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Development of Silica-Immobilized Vaccines for Improving Thermo-Tolerance and Shelf-Life

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ABSTRACT

Introduction. It is estimated that 50% of vaccines produced annually are wasted because effectivity is dependent on protein structure and heat exposure disrupts the intermolecular interactions that maintain this structure. Since 90% of vaccines require a temperature-controlled supply chain, it is necessary to create a cold chain system to minimize vaccine waste. We have developed a more sustainable technology via the adsorption of Invasion Plasmid Antigen D (IpaD) onto mesoporous silica gels, improving the thermal stability of protein-based therapeutics.

Methods. The solution depletion method using UV-Vis was utilized to study the adsorption of IpaD onto silica gels. The silica-IpaD complex is heated above the denaturing temperature of the protein and then the IpaD is removed using N,N-Dimethyldodecylamine N-oxide (LDAO) and their secondary structure is tested using circular dichroism (CD).

Results. Pore diameter, pore volume and surface area were characterized for seven different silica gels. Silica gels designated as 6389, 6378, and 6375 had an adsorption percentage above 95% at pore volumes of 2.2, 2.8 and 3.8 cm^3 mg-1, respectively. CD analyses confirmed that the adsorbed IpaD after the heat treatment displayed a similar "W" shape CD signal as the native IpaD, indicating the conservation of α-helices. In contrast, the unprotected IpaD after being exposed to high temperature shows a flat CD signal, demonstrating the loss of secondary structure.

Conclusion. We have successfully increased the thermo-tolerance for IpaD using mesoporous silica and continue to further optimize mesoporous silica's physiochemical properties to improve adsorption and desorption yields. ***Kans J Med 2020;13(Suppl 2):6-9.***

INTRODUCTION

Temperature-sensitive substances composed of proteins, enzymes, and antibodies are inherently unstable at ambient temperature because they lose their secondary structure and their activity over time. This is a particularly sensitive issue for protein-based medications, such as vaccines, as their storage and handling usually requires continuous cold chain transportation (2 to 8°C) to maintain their functional properties. Cold chain logistics are expensive and prone to disruptions, which often leads to rapid loss of protein potency and deactivation, creating serious concerns for global public health.[1] Although there have been several efforts to develop successful approaches for enhancing the long-term thermal-stability of proteins and vaccines, including pegylation,[2] addition of excipients,[3,4] biomineralization[5] and nanopatches.[6] These methods are unable to provide satisfactory thermal stability, while ensuring the delivery in a biologically active form at the point of care, particularly in developing countries. Thus, the development of innovative and cost-effective strategies capable of protecting thermo-sensitive substances would eliminate the need for an uninterruptible cold chain and help to solve one of the most important issues associated with vaccine wastage. Such advancement can help to ensure that proper treatment is delivered safely and efficiently to the patient, from vaccine production to the patient bedside.

Silica gels have demonstrated the ability to retain protein structure and functionality.[7,8] Some of the most relevant advantages of silicas in protein adsorption include: high thermal stability, useful porosity range for proteins, simple chemistry, mild pH, tunable surface chemistry, and encapsulated biomolecular samples that retain high activity.

Shigellosis is a gastrointestinal disease that causes over a million deaths annually,[9] especially in developing countries and among children.[10] *Shigella flexneri*, the bacteria that is responsible for causing Shigellosis, is spread through the fecal-oral route and only a very low dose is needed for causing an infection.[11] *S. flexneri* is acid-tolerant, and once it reaches the large intestines, it is transcytosed through M cells.[9,12] At this point, the bacteria induces apoptosis in macrophages and invades epithelial cells using a type-III secretion system (TTSS).[13] Invasion plasmid antigen D (IpaD) has a crucial role in TTSS, as it controls the secretion of IpaB and IpaC. All three Ipa proteins must be released for epithelial cell invasion.[14]

Currently, there is no existing vaccine for shigellosis[15] despite extensive attempts to formulate a vaccine against this bacteria.[16,17,18] This is because previously developed vaccines have shown high reactogenicity in human trials.[19] IpaD is a promising target for shigellosis vaccine development since it has already been demonstrated that IpaD antibodies are present in serum from infected patients.[20,21]

Circular dichroism (CD) and Fourier transform infrared (FTIR) have proven that IpaD is predominantly an alpha-helical protein.[13,22] IpaD has a high thermal stability with denaturing occurring above 80°C. Approximately 13% of IpaD is made up of serine amino acids,[23] which are able to form hydrogen bonds at the surface of the silica as shown in Figure 1. Therefore, it is possible to encapsulate IpaD within mesoporous silica and prevent protein denaturation.

METHODS

Adsorption. Silica gel particles were crushed and sifted to a particle size of 90 - 150 µm. Then 0.7 mL of IpaD in 1xPBS buffer at 1.5 mg/mL was added to 30 mg of silica. The solution was mixed on the ThermoMixer at 950 rpm and 25°C for 20 hours. The samples were centrifuged at 14,800 rpm for four minutes at ambient temperature to separate the silica-IpaD complex from the supernatant.

The solution depletion method was utilized to determine the amount of IpaD transferred from the solution into the silica, such that the supernatant concentration indirectly determined the proteins adsorbed. The concentration of the supernatant was measured at 280 nm with an IpaD extinction coefficient of 9.48 $M^{-1}cm^{-1}$ using the Nanodrop 2000 UV-Vis spectrophotometer. The supernatant concentration was used along with the original concentration of IpaD to quantify the adsorption process in Equation 1:

Percent adsorption = (1-(Supernatant IpaD concentration)/(Inital IpaD concentration))*100

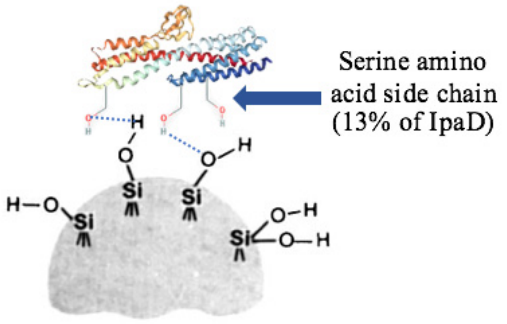

Figure 1. Hydrogen bonds between IpaD and mesoporous silica.

Washing. The unbound proteins in the supernatant were extracted by removing the supernatant from the silica-IpaD particles. A sample containing 0.6 mL of 1xPBS was added to the silica to remove any unattached IpaD trapped within the silica particles. The vials were mixed briefly to loosen the unbound proteins then centrifuged at 14,800 rpm for four minutes, and the wash supernatant was extracted. The washing method was repeated twice, with the exception that 1xPBS was not added back to the sample vial on the third wash. The supernatant of each wash was combined into a single vial, and the concentration was measured.

Heating. The samples were then placed on the ThermoMixer at 95°C for two hours at 950 rpm. The samples were allowed to cool down to room temperature for at least 30 minutes.

Desorption. To detach the proteins from the silica gel, 0.7 mL of 10% LDAO was added to each sample. The samples were then mixed at 950 rpm and 25°C for 20 hours. Finally, the samples were centrifuged at 14,800 rpm for four minutes and the percent IpaD desorption was calculated using the equation:

Percent desorption= ((Mass of desorbed IpaD)/(Mass of IpaD on silica after wash))*100

Circular Dichroism. A Jasco J-1500 Circular Dichroism Spectrophotometer instrument was used to evaluate the secondary structure of chiral protein samples at a temperature of 15°C over a wavelength range of 190 - 260 nm. Before CD measurements were performed, the samples were diluted to 0.4 mg/mL in 10% LDAO which is within the required concentration range of 0.2 - 0.5 mg/mL. The nitrogen flowrate was set at 30 SCFH. A 1 cm pathlength quartz cuvettes was cleaned using 2% Hellmanex II solution to remove any remaining biological specimen from previous runs. Then the cuvettes were thoroughly washed with milliQ water before purging with nitrogen to remove remaining moisture. The cuvettes were filled with 250 µL of sample and placed in the cuvette holder in the instrument. The raw data from CD was reported in Q (mdeg). This was converted to molar ellipticity to account for the concentration of the sample and properties of IpaD using the equation:

$$\theta=(Q\cdot 100\cdot MW)/(C\cdot l\cdot n\cdot 1000)$$

MW represents the molecular weight of the protein sample and n represents the number of amino acids. For IpaD, these values are 38825.29 Da and 352, respectively. *C* is the concentration of the sample in mg/mL, and it is found at 280 nm using UV-Vis absorbance spectrophotometry. The length (*l*) of the quartz cuvette is 1 cm for the J-1500 instrument.

RESULTS

Physiochemical Characterization. The physicochemical properties of seven mesoporous silica gel materials were characterized to generate a "tool box" with a range of physicochemical properties. The material selection criteria for the immobilization of IpaD were correlated between the physico-chemical properties of the support (pore sizes, volumes and shapes, and surface functional groups) and with those of the protein. The physico-chemical properties of selected mesoporous silica gel materials used as supports for the protein thermal-stabilization studies are provided in Table 1. *The identification of the specific silica gels (6360, 6369, 6765, 6389, 6378 and 6395) are confidential and a patent application is being prepared for filing.*

Table 1. Physicochemical characterization of selected mesoporous silica gel materials.

CEBC code	Average pore diameter (nm)	Accessible pore volume (cm^3/g)	Accessible surface area (m^2/g)
6339	17.7	1.41	319.3
6360	3.9	0.07	39.0
6369	8.1	0.30	125.6
6378	36.3	2.87	286.1
6389	24.2	2.18	370.7
6395	30.2	3.78	300.0
6765	15.0	1.28	332.0

Effect of silica pore volume on IpaD percent adsorption. After improving the protocol to adsorb IpaD onto various silica gel materials, the material properties with the most impact on the adsorption efficiency of IpaD were investigated. Our results show that the successful adsorption of IpaD protein is strongly influenced by the pore volume of the silica gel material (Figure 2). We define accessible pore volumes as the pores with diameters ≥ 5 nm.

IpaD secondary structure. To assess whether the adsorption into silica-based supports protects the protein against high temperatures, the IpaD adsorbed onto the silica gels was heated to 95°C for 2.5 h, desorbed from the support, and then analyzed by CD. Non-adsorbed (native) IpaD which was not heated was used as control. The CD analyses demonstrate that the adsorbed IpaD after the heat treatment (and desorption step) displays a similar "W" shape CD signal as the native unheated IpaD.

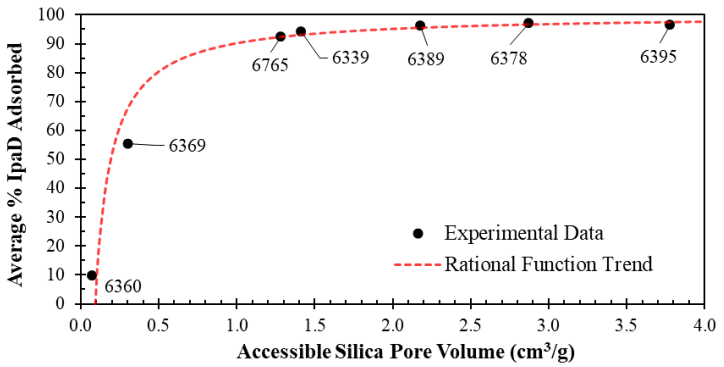

Figure 2. Average percent of IpaD adsorbed to mesoporous silica gel materials as a function of accessible pore volumes (cm^3/g).

DISCUSSION

Effect of silica pore volume on IpaD adsorption. The adsorption of IpaD onto various mesoporous silicas has been hypothesized to have similar surface chemistry for all silicas tested. The adsorption process is governed by electrostatic interactions and hydrogen bonding between the hydroxyls on the silica surface and the properties of the amino acid side chains on IpaD.[23] The most abundant amino acid in IpaD is serine, which makes up 13% of the structure. This creates the potential for a high level of hydrogen bonding between serine residues and the silica surface. As shown in Figure 2, there is a strong correlation between the pore volume of the silica gel to the percent of IpaD adsorbed. For example, silica gel 6765 has a pore volume of 1.29 cm^3/g and adsorbs an average of 92.2% IpaD, while silica gel 6360 has a pore volume of 0.20 cm^3/g and adsorbs an average of 9.7% IpaD. According to Figure 2, silicas with a pore volume greater than 1 cm^3/g are expected to adsorb more than 90% IpaD. The pore diameter of the silica gels followed a similar trend to the pore volume. Therefore, silicas with larger pore volumes and pore diameters tend to adsorb more IpaD. This indicates that the proteins are not only attaching to the surface of the silica, but also being adsorbed into the interior of the larger silica pores. IpaD's thermostability while attached to silica during heating further supports the idea that IpaD is confined within the pores during adsorption.

IpaD secondary structure. The purpose of this study was to demonstrate that silica gels improve the thermostability of IpaD. It is evident from Figure 3 that the presence of silica during heating enables IpaD to maintain its α-helical structure as shown by the characteristic "W" curve. The IpaD in solution completely denatures during heating, and the IpaD heated on silica has a small decrease in molar ellipticity from that of the native IpaD. We hypothesize that this decrease in molar ellipticity will not reduce IpaD's efficacy in a vaccine.

The source of the loss in structure for the IpaD heated on silica is most likely due to variables other than heating. It has been commonly reported that proteins lose some of their conformational structure upon adsorption to a solid surface.[24,25,26,27] Another possible alternative for this loss in structure is the effects of LDAO on IpaD. This zwitterionic detergent is used at a high concentration to remove IpaD from the silica in these studies. One problem is that LDAO increases the high tension (HT) voltage due to the high concentration (10% LDAO). During CD experimentation, it has been observed that the HT voltage is above the recommended value of 600 V at wavelengths below 200 nm. This indicates that the sample is absorbing too much light, which means it is overly concentrated. This effect creates noise in the CD wavelength range of 190 - 200 nm, which eliminates the expected Cotton effect peak at 195 nm that is seen in the native IpaD sample. Furthermore, the drop in molar ellipticity in Figure 3 is potentially due to the buffer concentration interference with the CD signal for the protein. While it is difficult to isolate the effect of LDAO on the CD signal from the physical effect of LDAO interactions with IpaD, further studies have indicated that LDAO is partially denaturing IpaD during the desorption process.

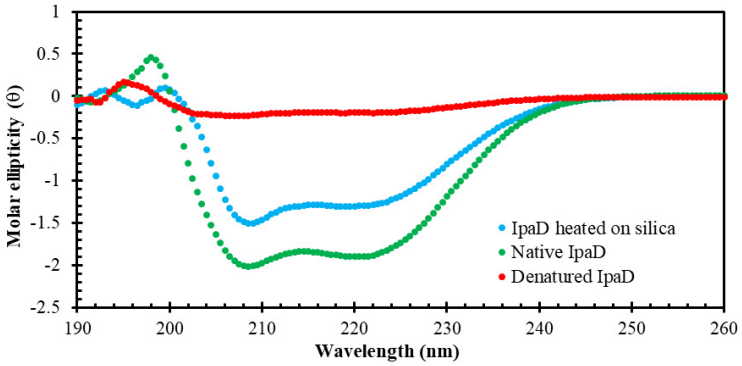

Figure 3. CD spectra for IpaD heated to 95°C for 150 minutes attached to silica gel 6339, native unheated IpaD, and IpaD denatured in PBS solution at 95°C for two hours. The blue curve is the average of 10 samples, and the green curve is the average of two samples.

CONCLUSION

This study has successfully characterized the physiochemical properties of different silica gels using the BJH method. With these physiochemical properties, it was possible to determine the relationship between pore volume and percent adsorption. In general, as pore volume increases, percent adsorption also increases. The most significant result is that IpaD adsorbed in mesoporous silica has a higher thermal stability than unadsorbed IpaD. This was confirmed by the characteristic W shape observed by CD, indicating the presence of alpha helices even after heat treatment.

Future research will explore alternative desorbing agents that are not as harsh as LDAO to reduce the small loss of protein conformational structure during desorption. The next steps will include the design of a device where a vaccine encapsulated in silica can be mixed with the desorbing agent for removing the bound protein and made ready for administration to a patient.

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