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Nanoencapsulation of Insulin into Zirconium Phosphate for Oral Delivery Applications

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Abstract

The encapsulation of insulin into different kinds of materials for non-invasive delivery is an important field of study because of the many drawbacks of painful needle and syringe delivery such as physiological stress, infection, and local hypertrophy, among others.¹ A stable, robust, non-toxic, and viable non-invasive carrier for insulin delivery is needed. We present a new approach for protein nanoencapsulation using layered zirconium phosphate (ZrP) nanoparticles produced without any preintercalator present. The use of ZrP without preintercalators produces a highly pure material, without any kinds of contaminants, such as the preintercalator, which can be noxious. Cytotoxicity cell viability *in vitro* experiments for the ZrP nanoparticles show that ZrP is not toxic, or harmful, in a biological environment, as previously reported for rats.² Contrary to previous preintercalator-based methods, we show that insulin can be nanoencapsulated in ZrP if a highly hydrate phase of ZrP with an interlayer distance of 10.3 Å (10.3 Å-ZrP or θ -ZrP) is used as precursor. The intercalation of insulin into ZrP produced a new insulin-intercalated ZrP phase with a ca. 27 Å interlayer distance, as determined by X-ray powder diffraction, demonstrating a successful nanoencapsulation of the hormone. The *in vitro* release profile of the hormone after the intercalation was determined and circular dichroism was used to study the hormone stability upon intercalation and release. The insulin remains stable in the layered material, at room temperature, for a considerable amount of time, improving the shelf life of the peptidic hormone. This type of materials represents a strong candidate to develop a non-invasive insulin carrier for the treatment of diabetes mellitus.

Keywords

Insulin; zirconium phosphate; drug delivery; controlled release

Introduction

Inorganic layered nanomaterials (ILN) are receiving great attention because of their size, structure, shape and possible biomedical applications.²⁻⁷ ILN have been studied in recent years as matrices for several chemical processes and have been proven to be good drug carriers and nonviral vectors; examples of these are layered double hydroxides (LDH) and

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Supporting Information Available: Diffuse reflectance spectra, molar ratios and loading percent table, and circular dichroism spectra of the intercalated material. This material is available free of charge via the Internet at <http://pubs.acs.org>.

zirconium or titanium phosphates, among others.²⁻⁷ Taking advantage of the expandable interlayer space of ILN researchers have been capable of encapsulating functional biomolecules into these inorganic matrices, protecting them from interacting with the environment, avoiding denaturation and enhancing their shelf life. Among the most studied ILN are zirconium phosphates (ZrP), which are inorganic cation exchange materials with high thermal stability, solid-state ion conductivity, resistance to ionizing radiation, and which are known as hosts capable of incorporating different types and sizes of guest molecules.⁸⁻¹¹ ZrP have also been used as host materials in biomedical applications such as in dialysis systems and most recently as chemical delivery materials.^{2, 12}

There are various kinds of zirconium phosphate materials that vary in their interlaminar spaces and their framework structures.¹³ Intercalation is a common approach that has been used to incorporate guest molecules into ILN. Kumar and coworkers reported the intercalation of several proteins and enzymes into ZrP using the exfoliation method and reported improvement in activities, stabilization, and thermal exposure.¹⁴⁻¹⁶ The major disadvantage of the exfoliation method is the need of a preintercalator to expand and delaminate the layered structure, since the protein/enzyme intercalated material is going to be mixed with the preintercalator.¹⁷ In many cases these preintercalators are toxic, disqualifying the resulting material from being used in biomedical approaches. Recently, Martí and Colón developed a new direct intercalation process that uses a hydrated phase of ZrP, known as the θ -ZrP ($\text{Zr}(\text{HPO}_4)_2 \cdot 6\text{H}_2\text{O}$).¹⁸ This new process makes it possible to intercalate a wide number of large size molecules, including bioactive species, without any preintercalation procedure that contaminates the product.¹⁸⁻²³

Since the intercalation process is reversible due the acid-base character of the interlaminar phosphate groups, as for other ILN, ZrP have the ability to sequester and release molecules of biological interest (such as drugs, hormones, and enzymes, among others) under certain conditions. Typically the release of the molecules of interest from the ILN matrixes is performed by a chemical switch, such as a concentration gradient, pH changes, and even biological stimuli.¹² ZrP crystals can be synthesized in different sizes and dimensions, with particle sizes ranging from micrometers to nanometers, which make facile the size tuning of the micro-nanoparticles for biological applications.²⁴ Furthermore, the ZrP surface can be modified to control the solubility, specific targeting, and *in vivo* release.²⁵ ZrP can be compared with the well studied porous silicon nanomaterials, since they have similar characteristics; however ZrP presents certain advantages over silicon nanomaterials.²⁶ Unlike spherical porous silicon nanoparticles, ZrP nanoparticles has a platelet-type shape. Ferrari and coworkers recently referred to spherical nanoparticles as having the worst possible shape in terms of their margination properties (have the capacity of do a lateral drifting toward the walls of blood vessels and interact with them), penetration through vascular fenestrations, and adherence to the endothelial walls.²⁷⁻³⁰ Moreover, ZrP is resistant to an acidic environment, making ZrP suitable to protect acid-sensitive biomolecules in the stomach and permits the absorption of the nanoparticle or the release of the biomolecule intercalated in ZrP in the intestine tract once the pH changes in the duodenum.

Currently much research effort is being devoted to stabilizing biomolecules of clinical, biomedical, and environmental interest through immobilization.¹ Insulin is one of the biomolecules that have currently attracted interest, due to the high number of patients that need the hormone and the many drawbacks of the insulin delivery by injection.^{1, 31-39} We intent in this publication to prove the concept that insulin encapsulated into ZrP nanoparticles can be used as an alternative delivery system for diabetic patients. The ideal support matrix should prevent protein aggregation or spontaneous denaturation, and leave the native properties of the immobilized protein intact.¹⁶ Encapsulating insulin into ZrP can

potentially impart stability to the hormone, resulting in a long-lasting shelf life. The proposed delivery system is stable at room temperature, which makes the insulin uptake more convenient and easier for the patient than the currently available treatment. Since our material is an acid resistant powdered nanomaterial, we envision that it could be potentially used for the administration of insulin in an oral way. This approach seems rational since ZrP is stable in acidic environments and the sequestered peptidic hormone is going to be protected from digestive enzymes such as pepsin in the stomach, or chymotrypsin, trypsin and carboxypeptidase in the small intestine.¹

Experimental Procedures

Reagents and materials

Zirconyl chloride octahydrate ($\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$), insulin from bovine pancreas and phosphoric acid were obtained from Sigma-Aldrich and used without further purification. Nanopure water was obtained using a Barnstead purification train (17.5 M Ω /cm). The θ -ZrP material was synthesized as previously reported by Martí and Colón.¹⁸ The typical procedure involves addition of 200 mL of a 0.05 M $\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$ aqueous solution to 200 mL of a 35% H_3PO_4 solution; the resulting mixture is refluxed with constant stirring at 94 °C for two days. The product was filtered and washed several times with nanopure water, resulting in a paste material. This material was characterized by X-ray powder diffraction (XRPD), resulting in an intense peak at low angles corresponding to an interlayer distance of 10.3 Å in addition to a second order diffraction peak corresponding to 5.1 Å. If this material is dried it will dehydrate and transform into the alpha phase (α -ZrP) which will have a diffraction peak corresponding to a distance of 7.6 Å in the XRPD analysis.⁴⁰

The intercalation process was performed by the batch method reported by Martí and Colón,¹⁸ with addition of the desired quantity of insulin to a water suspension of θ -ZrP at different molar ratios (1:15, 1:3, and 2:3). Insulin is difficult to dissolve in nanopure water, therefore a typical procedure for the solubilization of insulin is to decrease the pH of the solution with a diluted acidic solution until it reaches pH = 2. The pI of insulin is 5.4;⁴¹ at pH below 5.4 the net charge of the hormone is positive. Since ZrP is a cation exchanger, the pH in which the intercalation takes place has a profound effect on the intercalation process, thus all the intercalation was performed without changing the pH (~3) of the θ -ZrP solution. The suspensions were constantly stirred for three days and the pH value of each intercalation was monitored before and after the intercalation process. After this time the suspensions were filtered by vacuum using 0.22 μm filters (Millipore). The solids were washed with abundant water and dried in a vacuum drier for one day followed by pulverization for characterization and later release studies.

Controlled release experiments of insulin at pH 7.4 and 8.2, $\mu = 0.1$ M

A suspension of about 0.03% w/v of the insulin intercalated ZrP material (Ins:ZrP) was prepared with PBS with the desired pH. This suspension was stirred for a period of 1-5 h while aliquots of 10 ml were taken at different periods of time. The aliquots were centrifuged for a period of 1 min and the supernatants were separated from the precipitates. The release of insulin from the layers was monitored by measuring the absorbance of the supernatant with UV-vis spectrophotometry and a plot of cumulative release with time is reported, where Cumulative Release (%) = $[A_t/A_{max}] \times 100\%$ where A_t is the absorbance of the characteristic peak at 204 nm at time t and A_{max} is the maximum absorbance of this peak.

Exposure of cells to particles and cell viability assays

The human breast cancer cell line MCF-7 were plated at a density of 1×10^3 cells/well in a final volume of 100 μ L in 96-well plates and allowed to attach for 24 h before addition of particle suspensions. The respective dilution of the particle suspension were added in 100 μ L complete cell culture medium to reach final concentrations of 1×10^{-12} to 10×10^{-4} M for nanoparticles. Cells grown without the nanoparticles complete cell culture medium were included as controls. MCF-7 cells in the presence or absence of nanoparticle-containing solutions were grown for 24 hours. Cell viability was assessed colorimetrically with the MTT reagent (Sigma-Aldrich) following standard protocols provided by the manufacturer. The absorbance was read with a microplate reader at 595 nm. Each value recorded was the average of readings from 3 wells. The absorbance measured was normalized to the absorbance of control cells.

Instrumentation

The complete characterization of the materials was performed using several analytical techniques. XRPD experiments were performed from 2 to 40° (2θ) using a Siemens D5000 X-Ray diffractometer system with a copper anode source ($K_{\alpha 1}$, $\lambda = 1.5406 \text{ \AA}$) with a filtered flat LiF secondary beam monochromator. Transmission electron micrographs (TEM) of the samples were acquired using a JEOL 2010 transmission electron microscope at an acceleration voltage of 200 kV. Samples were prepared using copper grids from Ted Pella. Some TEM images were color manipulated using the ImageJ program with the bgrbcmyw LUT. IR spectra of the intercalated materials were recorded on KBr pellets (1%, w/w) with a NICOLET Magna 750 FTIR spectrometer. UV-vis absorption spectra were measured with a HP 8453 diode array spectrophotometer. Diffuse reflectance spectra were obtained using a Cary 1E UV-vis spectrophotometer. Thermogravimetry experiments were carried out on a TGA Q500 TA Instrument. The temperature was ramped at $5^\circ \text{C min}^{-1}$ under a flow of N_2 up to 800°C . The circular dichroism measurements were recorded on an Olis DSM-10 UV-Vis CD spectrophotometer on fresh insulin and six months old Ins:ZrP samples. Protein concentration was 0.6 mg/ml in 10 mM potassium phosphate buffer at pH 7.4. Spectra were recorded between 260–310 nm using a 1.0 cm path length quartz cell. Each spectrum was obtained by averaging five scans at 2 nm resolution. Solvent reference spectra were digitally subtracted from the protein CD spectra.

Results and Discussion

Insulin is a relatively small polypeptide hormone which is soluble in mild acidic media; these peculiarities make insulin the perfect candidate to be intercalated in ZrP, using θ -ZrP as precursor. Figure 1 shows the XRPD patterns of dry products of the intercalation reaction of insulin with ZrP at various Ins:ZrP molar ratios. The intercalation reactions with ZrP are known to be topotactic;⁴² the diffraction peak at the lowest 2θ angle corresponds to the interlayer distance. The XRPD patterns of insulin-exchanged ZrP at molar ratios 2:3 and 1:3 show that a new phase has formed whose first-order diffraction peak corresponds to an interlayer distance of ca. 26–27 $\text{\AA}$. In addition, those two XRPD patterns show a diffraction peak corresponding to a distance of 13.4–13.2 $\text{\AA}$, another peak for the 2:3 Ins:ZrP material corresponding to a distance of ca. 9.4 $\text{\AA}$, and a peak at 7.6 $\text{\AA}$, the characteristic interlayer distance of α -ZrP.

The dimensions of insulin are ca. $33.4 \times 31.0 \times 21.3 \text{ \AA}$,⁴³ while the thickness of a ZrP sheet is 6.6 $\text{\AA}$.^{44–45} Therefore, a minimum interlayer distance of 27.9 $\text{\AA}$ is predicted corresponding to the distance between (001) planes if successful intercalation of insulin into θ -ZrP is accomplished (assuming that insulin maintains its crystal structure dimensions). The slightly smaller experimental interlayer distance obtained is consistent with the predicted one,

particularly since small reversible conformational changes in the overall structure of insulin may occur upon intercalation and some of the phosphate groups can penetrate within pockets in the hormone surface, producing a slight reduction from the predicted interlayer distance.

Therefore, we assign the peak at 27.3 Å for the 2:3 molar ratio and at 26.2 Å for the 1:3 molar ratio intercalation products to a first order diffraction peak from the (001) planes, the peak at ca. 13 Å to a second order (002) diffraction peak, and the peak at ca. 9 Å to a third order (003) diffraction peak. The products obtained for the 2:3 and the 1:3 Ins:ZrP molar ratio intercalation reactions are clearly mixed-phase products, as evidenced by the presence of unintercalated α -ZrP with its characteristic peak at 7.6 Å. As the Ins:ZrP molar ratio increases, the α -ZrP characteristic peak becomes broader and less intense, indicating that the proportion of the α -ZrP phase in the produced material is decreasing. The intralayer ZrP structure is retained in all samples, as evidenced by the preservation of the diffraction peaks at 33.8 and 34.1 Å corresponding to the (0,2,0) and (3,1,-2) planes.

The XRPD pattern obtained for the Ins:ZrP material at the low 1:15 molar ratio (Figure 1) is that of unintercalated α -ZrP with an interlayer distance of 7.6 Å. The α -ZrP phase is formed upon dehydration of θ -ZrP without any intercalated species present.⁴⁶ This result indicates that at that low insulin loading level a new phase is not formed in the materials and any exchanged insulin in this sample is mainly bound on the surface of the agglomerated ZrP nanoparticles; intercalation would have reduced agglomeration. This explanation is supported by the TEM data (Figure 2) that show higher agglomerations of nanoparticles at lower Ins:ZrP molar ratios.

The TEM images of samples correlate remarkably well with the structure that the XRPD patterns suggest. TEM images of the 2:3 and 1:3 Ins:ZrP materials show marked differences in morphology and homogeneity between them (Figure 2). The TEM image of the 1:3 Ins:ZrP material at lower magnification (Figure 2a) shows the heterogeneity of the sample and a considerable amount of particle agglomeration. Hexagonal nanocrystals, characteristic of α -ZrP, can be identified in the TEM images of the 1:3 sample at intermediate magnification (Figure 2b) in agreement with the 7.6 Å diffraction peak observed in the XRPD patterns (Figure 1). The highly transparent particles shown in the TEM images are most likely the Ins:ZrP nanoparticles; the transparency is due to the presence of a large amount of organic molecules in the materials. These transparent nanoparticles shown in the TEM images should be less crystalline, as indicated by the broad peaks shown in the XRPD pattern (Figure 1), also suggesting that these nanoparticles correspond to the Ins:ZrP intercalation product.

In the case of the 2:3 Ins:ZrP material the TEM images (Figures 2d, e, and f) show a more homogeneous material, with little agglomeration and a well defined nanostructure compared with the 1:3 Ins:ZrP material. Figure 3 shows a magnification of the circled area in Figure 2f; it appears to be a small nanoparticle that broke apart from a larger one, ending up tilted, so that the thickness of the material can be measured. That particular nanoparticle presents three layers of ZrP with an estimated interlayer distance of ca. 2.9 nm, which is in agreement with the interlayer distance of the insulin-intercalation ZrP product obtained from the XRPD patterns. In addition, the white spots in the TEM image, which appear in the interlayer space, have a diameter of ca. 21 Å consistent with the dimension of the intercalated insulin.

The UV-vis diffuse reflectance spectra of insulin intercalated ZrP (Supporting Information Figure SI-1) shows the characteristic absorptions bands around 220 nm and 280 nm expected for insulin, but with a slight red shift (ca. 2 nm); no new bands are observed. As expected, the intensity of those bands increases as the Ins:ZrP molar ratio increases from

1:15 to 2:3, indicating a higher concentration of insulin within the 2:3 Ins:ZrP material. Those characteristic absorption bands of insulin are also observed for the 1:15 Ins:ZrP material, even though the XRPD result shows no discernible intercalation. Therefore, the absorption bands observed in the 1:15 Ins:ZrP sample are due to the insulin adsorbed on the exterior surface of ZrP at lower molar ratios.

To determine if any chemical change has occurred to the hormone during the intercalation process, FTIR measurements of the intercalated materials were performed. Figure 4 shows the FTIR spectra of insulin intercalated into ZrP at different molar ratios. The IR spectra of the intercalation products with Ins:ZrP molar ratios of 2:3 and 1:3 show the characteristics amide bands of insulin at the $\sim 1500\text{ cm}^{-1}$ region. On the other hand, the IR spectrum of the intercalation product with Ins:ZrP molar ratio of 1:15 shows very weak amide region bands, suggesting that although there is insulin adsorbed on the surface of the aggregated nanoparticles, the overall concentration of the hormone is low, which is in agreement with the UV-vis diffuse reflectance spectroscopy and XRPD results. As expected, the orthogonal phosphate bands at $\sim 1050\text{ cm}^{-1}$ have disappeared in the 2:3 and 1:3 Ins:ZrP intercalation products, indicative of a successful intercalation.⁴⁷ Since there are no significant shifts of the IR bands of amide I to higher frequencies or amide II to lower frequencies, these results suggest that there is no significant denaturation of the hormone after intercalation.¹⁶

In order to obtain the actual loading of the intercalated solids, thermogravimetric analysis (TGA) was performed (Supplemental Information Table 1-SI). The TGA results indicate that the experimental Ins:ZrP molar ratio is 1 mol of insulin for every 42 formula units of ZrP, for the 1:3 intercalation product (24% loading) and 1 mol of insulin for every 32 formula units of ZrP for the 2:3 intercalation product (28% loading). Taking into account the dimensions of insulin and the interlayer distance, obtained by XRPD, we can determine the cross-sectional area of the insulin that will be parallel to the layer ($31.0 \times 33.4\text{ \AA}^2$). Since the area of a single ZrP formula unit is $24\text{ \AA}^2$, the product of the ratio between the insulin area and the ZrP unit area should produce the theoretical full loading of Ins:ZrP. The value of this calculation is 1 to 43, in other words, theoretically at full loading there should be 1 insulin molecule for every 43 ZrP formula units. This result is in agreement with all the TGA results; the 1:3 loading level is a full loaded material, and the 2:3 is fully loaded with excess of insulin adsorbed on the surface.

The controlled release experiments of Ins:ZrP suspensions were carried out between pH 8.2 and 7.4 to study the release of the hormone from the layers using a pH stimuli. The release was monitored with UV-vis spectrophotometry by observing the change in absorbance of the characteristic band of insulin at 280 nm of the centrifuge aliquots of the suspensions. Figure 5 shows the release profile of Ins:ZrP at pH 8.2 and 7.4. At pH 8.2 insulin is released from the layers at a fast pace (~ 5 minutes) until it reaches a plateau; at pH 7.4 the release is slower (~ 30 minutes). Since the isoelectric point of insulin is 5.4, at pH between 8.2 - 7.4 the six carboxylic acid groups of the hormone are deprotonated. The produced carboxylate groups disrupt the hydrogen bond interactions between the insulin and the phosphate groups of the layers producing a permanent negatively charged insulin. The overall negative charge of insulin at pH 8.2-7.4 will be repelled by the negatively charged phosphate groups of the ZrP layers, resulting in the rapid release of insulin to the solution and an uptake of the Na^+ ions from the buffer. This release mechanism is very similar to the biological release of insulin, where the insulin is in the hexamer conformation bonded by a Zn^{2+} cation in the storage vesicle; once the insulin hexamer is released to the serum it experiences a jump in pH from ~ 5.5 to 7.4. The pH jump causes the carboxylic acid groups to deprotonate and the repulsions lead to a rapid dissociation from the metal complex hexamer; the insulin ends up as the monomer in the blood stream.⁴⁸ The rapid release of the hormone from the ZrP galleries at pH 7.4 is comparable to the time it takes for the insulin level to rise in the body

between meals (30 minutes).⁴⁹ The acidic nature of the ZrP material and the relatively high pH of the buffer are the keys for the controlled release mechanism of the nanoparticle, making possible a new therapy for patients of diabetes.

Circular dichroism in the UV region was performed for the samples of insulin-ZrP intercalated material and samples collected during the release experiment at pH 7.4 (Supplemental Information Figure 2-SI). The spectra show the characteristic bands of native insulin in the region of 180-250 nm, indicating that the secondary structure of the hormone is retained on the intercalated material upon release from the ZrP framework. The CD spectrum of the released hormone is practically superimposable with that of the native insulin, implying little or no structural change after immobilization and the subsequent release of the hormone from the ZrP framework.

To determine the potential toxicity of the ZrP nanoparticles to human cells, we measured the cell viability of a human breast carcinoma cell line MCF-7 grown for 24 hours in the presence of different concentrations of nanoparticles (Supplementary Information Figure 3-SI). The MTT cell viability assay revealed an absence of overt toxicity to MCF-7 cells following exposure to the formulations containing ZrP for up to 24 hours.

The release of insulin into the duodenal duct will cause the absorption of the hormone into the blood stream. The activity of the insulin is going to be less compromised via this route since the initial steps of digestion are avoided. Based on previous methods of gastrointestinal insulin delivery the insulin uptake should be around 5%,^{1, 31-34} which would lead to an oral administration of 162 mg and 139 mg, for the 1:3 and 2:3 Ins:ZrP intercalation product, respectively, per meal. This amount should be equivalent to the usual insulin dose per meal when injected by the conventional injection route.

Conclusions

A new approach for protein nanoencapsulation has been demonstrated via direct ion exchange using θ -ZrP. For the first time the cytotoxicity of ZrP to human cells *in vitro* has been investigated and no toxicity was demonstrated. A controlled release study of nanoencapsulated insulin using pH changes as stimuli has been proposed and investigated. The hormone remains stable during the entire process and does not appear to have any significant chemical or structural changes over a period of time (up to six months). Although more detailed studies are warranted, this system may pose an alternative to the painful and tedious administration of insulin through injection that diabetic patients suffer every day.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. XRPD patterns of α -ZrP, insulin, and insulin:ZrP at different molar ratios.

Figure 2.
TEM images of insulin:ZrP at 1:3 (a,b,c) and 2:3 (d,e,f) molar ratios at different magnifications.

Figure 3.

Magnifications of the white circle area of the TEM image in Figure 2f. The left image is the magnification of a nanoparticle of insulin:ZrP at the 2:3 molar ratio. The right image is the same image on the left with a color manipulation using the ImageJ program to enhance the gray scale. The white rectangles represent the ZrP layers, the double head arrows the interlayer distance, and the white circles represent the insulin intercalated into ZrP.

Figure 4. FTIR spectra of (upper to lower) Insulin, α -ZrP, and insulin:ZrP framework at different molar ratios.

Figure 5. Insulin release profiles for insulin:ZrP upon agitation in NaPi pH 8.2 (◇) and 7.4 (□) $\mu = 0.1$ buffer at room temperature.