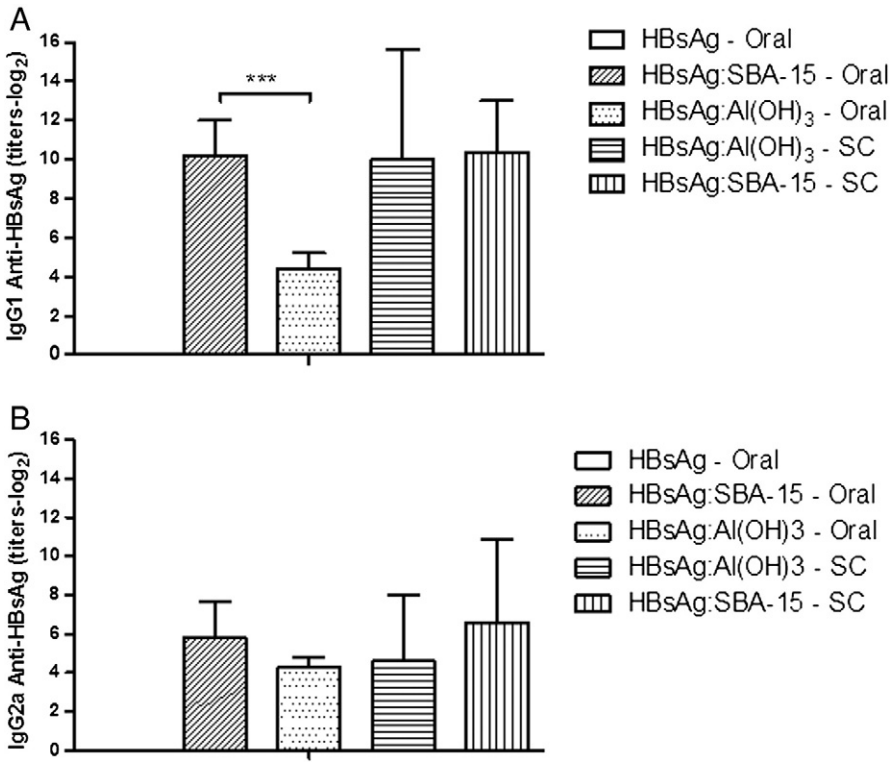

Figure 4. Secondary IgG1 and IgG2a anti-HBsAg production in BALB/c mice subcutaneously or orally immunized.BALB/c mice subcutaneously or orally immunized with 0.5 μg HBsAg, 0.5 μg of HBsAg: 5 μg SBA-15 or 0.5 μg of HBsAg: 6.25 Al $(OH)_3$. (**A**) IgG1 [***$P < 0.001$] (**B**) IgG2a antibody titers.

both being highly immunogenic attenuated vaccines.[20,22,27] The development of mucosal adjuvants or carrier systems is extremely important to protect antigens from degradation within the gastrointestinal tract, allowing them to reach immunological sites. The effective stimulation of the APC, mainly dendritic cells, will ascertain the positive modulation of antigen-specific B and T lymphocytes and ensure a long-term and systemic immunity.[18–29,31,33,43,44] Previous studies demonstrated the effectiveness of SBA-15 silica as adjuvant and the proposal of using it in oral immunizations was due to its structural characteristics and physicochemical properties.[1,7,8] Here, the efficiency of this macro and mesoporous silica as a vehicle for this route was presented, and the environmental variances were lower in comparison with those of the subcutaneously immunized groups (Figure 4).

The incorporation analysis showed that the protein HBsAg with more than 3000 kDa and nearly 22 nm in diameter was efficiently encapsulated and retained in the macroporosity of SBA-15 silica, which improved the specific immune response against such antigens.

Another interesting aspect to be analyzed is that particles with sizes between 2 and 5 μm are easily captured by the M cells at PP, while particles measuring 2 μm or less and the ones larger than 10 μm are quickly drained to the MLN.[18,19] SBA-15 silica particles have a mean diameter of approximately 20 μm,[1] suggesting that they are not captured by M cells and can be applied exclusively as a carrier system, thus allowing the capture of antigens by the APC from *lamina propria* and MLN. When drained to these organs, antigens encapsulated/adsorbed to SBA-15 can be protected and released, stimulating the development of local immune responses.

Increased levels of specific systemic antibodies establish that this silica is effective in inducing a general and local responsiveness without polarizing it to a certain class of T_H lymphocytes. The differentiation of T_H lymphocytes in distinct subsets of effectors cells depends on cytokines, the nature of the antigens, the infection route, and the activation of innate immune cells as well as the use of adjuvants.[30,37] With regard to immunizations, the search for an adjuvant that does not affect the polarization of the immune response is of great interest. Regularly, the oral mucosal microenvironment tends to induce a T_H2 response due to the predominant concentration of cytokines such IL-4, IL-5 and IL-10, especially after infectious processes or immunizations with proteins. Nevertheless, this polarization may not be suitable in protecting against certain infections like those that predominantly require a T_H1-type response. In the absence of infections, mucosal $CD103^+$ DC produces IL-10 and TGF-β keeping the T_H3 profile and suppressing the T_H2 responses.[24]

Moreover, commensal microorganisms and antigens from constant feeding stimulate the immune system resulting in the production of antibodies, sometimes undetected or creating oral tolerance.[25] Thus, it is expected that vaccination processes stimulate the production of protective antibodies and the induction of specific and long lasting immunological memory.

Naturally, the immune response against pathogens also happens at PP. The interactions between B and T lymphocytes occur in its germinal centers stimulated by cytokines responsible

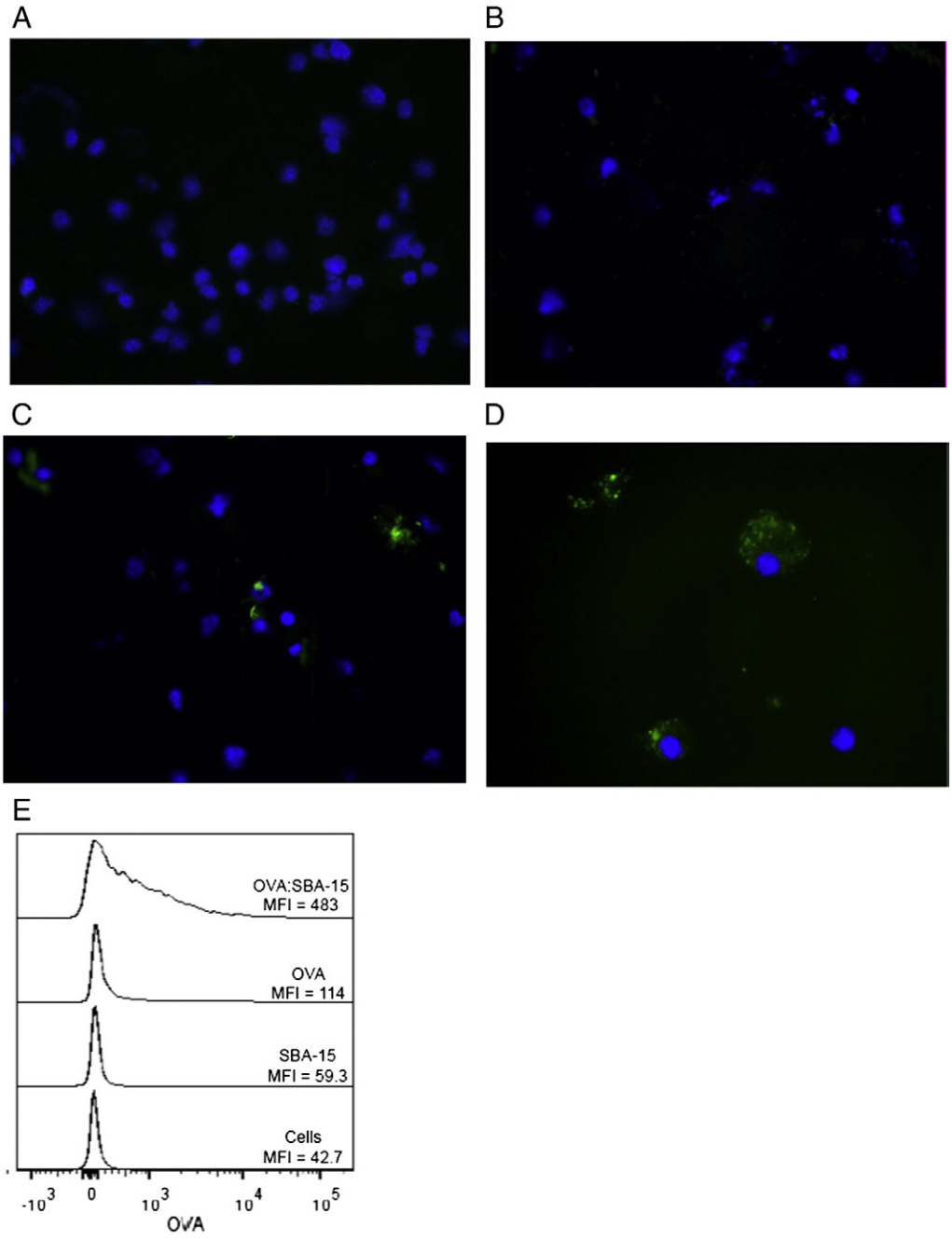

Figure 5. FITC-labeled OVA phagocytosis assay. BMDCs (5×10^5 cells) were cultured with (**A**) RPMI medium, (**B**) 125 μg/mL SBA-15, (**C**) 5 μg/mL FITC-labeled OVA or (**D**) 5 μg/mL FITC-labeled OVA: 125 μg/mL SBA-15 in 1 mL of complete RPMI for 2 h at 37 °C, 5% CO_2. (**E**) Results expressed as histograms with the mean fluorescence intensity (MFI) of each group.

for supporting the clonal expansion of plasma cells, producing these antibodies even in a non-inflammatory milieu and independent of T lymphocytes. Besides, there are distinct subsets of DC at the small intestine, and they are essential for beginning a mucosal immune response against pathogens as well as the activity of regulatory T cells, participating in the generation, maintenance and down-regulation of mucosal inflammation induced by allergies or chronic diseases.[41,43–46]

Supported by the high titers of specific antibodies, the elevated numbers of APC observed in lymphoid tissues, such as Peyer's patches and mesenteric lymph-nodes from mice immunized with the antigens in silica, indicate the enhancement of the immune response after the use of this particle as an oral adjuvant.[8,47]

The overall analysis of our results determines the promising use of SBA-15 silica as an adjuvant to be used in oral immunizations. Proteins usually fail in generating detectable mucosal immune responses since antigens are degraded along the gastrointestinal tract. It is believed that this particle acts on the physical protection of antigens or important epitopes, thus

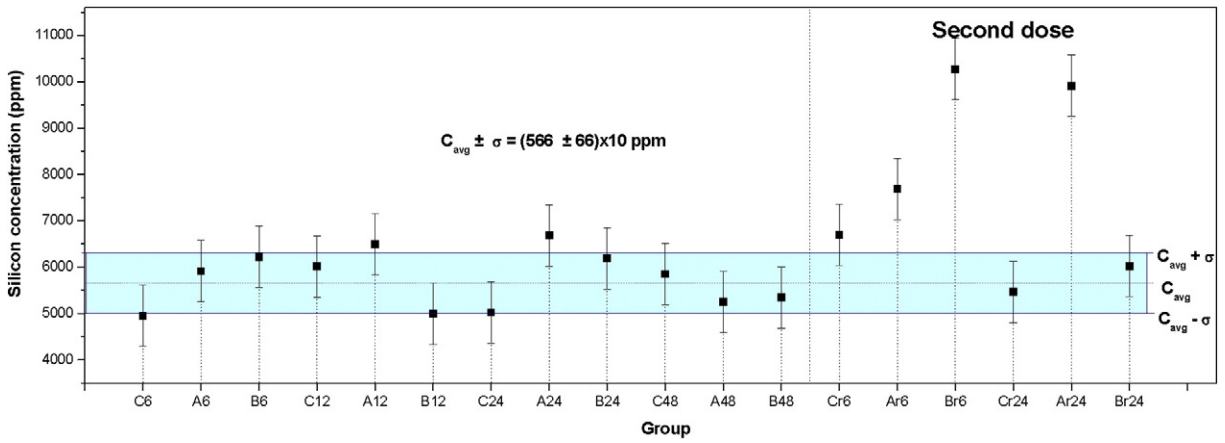

Figure 6. Proton induced X-ray emission of silicon in mice excrements.Silicon concentration in mice excrements. Groups A and B received SBA-15 and the control Group C received PBS. The symbol *r* stands for extra silica administration. The colored area corresponds to the concentration detected for the control group.

helping in their release and efficiently activating the immune system.

The previous results that demonstrated the total release of silicon from mice organs after 70 days,[1] together with the present PIXE results, evidenced that silicon is constantly excreted, supporting the safety of mesoporous SBA-15 silica as a non-toxic powerful oral adjuvant.

There are strong evidences that the mesoporous silica matrix acts to physically protect the antigens or relevant epitopes, enhancing the phagocytosis of antigens by APC and promoting an efficient activation of the immunological system and the proficient induction of immunological memory.[47] Silica nanoparticles (10-20 nm) were recently tested as adjuvant for Hepatitis B virus core (HBc) protein using intraperitoneal or subcutaneously injection, showing the induction of strong Th1-biased immune responses in mice.[48] Recent reviews on nanoparticle adjuvants also pointed out that those mesoporous silica nanoparticles have a "bright future as adjuvants for vaccine delivery"[49] and that "nanocarriers possess immense potential in successful vaccine delivery".[50]

The present work presents two advantages of the Hepatitis B vaccination strategy: (i) the use of HBsAg, the surface antigen of the Hepatitis B virus; and (ii) oral administration.

References

1. Mariano-Neto F, Matos JR, da Silva LC Cides, Carvalho LV, Scaramuzzi K, Tanaka GD, et al. Physical properties of ordered mesoporous SBA-15 silica as immunological adjuvant. *J Phys D* 2014;**47**:425402-11.
2. Kresge CT, Leonowiez ME, Roth WJ, Vartuli JC, Beck JS. Ordered mesoporous molecular sieves synthesized by a liquid-crystal template mechanism. *Nature* 1992;**359**:710-2.
3. Matos JR, Mercuri LP, Kruk M, Jaroniec M. Toward the synthesis of extra-large-pore MCM-41 analogues. *Chem Mater* 2001;**13**:1726-31.
4. Yang H, Coombs N, Ozin GA. Morphogenesis of shapes and surface patterns in mesoporous silica. *Nature* 1997;**386**:692-5.
5. Zhao D, Feng J, Huo Q, Melosh N, Fredrickson GH, Chmelka BF, et al. Triblock copolymer syntheses of mesoporous silica with periodic 50 to 300 angstrom pores. *Science* 1998;**279**:548-52.
6. Biozzi G, Mouton D, Sant'Anna OA, Passos HC, Gennari M, Reis MH, et al. Genetics of immunoresponsiveness to natural antigens in the mouse. *Curr Top Microbiol Immunol* 1979;**85**:31-98.
7. Mercuri LP, Carvalho LV, Lima FA, Quayle C, Fantini MC, Tanaka GS, et al. Ordered mesoporous silica SBA-15: a new effective adjuvant to induce antibody response. *Small* 2006;**2**:254-6.
8. Carvalho LV, Ruiz RC, Scaramuzzi K, Marengo EB, Matos JR, Tambougi DV, et al. Immunological parameters related to the adjuvant effect of the ordered mesoporous silica SBA-15. *Vaccine* 2010;**28**:7829-36.
9. Ajitha S, Sugunan S. Tuning mesoporous molecular sieve sba-15 for the immobilization of a-amylase. *J Porous Mater* 2010;**17**:341-349.
10. Halamová D, Badanicová M, Zelenák V, Gondová T, Vainio U. Naproxen drug delivery using periodic mesoporous silica SBA-15. *Appl Surf Sci* 2010;**256**:6489-6494.
11. Rosenholm J, Meinander A, Peuhu E, Neimi R, Eriksson J, Sahlgren C, et al. Targeting of porous hybrid silica nanoparticles fo cancer cells. *ACS Nano* 2009;**3**:197-206.
12. Hartono SB, Gu W, Kleitz F, Liu J, He L, Middelberg APJ, et al. Poly-l-lysine functionalized large pore cubic mesostructured silica nanoparticles as biocompatible carriers for gene delivery. *ACS Nano* 2012;**6**:2104-2117.
13. Hartono SB, Qiao SZ, Jack K, Ladewig BP, Hao Z, Lu GQM. Improving adsorbent properties of cage-like ordered amine functionalized mesoporous silica with very large pores for bioadsorption. *Langmuir* 2009;**25**:6413-6424.
14. Hartono SB, Qiao SZ, Liu J, Jack K, Ladewig BP, Hao Z, et al. Functionalized mesoporous silica with very large pores for cellulase immobilization. *J Phys Chem C* 2010;**114**:8353-8362.
15. Rosenholm JM, Sahlgren C, Lindén M. Towards multifunctional, targeted drug delivery systems using mesoporous silica nanoparticles—opportunities & challenges. *Nanoscale* 2010;**2**:1870-1883.
16. Lu J, Lion M, Zink J, Tamanoi F. Biocompatibility, biodistribution and drug-delivery efficiency of mesoporous silica nanoparticles for cancer therapy in animals. *Small* 2010;**6**(16):1794-1805.
17. He Q, Zhang Z, Gao F, Li Y, Shi J. *In vivo* biodistribution and urinary excretion of mesoporous silica nanoparticles: effects of particle size and pegylation. *Small* 2011;**7**(2):271-280.
18. Brandtzaeg P, Baekkevold ES, Farstad IN, Jahnsen FL, Johansen FE, Nilsen EM, et al. Regional specialization in the mucosal immune system: what happens in the microcompartments? *Immunol Today* 1999;**20**:141-51.
19. Faria AM, Weiner HL. Oral tolerance. *Immunol Rev* 2005;**206**:232-59.
20. Furrie E, Smith RE, Turner MW, Strobel S, Mowat AM. Induction of local innate immune responses and modulation of antigen uptake as mechanisms underlying the mucosal adjuvant properties of immune stimulating complexes (ISCOMS). *Vaccine* 2002;**20**:2254-62.

21. Jepson MA, Clark MA, Foster N, Mason CM, Bennett MK, Simmons NL, et al. Targeting to intestinal M cells. *J Anat* 1996;**189**(Pt 3):507-16.
22. Kraehenbuhl JP, Corbett M. Immunology. Keeping the gut microflora at bay. *Science* 2004;**303**:1624-5.
23. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 2003;**3**:331-41.
24. Mowat AM, Parker LA, Beacock-Sharp H, Millington OR, Chirdo F. Oral tolerance: overview and historical perspectives. *Ann N Y Acad Sci* 2004;**1029**:1-8.
25. Shalaby WS. Development of oral vaccines to stimulate mucosal and systemic immunity: barriers and novel strategies. *Clin Immunol Immunopathol* 1995;**74**:127-34.
26. Brewer JM. (How) do aluminium adjuvants work? *Immunol Lett* 2006;**102**:10-5.
27. Coffman RL. Origins of the T(H)1-T(H)2 model: a personal perspective. *Nat Immunol* 2006;**7**:539-41.
28. Cox JC, Coulter AR. Adjuvants—a classification and review of their modes of action. *Vaccine* 1997;**15**:248-56.
29. Edelman R, Tacket CO. Adjuvants. *Int Rev Immunol* 1990;**7**:51-66.
30. Hannesdottir SG, Olafsdottir TA, Giudice GD, Jonsdottir I. Adjuvants LT-K63 and CpG enhance the activation of dendritic cells in neonatal mice. *Immunol* 2008;**68**:469-75.
31. Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med* 2008;**205**:869-82.
32. Lambrecht BN, Kool M, Willart MA, Hammad H. Mechanism of action of clinically approved adjuvants. *Curr Opin Immunol* 2009;**21**:23-9.
33. Malik B, Goyal AK, Mangal S, Zakir F, Vyas SP. Implication of gut immunology in the design of oral vaccines. *Curr Mol Med* 2010;**10**:47-70.
34. Mosca F, Tritto E, Muzzi A, Monaci E, Bagnoli F, Iavarone C, et al. Molecular and cellular signatures of human vaccine adjuvants. *Proc Natl Acad Sci U S A* 2008;**105**:10501-6.
35. Schijns VE, Degen WG. Vaccine immunopotentiators of the future. *Clin Pharmacol Ther* 2007;**82**:750-5.
36. Yeh P, Ellens H, Smith PL. Physiological considerations in the design of particulate dosage forms for oral vaccine delivery. *Adv Drug Deliv Rev* 1998;**34**:123-33.
37. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol* 2006;**6**:148-58.
38. Giles AR. Guidelines for the use of animals in biomedical research. *Thromb Haemost* 1987;**58**:1078-84.
39. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* 1971;**8**:871-4.
40. Sardinha LR, Elias RM, Mosca T, Bastos KR, Marinho CR, D'Império Lima MR, et al. Contribution of NK, NK T, gamma delta T, and alpha beta T cells to the gamma interferon response required for liver protection against *Trypanosoma cruzi*. *Infect Immun* 2006;**74**:2031-42.
41. Mengran Y, Yan L, Songping Z, Xiunan L, Yanli Y, Yi C, et al. Improving stability of virus-like particles by ion-exchange chromatographic supports with large pore size: advantages of gigaporous media beyond enhanced binding capacity. *J Chromatogr A* 2014;**1331**:69-79.
42. Berkover I, Spadaccini A, Chen H, Al-Awadi D, Muller J, Gao Y, et al. Hepatitis B virus surface antigen assembly function persists when entire transmembrane domains 1 and 3 are replaced by heterologous transmembrane sequence. *J Virol* 2011;**85**:2439-48.
43. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol* 2008;**8**:435-46.
44. Iwasaki A, Kelsall BL. Mucosal immunity and inflammation. I. Mucosal dendritic cells: their specialized role in initiating T cell responses. *Physiol* 1999;**276**:G1074-8.
45. Johansson C, Kelsall BL. Phenotype and function of intestinal dendritic cells. *Semin Immunol* 2005;**17**:284-94.
46. Cerutti A. The regulation of IgA class switching. *Nat Rev Immunol* 2008;**8**:421-34.
47. Scaramuzzi K, Oliveira DCA, Carvalho LV, Tambourgi DV, Tenorio ECN, Rizzi M, et al. Nanostructured SBA-15 silica as an adjuvant in immunizations with hepatitis B vaccine. *Einstein* 2011;**9**:436-41.
48. Skrastina D, Petrovskis I, Lieknina I, Bogans J, Renhofa R, Ose V, et al. Silica nanoparticles as the adjuvant for the immunisation of mice using hepatitis B core virus-like particles. *PLoS One* 2014;**9**(12):e114006.
49. Prashant CK, Kumar M, Dinda AK. Nanoparticle based tailoring of adjuvant function: the role in vaccine development. *J Biomed Nanotechnol* 2014;**10**:2317-31.
50. Prabhu P, Patravale V. Potential of nanocarriers in antigen delivery: the path to successful vaccine delivery. *Nanocarriers* 2014;**1**:10-45.