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ADVANCES IN ZEIN- BASED HYBRID BIO-NANOCOMPOSITE: A SHORT REVIEW

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ABSTRACT

This paper contains synthetic approach and characterization study of bio-hybrid material based on zein. A novel zein is found as a mixture of protein complexes whose molecular size, solubility and charge depend on the corn variety and the separation method used for extraction. Zein is soluble in various primary, binary and ternary solvent systems, mainly determined by the balance between the polar/nonpolar groups in those systems. Because of the large proportion of hydrophobic residues in zein, this protein is not soluble in pure water. Due to their biocompatibility and null toxicity, bionanocomposites based on zein and conformed as beads were also investigated as slow-release systems of bioactive molecules, such as drugs. montmorillonite containing Na ions or quaternary alkylammonium cations in the interlayer region was used. By different strategies of synthesis and the use of several techniques, it was possible to investigate the zein intercalation mechanism into montmorillonite, as well as the structure and properties of the resulting zein-montmorillonite bio-hybrids. It was deduced that the intercalation processes, as well as the cation located in the interlayer region of the clay, are strongly influenced by the solvent used for zein solubilization. The resulting bionanocomposite films loaded with zein-based bio-hybrids exhibited good compatibility, homogeneity and mechanical properties. These introduced results point out that these new bio-hybrid materials prepared from different approaches could be associated with other polymers of different nature, being a promising ecological alternative to common organoclays based on alkyl ammonium cations, increasing the possibilities of research and applications in this innovative field.

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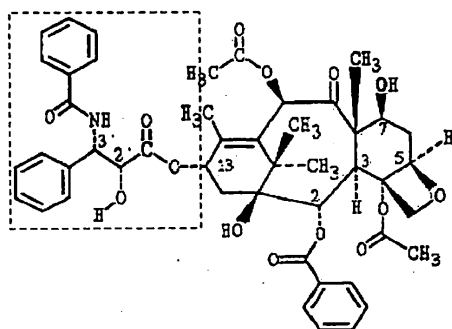
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INTRODUCTION

Zein a water-insoluble prolamine found from corn gluten and manufactured initially as in the form a concentrated powder. It is unique in its ability to form odourless, tasteless, clear, hard and almost invisible edible films. Since Zein films are completely safe to ingest, it is a perfect coating for foods and some pharmaceutical ingredients. Zein is extracted from gluten by physical process rather than chemical means and is, therefore, totally natural. It is a food ingredient but not an additive of food. Zein is observed to be remarkably resistant to bacterial attack, which frequently decomposes other proteinaceous material.

It seems to repel many insects such as the Indian Meal Moth, which often infests nut and grain products that are unprotected. Zein is presently used to coat candy, flavours, pharmaceutical tablets and ingredients which are encapsulated in time-release, granulation and seeds. The coating forms used can be general film coating and enteric coating. In the food and confection industries, Zein is used for coating enriched rice, candies, such as chocolates and jelly beans, dried fruits, nuts, nut meats, and also for the encapsulation of flavours and sweeteners. Zein can be combined with many other ingredients and gives different coating qualities and with different colours producing a wide range of colour coatings. This type of coating is considered a better coating than shellac (confectioner's or pharmaceutical glaze) because Zein coating solutions dry faster than shellac

offering extended shelf-life, mostly under high-humidity and high-heat conditions. Corn or maize is the only cereal crop indigenous of the Americas and one of the most important food industrial crops in the world (Bicudo *et al.*, 2006). In corn, the proteins content is approximately 10% of the dry weight of the grain, while starch, soluble sugars and oil content range between 70-80%, 1-4% and 3-6%, respectively (Anderson, 2011). The corn proteins are constituted of about 20% of globulins and albumins (water soluble proteins or salt solutions), 40% prolamins (water insoluble proteins and 70% v/v alcohol soluble) and 40% of glutelins (water and alcohol insoluble proteins) (Bicudo *et al.*, 2006). The proteins of these two latter groups are also known as storage proteins and in corn, the class of proteins belonging to the prolamins group is called as zein. Prolamin proteins occur specifically in cereals, and, in the corn, they are found exclusively in the endosperm (Mohammad, 1990). Zein is used as nitrogen source during germination and early seedling growth and as nitrogen sink during seed development (Shewry, 1990). Thus, zein is considered as the major storage protein of corn and an important source of protein in the human diet, since it is present in human food due to direct consumption or by consumption of animals whose diet is based on corn, such as poultry or swine's (Cabra *et al.*, 2005). Although zein is known since its first isolation in 1821 by the scientist John Gorham (Sessa *et al.*, 2003) which is remarkable in the study of this protein not only because of its nutritional value, but also due to its potential technological use. Zein is rich in non-polar amino acid residues such as valine, leucine, proline, isoleucine, alanine, and phenylalanine, therefore it has hydrophobic character to the protein and thus, zein is insoluble in water but soluble in aqueous ethanol (60-95%v/v), and some organic polar solvents such as propylene glycol and acetic acid. Zein is also soluble in aqueous alkaline solution having pH above 11, in the presence of sodium dodecyl sulfate (SDS) and in water solutions that contain urea at high concentrations. Such features of zein are determined by its structure, on the protein fraction, processing, chemical modifications and other factors. Thus, a good knowledge of structural and solubility properties of zein becomes essential for the preparation of materials based on this protein. Molecular structure of Zein shown below.



(1)

(Structure of zein)

SYNTHESIS AND PREPARATION METHODS

Zein-layered clays bio-hybrids

Preparation of zein-Cloisite-Na bio-hybrid

For the preparation of zein-CloisiteNa bio-hybrids (Zein-Cloisite-Na), three synthesis routes were known, which differ

in the main solvent used to disperse the clay and protein, and in the order of mixing of both components.

Synthesis 1

For the preparation of zein-Cloisite-Na bio-hybrids by this method, 50 mL of an 80%v/v ethanol solution in water was added in 300 mg of Cloisite-Na, and vigorously stirred by means of a mixer (G₂ model, Lomi) in order to properly disperse the clay. On the other hand, solutions of zein (80%v/v ethanol/water) with different content in protein (30, 60, 120, 200, 300, 500, 1000 and 1500 mg) were prepared in 50 mL, in order to achieve different weight proportions of zein to the Cloisite-Na in the bio-hybrid materials (0.1:1, 0.2:1, 0.4, 0.67:1, 1:1, 1.6:1, 3.3:1 and 5:1, respectively). Each zein solution was added to 50 mL of the Cloisite-Na dispersion and the resulting mixture was stirred for 48h at room temperature (approx. 23°C). Then, the solid product obtained was isolated by centrifugation (40 minutes at 8000 rpm) and dried overnight at temperature of 40 °C. The bio hybrid composite materials prepared from Cloisite-Na through this method were named as Zein- Cloisite-S₁ (Sessa, 2003; Lawton , 2002).

Synthesis 2

In this second synthetic method, 300 mg of CloisiteNa were firstly swollen in 20 mL of water. On the other hand, different amounts of zein (30-1500 mg) are added in 80 mL of ethanol. Both volume of ethanol and water was calculated to reach up to 80:20 final ratio of ethanol & water, after mixing both zein and clay suspensions. It was observed that in pure ethanol, zein was not completely solved, but a separation process of different components of the protein took place (Figure-1), showing the presence of an extracted phase (soluble in alcohol) and another solid phase (insoluble in alcohol). The aqueous clay suspension was then added to these two phases system of zein in absolute ethanol. The solid phase of the protein began to solubilize as the liquid phase reached (80:20) ethanol: water ratio, forming at this point a homogeneous Z-Cloisite-Na suspension. The system was maintained under magnetic stirring for 48h at room temperature, and then the solid was separated by centrifugation and dried similarly as in synthesis- 1. The bio-hybrids resulting from this synthetic method were denoted as Z- Cloisite-S₂



Synthesis 3

The third synthetic method used for the preparation of Z-Cloisite-Na bio-hybrid composite is based on the fact that zein can be dissolved in strongly alkaline solution. Thereby, different amounts of zein (30-1500 mg) were dissolved in 35 mL of NaOH 0.1 M (pH 13), while 300 mg of Clois-Na were swollen in 65 mL of water, both at room temperature. After complete dissolution of the protein, this was added to the suspension of the swollen clay, forming a single batch that was kept under magnetic stirring for 48 h at room temperature. The solid product was separated by centrifugation and washed

several times with distilled water for removal of residual NaOH until neutral pH, and then dried overnight at 40 °C.

The bio-hybrid materials resulting from this synthetic procedure were denoted as Z- CloisNa₃.

Preparation of Zein- Cloisite 30B bio-hybrids

The preparation of the bio-hybrids based on zein protein and Cloisite30B was carried out in the same procedure followed to prepare Z-Cloisite-Na materials according to method -1 (synthesis -1), using 80%v/v ethanol/water as solvent, but substituting Cloisite-Na by the Cloisite30B organomontmorillonite(MMT)clay as inorganic counterpart. The resulting bio-hybrid materials prepared with Cloisite30B were denoted Z-Cloisite-30B.

Use of zein-layered clays as compatibilisers in biopolymer films preparation

The zein-layered clays bio-hybrids synthesized were employed as compatibilisers in the preparation of biopolymer films. Thus, Z-Cloisite-Na-S₃ and Z- Cloisite-30B bio-hybrids of different compositions were used in the preparation of zein and starch films with different bio-hybrid content. In the case of zein films loaded with zein-layered clays, 2.5 g of zein were solubilized in 45 mL of aqueous ethanol solution (80% v/v), under vigorous magnetic stirring and at temperature of 80 °C. Then, 5 mL of zein-layered clay dispersion in water were added to the zein solution at 80 °C forming a single batch and that was kept under stirring for approximately 35 mins to reach room temperature. After total homogenization, the resulting dispersion was placed in a methacrylate box, and it was dried at room temperature. The starch films based on zein-layered clays bio-hybrids were prepared similarly to the zein films discussed above, except that in this case, the solvent used for the solubilisation of the starch polysaccharide was pure water heated at 80°C. For comparison, zein and starch films containing Cloisite-Na and Clois30B were also prepared in the same conditions than those used for the preparation of the bionanocomposite films filled with bio-hybrids. Blank films of zein and starch (i.e. without clay or bio-hybrid material) were prepared by dissolving 2.5 g of zein or starch in 50 mL of ethanol solution at 80% (v/v) or pure water, respectively. In both systems it was necessary to add 0.5 g of glycerol as plasticizer, keeping the mixtures under magnetic stirring at 80 °C until complete homogenization of the components.

Zein-fibrous clays bio-hybrids

Preparation of zein-sepiolite and zein-palygorskite bio-hybrids

The method employed in this synthesis was analogous to synthesis- 1 used for the preparation of zein-Cloisite-Na bio-hybrids. Thus, suspensions of sepiolite or palygorskite (6% g L⁻¹) were prepared in ethanol/water (80%, v/v), being vigorously stirred by means of a mixer grinder in order to properly disperse the clay in the medium. Different amounts of zein were dissolved in 50 mL of ethanol/water (80%, v/v) in order to prepare a set of alcoholic solutions with zein concentration ranging between 0.6 and 30 g L⁻¹. Each zein solution was then added to the sepiolite or palygorskite dispersion, forming a single batch that was stirred for 48 h at room temperature (approximately 23 °C). After that period of time, the solid product was isolated by centrifugation (40 minutes, 8000 rpm)

and subsequently dried overnight at 40 °C. The resulting zein-sepiolite and zein-palygorskite bio-hybrid materials were denoted as ZSEP and Z-PALY, respectively.

Bionanocomposite membranes using zein-fibrous clays as filler

Zein-sepiolite or zein-palygorskite bio-hybrids were used as fillers of alginate polysaccharide in the preparation of bionanocomposite membranes. Thus, alginate films loaded with different amounts of zein-fibrous clays were produced employing the solution blending method (Cong *et al.*, 2007), where a necessary amount of bio-hybrid was dispersed in deionized water by vigorous stirring for 24 h at room temperature, in order to achieve alginate: bio-hybrid weight ratios of 1:1, 1:2 and 1:3. The bio-hybrid suspension was gradually added to the previously prepared alginate dispersion in water (2%w/v), forming a single batch that is kept under constant stirring overnight. Finally, the resulting bionanocomposites were placed onto a glass plate and allowed to dry at room temperature. The films prepared from alginate incorporating zein-sepiolite or zein-palygorskite bio-hybrid filler were denoted as ALG/Z-SEP and ALG/Z-PALY, respectively. For comparison, pure alginate membranes were to be produced in the same way from a 2% sodium alginate aqueous solution. After casting onto a glass plate, the resulting dried membranes were in some cases immersed in 5% CaCl₂ for 15 min to procure a crosslinking of the alginate chains. After the cross-linking process, the membranes were washed with doubly distilled water to remove residual Ca²⁺ ions and allowed to dry at room temperature.

Zein-sepiolite bionanocomposite foams Tridimensional

Macroporous zein-sepiolite foams were prepared profiting from the different solubility of zein components in water and ethanol.

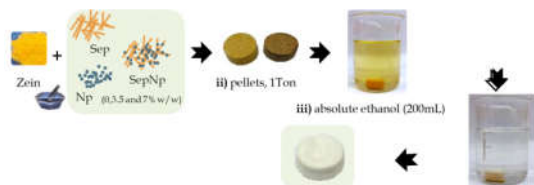
Zein-sepiolite foams

Zein-sepiolite bionanocomposite foams were prepared using the following general procedure: i) an amount of zein is mechanically mixed with different amounts of pure sepiolite (SEP) (0, 3.5 and 7% with respect to the protein weight); ii) after homogenization, the resulting material was conformed as pellets of 12 mm in diameter, applying a pressure of 1 Ton; iii) the pellets were immersed in 200 mL of absolute ethanol during 24h, provoking the extraction of the soluble fraction of zein; iv) the hybrid materials were removed from the ethanol system and immediately immersed in 200 mL of water, where they were maintained for 24 h more; v) the pellets then were frozen at -20 °C and subsequently lyophilized in a freeze-drier (Cryodos, Telstar). The zein-sepiolite foams prepared from this method were denoted

Zein-sepiolite magnetic foams

The preparation of the zein-sepiolite magnetic bionanocomposite foams were carried out following the same protocol adopted in the synthesis of zein-sepiolite bionanocomposite foams (Figure- 2), but in this case the sepiolite employed was previously modified with magnetic nanoparticles (SepNP). The composition of this heterostructure was 50% of sepiolite and 50% of oleic acid-capped magnetite nanoparticles. The resulting magnetic bionanocomposite foams were denoted as Z-SepNP. Magnetic blank foams were also prepared following the same experimental procedure,

except that in this case the heterostructure was substituted by pure magnetite nanoparticles (NP), being denoted as Z-NP. Magnetic nanoparticles (Fe_3O_4) provided with superparamagnetic behavior were prepared in the presence of oleic acid. These nanoparticles were used to prepare ferrofluids that were then employed to prepare superparamagnetic sepiolite according to the procedure patented by our research group (Lawton, 2002). This superparamagnetic sepiolite was applied as adsorbent and also to prepare other multifunctional materials (Kale, 2009) and it was used in the preparation of various zein-based magnetic foams in this work.



(Figure -2) Scheme of the general procedure employed for the preparation of the zein-based bionanocomposite foams

mechanical mixing 24h
freezing -20°C ;

lyophilisation zein-based on foam
materials (24h) iv) water (200mL)

(Figure -2) Scheme of the general procedure employed for the preparation of the zein-based bionanocomposite foams.

Zein-layered hydroxide bio-hybrids

Diverse approaches were explored using single layered hydroxide (LSH) and layered double hydroxides (LDH) containing different inorganic anions in the interlayer region in order to obtain zein-layered hydroxide bio-hybrid materials. The syntheses of these materials are described below.

Zein-layered double hydroxide bio-hybrids

Synthesis of $\text{Mg}^{2+}\text{Al}^{3+}$ -LDH with different inorganic anions in the interlayer region

For the synthesis of layered double hydroxides (LDH) of Mg and Al in the 2:1 ratio, containing Cl^- ($\text{Mg}_2\text{Al}-\text{Cl}$), NO_3^- ($\text{Mg}_2\text{Al}-\text{Nit}$) and CO_3^{2-} ($\text{Mg}_2\text{Al}-\text{Carb}$) anions, the coprecipitation reaction was followed at pH constant, according to the procedure described by Constantino and Pinnavaia (Constantino and Pinnavaia, 1995). For the preparation of the $\text{Mg}_2\text{Al}-\text{Cl}$ LDH, a mixture of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ was dissolved in 250 mL of decarbonized water. This aqueous solution was added dropwise with a peristaltic pump to 100 mL deionized water kept under a nitrogen flow for removing CO_2 . Simultaneously, a solution of 1 M NaOH was also added dropwise to the aqueous system through an automatic dispenser (Dosimat 765 with an 806 Exchange Unit, from Metrohm) controlled by a 781 pH/Ion Meter (Metrohm) to keep a constant pH of 11 during the synthesis. The resulting suspension was vigorously magnetically stirred for 24 h under a nitrogen flow. The solid product was isolated by centrifugation, washed three times with bidistilled and degassed water, and dried overnight at 60°C . The Mg:Al LDH containing CO_3^{2-} ions ($\text{Mg}_2\text{Al}-\text{Carb}$) was prepared analogously to the $\text{Mg}_2\text{Al}-\text{Cl}$ LDH described above, including the same salts and their respective concentration, except that in the different stages of synthesis the nitrogen flow was not employed and the precipitation pH was 9.0 ± 0.1 , adjusted with 0.2 M Na_2CO_3 aqueous solution. Once formed, the $\text{Mg}_2\text{Al}-\text{Carb}$ LDH was recovered, washed and dried in an

oven at 60°C . The ($\text{Mg}_{0.67}\text{Al}_{0.33}(\text{OH})_2$) $\text{CO}_3 \cdot 0.33 \cdot \text{nH}_2\text{O}$ was denoted as MgAl-Carb LDH. The $\text{Mg}_2\text{Al}-\text{NO}_3$ LDH nitrate was also prepared by a method analogous to the LDH chloride described above, except that $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (17.5 mmol) and $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (8.74 mmol) were used as the source of magnesium and aluminium. The pH of the solution was maintained at 11.0 by adding 1 M NaOH solution. The resulting white precipitate was aged for 24 hours under nitrogen flow then it was filtered and washed several times with huge amounts of deionized and degassed water and finally dried at about 60°C in a hot oven. The ($\text{Mg}_{0.69}\text{Al}_{0.34}(\text{OH})_2$) $\text{NO}_3 \cdot 0.34 \cdot \text{nH}_2\text{O}$ was denoted as MgAl-Nit LDH.

Zein-LDH intercalation compound preparation

Ion exchange method

For the preparation of bio-hybrids by the ion exchange method, briefly, 0.5 g of zein was dissolved in 50 mL of 0.1 M NaOH through magnetic stirring or by means of an ultrasound tip (VC750 Sonics Vibra-Cell, operating 20KHz), using a tip of 13 mm and applying intermittent pulses of 10 s followed by a standby step of 10 s up to a total applied energy of 60 kJ/0.5 g of zein. The pH of the protein solution was adjusted to 11 by controlled addition of 0.1 M HCl. This solution was slowly added to a suspension containing 2 g of MgAl-Cl or MgAl-Nit LDH freshly prepared in 50 mL of degassed bidistilled water. The pH of the system was reset back to 11 and then maintained under magnetic stirring at 50°C under nitrogen flow for 4 days. Afterwards, the solid product was isolated by centrifugation, washed with distilled water and dried at 40°C . The resulting hybrid materials derived from MgAl-Cl and MgAl-Nit LDH were denoted as Z-LDH-Cl_{ie} and Z-LDH-Nit_{ie}, respectively.

Co-precipitation method

Intercalation of zein into the MgAl-Cl or MgAl- NO_3 LDH by co-precipitation method was carried out by adding slowly 250 mL of an aqueous solution containing the Mg and Al salts to 100 mL of a zein protein solution (0.5 g of protein/100 mL of 0.1 M NaOH), previously prepared by magnetic stirring or ultrasonication, similarly as described for the ion-exchange method. The metal ions and the protein solutions were constantly purged with N_2 and the pH of the system was controlled at 11.0 using the Metrohm 765 Dosimat. The resultant suspension was stirred for 24 h at room temperature under nitrogen flow. The solid fraction was separated by centrifugation (4500 rpm, 15 min), washed with bidistilled water, and dried overnight at 40°C . The bio-hybrid materials synthesized by the co-precipitation method derived from MgAlCl and MgAl-Nit LDH were denoted as Z-LDH-Cl_{cppt} and Z-LDH- NO_3 _{cppt}, respectively.

SYNTHESIS AND CHARACTERIZATION OF ZEIN-LAYERED CLAY BIO-HYBRIDS

Zein-layered clay hybrid bio-materials were prepared by the direct adsorption of zein protein in the solution of a montmorillonite exchanged with two type of cations, the commercial products Cloisite Na (CloisNa) and the organically modified Cloisite 30B (Clois30B), which contain Na^+ ions and quaternary alkylammonium cations (2ethyl hexylhydrogenated tallow alkylammonium cation) in the interlayer region, respectively. In the case of Clois30B systems, zein adsorption was easily achieved from zein water/ethanol solutions.

However, regarding the adsorption of zein on CloisNa, other strategies of synthesis, such as the use of alkaline medium, were employed in addition to the hydroalcoholic media to reach protein intercalation. Taking into account that ethanol/water mixtures are the most usual solvent for dissolving zein, a protocol that uses 80%(v/v) ethanol as solvent was firstly tried for the preparation of the bio-hybrids based on Clois30B and CloisNa. In contrast, other approaches for zein adsorption were carried out to reach the goal when using CloisNa, for instance, by using either the protein extracted by phase separation in pure ethanol or that solubilized in alkaline medium (both described in the experimental section, § 2.3.1a, synthesis 2 and 3, respectively).

Zein-montmorillonite bio-hybrids from zein in aqueous ethanol solution (80% v/v)

Characterization of the zein ethanol solution

The inorganic counterpart used in the preparation of these bio-hybrids is a lamellar clay that shows cation exchange properties, it was firstly intended to verify the pH of the starting zein solution in 80% (v/v) ethanol in order to know the global charge of the protein. This hydro-alcoholic zein solution shows a pH value of 5.3. Since the isoelectric point of the protein is around 6.2 (8) The protein will present predominantly protonated groups in its structure at the pH of this synthesis process, thus it will be susceptible to accomplish ion exchange reactions with montmorillonite clays. On the other hand, the molecular weight of the protein was investigated by electrophoresis (SDS-PAGE) (Figure 3.1), indicating the presence of α zein conformation with two typical bands of approximately 23 and 25 kDa, which correspond to Z19 and Z22 proteins (Kale, 2009) respectively. In this SDS-PAGE gel, it is possible to even observe the presence of α -zein dimers, which are characterized by the presence of bands around 50kDa (Muller *et al.*, 2009; Wang, 2010) In these conditions, zein chains are quite disaggregated, being thus susceptible for intercalation.

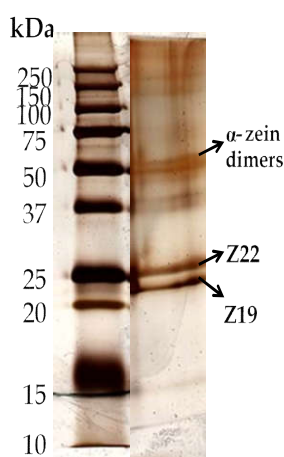


Figure 3 SDS-PAGE in acrylamide 20% of zein 80% (v/v) ethanol/water solution. The gel was silver stained

Solid state NMR and FTIR spectroscopy were also used in order to characterize the protein before and after solubilisation in 80% (v/v) Ethanol/water, and the results are shown in Figure 3 .

The FTIR spectrum of zein (Figure 3.2) presents the characteristic bands of proteins at 3308, 1658, and 1538 cm^{-1} , which are assigned to the NH stretching vibration modes of the

so-called amide A groups, to the ν_{CO} vibrations of C=O of amide-I, and to the ν_{CN} vibrations of C-N-H bonds of amide -II from the peptide groups, respectively. The observation of these bands is associated with the presence of zein predominantly in the α -helix conformation (Giles *et al.*, 1960) In the same spectrum, the stretching vibration bands appearing in the 2950-2850 cm^{-1} range are assigned to C-H groups. It is noted that zein dissolved in 80% (v/v) ethanol/water solution shows a similar IR spectrum, confirming that this solvent does not affect the main characteristics of zein (Figure 3.2), appearing only a broad band at 3341 cm^{-1} that can be associated with the OH groups from the remaining solvent. The ^{13}C NMR spectrum of zein (Figure 3.3) shows the typical signals of the protein at 174 ppm assigned to carbonyl carbons. There are also signals in the 140 to 100 ppm range corresponding to amino acid aromatic side chains residues, in the 70 to 45 ppm range due to α -carbons linked to amino groups, and also signals from 45 to 15 ppm assigned to carbon of the amino acid aliphatic side chains (Giles *et al.*, 1960; Nedi *et al.*, 2012) The observed chemical shift of carbonyl groups at 174 ppm, which is sensitive to the secondary structure, could indicate high content in α -helix, corroborating the results from the FTIR studies (Ruiz Hitzky *et al.*, 2010). The morphology of zein before and after dissolution in 80% (v/v) ethanol/ water was investigated by FE-SEM (Figure 3.1). The commercial zein powder used in this work shows a compact and smooth texture formed by thick sheets (Figure 3.1 a and b). However, once the aqueous ethanol solution of zein was left to dry, the morphology of corroborating results from the FTIR studies (Giles, 1960).

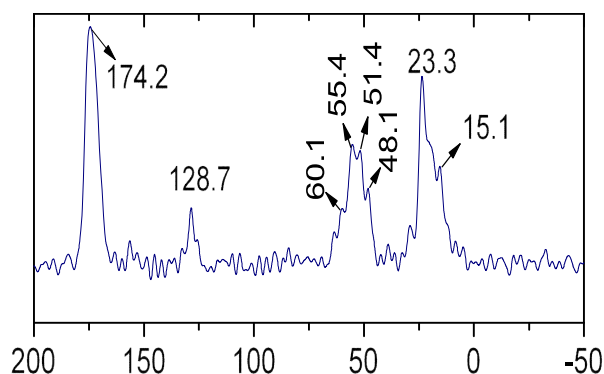


Figure 3.1 ^{13}C NMR spectra of starting zein protein

This sample was observed again by FE-SEM. Such images (Figure 3.1 b and c) show the presence of globular aggregates, which may occur from aggregates of small size of around 176 nm up to few microns (approx. 4.0 μm). This morphology may result from the rearrangement of zein molecules during the drying process. The fast evaporation of ethanol may lead to a solution with an increasing content in water that contributes to enhance the zein-zein interactions, minimizing the number of hydrophobic chains exposed to water and, thus, reducing their undesirable interaction with the water molecules after ethanol evaporation. The formation of zein aggregates during the drying process was also reported by Wang *et al.* in studies focused on the microphases formed from zein in ethanol-water solution (Sorrentino *et al.*, 2007; Luecha *et al.*, 2011).

Zein-Cloisite Na and Zein-Cloisite 30B bio-hybrids prepared from 80%(v/v) ethanol/water solution

As commented in the experimental section (§2.3.1a), suspensions of CloisNa and Clois30B layered clays in 80%

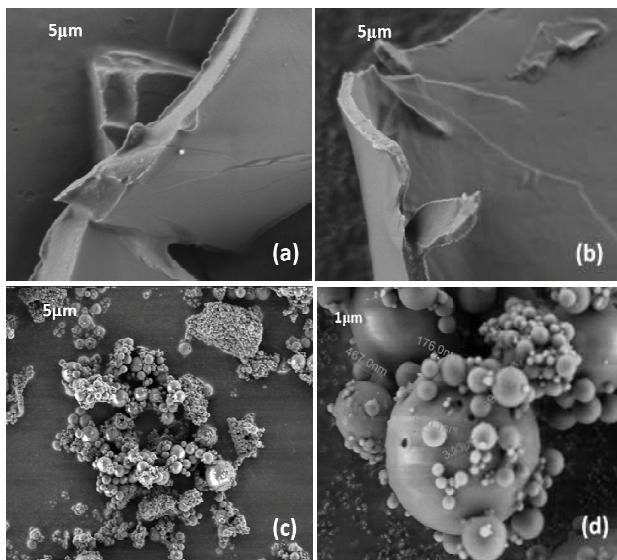
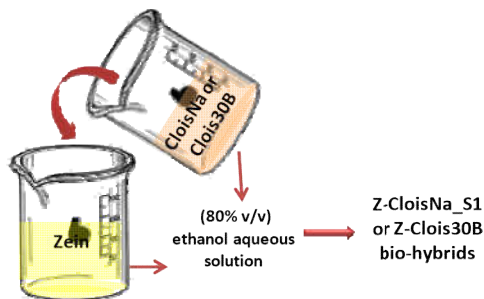


Figure 3.1 FE-SEM images of pristine zein (a and b) and after (c and d) drying a protein solution in 80%(v/v) ethanol/water

(v/v) ethanol/water were incorporated to solutions of increasing concentration of protein, resulting in a series of bio-hybrids denoted as ZCloisNa_S1 and Z-Clois30B, respectively (Scheme 3.1).



Scheme 3.2 Synthesis of Z-CloisNa_S1 and Z-Clois30B prepared from 80%(v/v) ethanol/water

The adsorption isotherms of zein on CloisNa and organomodified Clois30B montmorillonites from 80%(v/v) ethanol/water are presented in Figure 3.5. From this graphic, it is clearly observed that the amount of protein adsorbed on Clois30B is higher than on CloisNa. Such differences in the adsorption may be related to a stronger affinity of the zein adsorbate toward the organophilic clay, where the presence of organophilic cations promote the compatibility between the protein and the clay, and thus enlarge the amount of incorporated zein. Thereby, the great disparity observed in the present case in zein adsorption on the two layered clays, suggests that a different adsorption mechanism is taking place in each case. According to the Giles classification (16) the adsorption isotherm of zein on CloisNa (Figure 3.2 a) resembles to an H-type isotherm, a special case of the Langmuir isotherm, where the adsorption increases rapidly at low equilibrium protein concentration, reaching a plateau at around 0.9 g L^{-1} with a maximum of adsorbed protein of 10.8 g of zein/100 g of CloisNa. From this equilibrium concentration, the amount of adsorbed zein increases rapidly (see Table 3.1), probably due to the formation of zein aggregates in solution at high zein concentrations, similarly as observed in the study of zein-fibrous clays(17,18). In contrast, zein adsorption on Clois30B (Figure 3.5 b) fits to the typical L-type curve (Langmuir isotherm) of Giles classification (19) where the

adsorbed zein increases gradually with the increase in the starting protein concentrations, reaching a maximum adsorbed protein of 80 g of zein/100 g of organoclay at equilibrium concentrations values around 12.6 g L^{-1} . It is evident that the values of zein adsorption in Clois30B are almost three times higher than those in CloisNa, which could indicate that the protein has a better affinity toward the organoclay substrate, and that the mechanism of adsorption of zein in both clays is different.

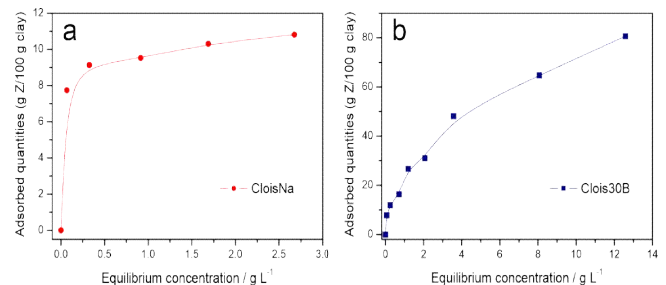


Figure 3.3 Adsorption isotherms at 23 °C of zein from 80%(v/v) ethanol/water solution on (a) CloisNa and (b) Clois30B layered clays

Zein-Cloisite Na bio-hybrids prepared from absolute ethanol

Characterization of the zein fractions formed in absolute ethanol

It is well-known that zein is not soluble in water or pure alcohol. However, it was observed in this study that, although zein cannot be dissolved in pure ethanol, a separation process of different components of the protein in a soluble phase and a precipitate occurs in these conditions. The two phases correspond to the soluble and insoluble zein fractions in alcohol, being henceforth denominated as extracted and precipitated respectively. Colorimetric tests, using a ninhydrin spray solution as revealing agent, were carried out in order to detect the presence of protein in the extracted liquid phase (Qu *et al.*, 2008). In fact, the presence of the protein in the ethanol phase was indicated by the purplish colour resulting from reaction between ninhydrin and free amino groups from amino acids of solubilized zein (Figure 3.3).

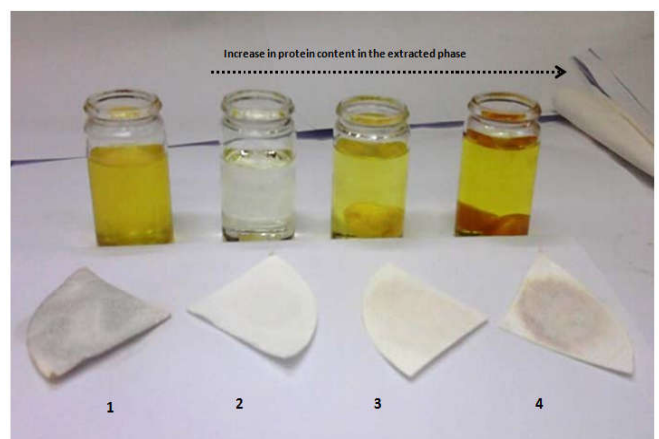


Figure-3.4 Colorimetric assay using ninhydrin spray solution as protein revealing agent in different zein preparations: (1) 0.3%(w/v) zein solution in 80%(v/v) ethanol/water as reference, and (2-4) zein incorporated in pure ethanol in 0.018, 0.15 and 0.3%(w/v) concentration

Table 3.1 Bio-hybrids of the Z-CloisNa_S1 and Z-Clois30B, prepared by adsorption of zein from 80%(v/v) ethanol/water solutions containing different initial amounts of zein, and their composite Protein content determined by chemical analysis

starting amounts (g zein /100 g clay)	zein-cloisiteNa hybrids code	bio- adsorbed zein /100 g CloisNa)	(gzein-Cloisite30B code)	B bio-hybrids adsorbed zein (g of zein /100g Clois30B)
10.0		7.74	Z-Clois30B-7	7.54
20.0	Z-CloisNa_S1-9	9.13	Z-Clois30B-12	11.9
40.0	Z-CloisNa_S1-9.5	9.52	Z-Clois30B-16	16.7
66.6	Z-CloisNa_S1-10	10.3	Z-Clois30B-26	26.1
100.0	Z-CloisNa_S1-11	10.8	Z-Clois30B-31	31.7
166.0	Z-CloisNa_S1-14	14.6	Z-Clois30B-50	49.4
333.3	Z-CloisNa_S1-20	20.8	Z-Clois30B-64	64.3

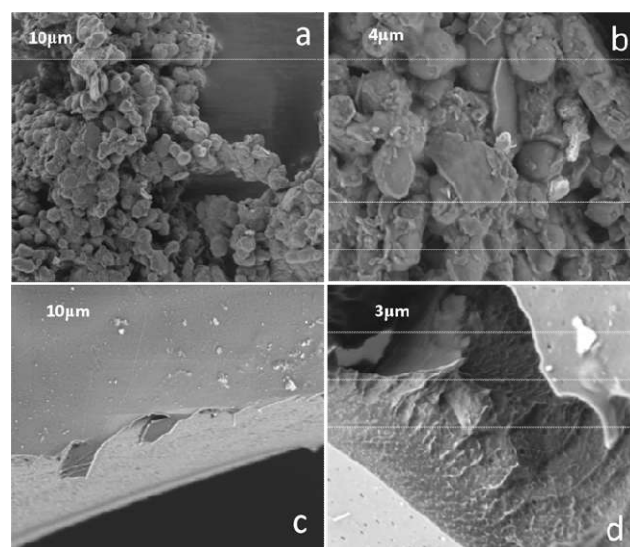
Table 4.1 Zein-sepiolite and zein-palygorskite bio-organoclays prepared in this work by adsorption of zein from ethanol/water (80% v/v) solutions containing different initial amounts of zein

starting amounts (g zein /100 g clay)	zein-cloisiteNa hybrids code	bio- zein content) /100 g SEP)*	(g zeinzein-palygorskite bio- hybrids codes)	Zein content (g of zein/ 100g PALY)*
10.0	Z-SEP10	9.85	Z-PALY9	9.30
20.0	Z-SEP16	16.31	Z-PALY12	11.93
40.0	Z-SEP20	19.74	Z-PALY13	13.07
66.6	Z-SEP24	23.53	Z-PALY14	14.05
100.0	Z-SEP25	25.02	Z-PALY15	14.78
166.0	Z-SEP29	29.21	Z-PALY18	17.80
333.3	Z-SEP54	53.59	Z-PALY21	20.80
500.0	Z-SEP48	47.88	Z-PALY28	28.40

Electrophoresis measurements (SDS-PAGE) were also conducted in these two phases. The protein patterns of the PCT phase, after dissolving it in 80%(v/v) ethanol/water, are very similar to those of neat zein also dissolved in the aqueous ethanol solution. The characteristic bands of α -zein and its dimers are observed at approximately 21-25 kDa and 50 kDa, respectively. In this PCT gel, a band set between 150 kDa and 250 kDa is also observed, which could correspond to the presence of other protein aggregates, such as trimers, tetramers and/or oligomers. Together with the bands corresponding to α -zein, SDS-PAGE of the EXT phase presents a band at around 10 kDa. An analogous band was reported by Sessa and co-authors, who extracted zein pigments using ethanol in a Sephadex LH-60 chromatographic column (Ruiz Hitzky).

These pigments are yellow-orange coloured oxygenated carotenoids known as xanthophylls, such as lutein and zeaxanthin, which show a molecular weight of around 565 Da (Sorrentino *et al.*, 2007) and It has been also reported that these zein pigments, which are responsible of its yellow colour, are located in the core of the triple-helical segments, being strongly linked to the Z19 monomer (Luecha *et al.*, 2011). Hence, the 10 kDa band could be related to ethanol-soluble protein components associated with the xanthophyll pigments as reported by Sessa and co-workers (Radebaugh *et al.*, 1988). The morphology of these zein fractions was also investigated by FE-SEM (Figure 3.4). It is observed that the EXT phase consists in distorted molecular aggregates, presenting also some plate-like shape agglomerates. In contrast to the typical zein morphology, the PCT fraction does not show agglomerates, being characterized by the presence of a continuous and homogeneous film of compact texture very similar to the commercial zein morphology (Figure 3.4 a and b). These different morphologies in zein can be associated with the amount of protein in each one of the separated phases. The effect of zein concentration in the texture of zein was reported by Padua and coauthors for zein samples dried from ethanol-water systems (Rives, 2002). Thus, diluted zein solutions showed the formation of free microspheres occasioned by hydrophobic associations, which is the case of the EXT phase.

In contrast, zein in higher concentration has a tendency to form narrow packed aggregates that subsequently fused into a film during the drying process, which may be a similar situation to that of the PCT in the current study.

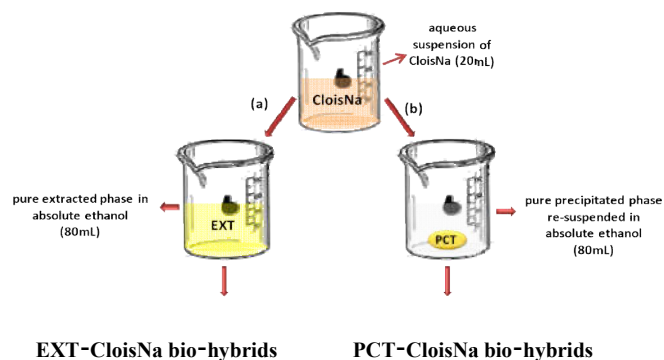
**Figure 3.4. FE-SEM images of (a, b) the EXT and (c, d) the PCT fractions separated from 1 18.7mg/mL zein incorporated in pure ethanol**

Study of the intercalation mechanism of zein in CloisNa from absolute ethanol

In order to understand the mechanism underlying this complex intercalation process, a systematic study of the individual steps followed to achieve zein intercalation in CloisNa were investigated. For this purpose, two stages were considered:

- The formation of a first bio-hybrid based on the zein components present in the soluble fraction in pure ethanol.
- The incorporation of the other zein molecules coming from PCT fraction after being solubilized in the ethanol/water mixture.

With the aim of reconstructing such steps, on the one hand, zein precipitate fraction (PCT) was separated and the extracted phase (EXT) was used to prepare bio-hybrids by addition of an aqueous clay suspension, resulting in a series of materials denoted as EXT-CloisNa bio-hybrids (Scheme 3.3). On the other hand, bio-hybrids based only on the precipitate phase and CloisNa were prepared by direct mixture of the CloisNa aqueous suspension and the precipitate in pure ethanol, forming the so-called PCTCloisNa bio-hybrids (Scheme 3.3). In both cases, the series of bio-hybrids were formed in a final liquid phase of 80:20 (v/v) of ethanol:water final ratio.



Scheme 3.3 Individual steps to achieve zein intercalation into CloisNa from (a) the extracted phase formed in pure ethanol and (b) the precipitate phase after re-suspension in absolute ethanol

These EXT and PCT phases were obtained from three different initial amounts of zein dissolved in pure ethanol chosen to reach 20, 100 and 500g per 100g of CloisNa final ratio. As mentioned above, CloisNa aqueous suspensions are added to each one of these EXT and PCT fractions present in pure ethanol, obtaining their respective EXTCloisNa and PCT-CloisNa bio-hybrids. The amount of protein adsorbed in CloisNa in each case was determined by CHNS chemical analysis (Table 3.4). From these values, it can be observed that the bio-hybrids based on the EXT phase show a low amount of adsorbed protein compared to those materials prepared from the PCT. This fact may be related to the adsorption of zein oligomers, shown in the SDS-PAGE results, which are present in the PCT fraction and become soluble in the aqueous ethanol solution formed with the water of the clay suspension. The adsorption of the extracted phase on CloisNa is also confirmed by UV-Vis spectroscopy of the supernatant separated after formation of the EXT-CloisNa bio-hybrids (Appendix A, Figure A.1), being clearly observed the decrease of the protein and carotenoid bands present in the EXT phase after the adsorption assay on the sodium montmorillonite.

Zein-CloisiteNa bio-hybrids assembled from zein dissolved in alkaline medium

Characterization of the zein in alkaline medium

Although the main solvent for zein is usually based on ethanol/water mixtures, solutions of alkali metal hydroxides (pH > 12) can be also dispersing agents of this protein. In alkaline medium, the solubility of zein is considerably increased due to the basic deamidation reaction that takes place in glutamine and asparagine amino acids (Ruiz-Hitzky, 2009; Ruiz-Hitzky *et al.*, 2008). In other studies, the alkaline solubility of zein is attributed to the formation of alkali salts of

phenolic-hydroxyl groups of tyrosine (Ruiz-Hitzky *et al.*, 2010). Anyway, the important thing is that in such conditions it is possible to achieve the complete solubilization of zein in aqueous medium. FE-SEM images display the morphology of the dried zein sample previously treated with 0.1 M NaOH. These images show the presence of zein as thick layers without evidence of the typical agglomerates of this protein. In this case, the layered morphology may result from the negative charges on the zein surface as a consequence of the basic treatment, which could help to minimize the hydrophobic protein-protein interaction and promote the protein-water interaction.

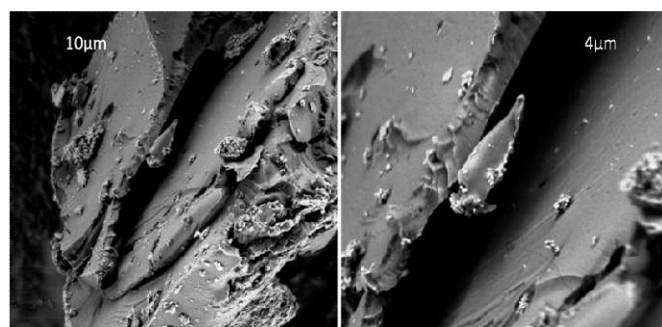


Figure 3.23 FE-SEM images of zein treated in alkaline medium (0.1 M NaOH)

Characterization of Zein-Fibrous Clays Bio-Hybrids

Zein-clay bio-hybrid materials were prepared by direct adsorption of the protein on the sepiolite and palygorskite fibrous silicates, according to the procedure described in Chapter 2, section 2.3.2. The knowledge of the affinity between zein and clays is fundamental for the preparation of the bio-hybrid material and their use as bioorganoclays. Figure 4.1 shows the adsorption isotherms (23°C) of zein in sepiolite and palygorskite from ethanol/aqueous solutions (80% v/v). In both cases, the adsorption isotherms show a sharp slope at low equilibrium concentration values fitting to a H-type isotherm, a special case of the L-type curve according to the Giles classification of isotherms (Ruiz-Hitzky *et al.*, 2005). This behaviour is indicative of a high affinity between the sepiolite and palygorskite substrates and the zein adsorbate. Both curves reach a plateau corresponding to values of 25.0 g and 14.7 g of zein per 100 g of sepiolite and palygorskite, respectively. Taking into account that zein structure is a compact rectangular prism with dimensions of 16 x 4.6 x 1.2 nm³ (Ruiz-Hitzky *et al.*, 2005), it is expected that the adsorption process takes place only at the external surface of fibrous clays and not inside the nanosized tunnels of the fibres. Thus, the corresponding plateau values suggest a complete coverage of the silicate surface in both clays with by zein. It has been observed that for equilibrium concentrations higher than 3 g/L, the adsorbed zein amounts are greater than the values in the plateau. This behaviour may be related to the formation of molecular aggregates of zein in solution, Table 4.1 summarizes the zein-sepiolite and zein-palygorskite bio-hybrids prepared in this Thesis, including the initial amounts of zein employed in their synthesis as well as the respective amount of adsorbed protein on each one. The code number assigned to each sample indicates the approximate content of zein in grams per 100 grams of inorganic solid. Comparing the values in Table 4.1, it is clearly observed that at equal initial concentrations of zein, the amount of retained protein is greater when the substrate is sepiolite.

This result can be explained by the higher specific surface area of this clay mineral compared with palygorskite, providing a larger specific area for the protein adsorption. Samples prepared from solutions with very high zein content result in bio-hybrid materials with adsorbed zein that may exceed 50.0 g and 28.0 g in sepiolite and palygorskite, respectively. These values are considerably higher than those corresponding to the plateau in the adsorption isotherms. This fact can be attributed not only to the coverage of the clays surface by several layers of protein, but also to the formation of molecular aggregates in solution at high concentrations of zein, which is a characteristic ability of this protein (Salerno *et al.*, 2010) that may be directly adsorbed on the silicate surface, or to the existence of more complex processes related to formation of zein microphases in ethanol-water media (Sanchez *et al.*, 2005).

Taking into account that a large part of the clay surface may be covered by the protein (according to the adsorption isotherms deduced from CHNS analysis), the N₂ adsorption-desorption technique was carried out in some bio-hybrids in order to determine their specific surface area (BET). The BET values of these zein-fibrous clays bio-hybrids materials are listed in the Table 4.2. The starting clays show a specific surface area of 340 and 186 m²/g for sepiolite and palygorskite, respectively. However, a considerably reduction of the specific surface in the bio-hybrid materials is evidenced, most likely due to the protein adsorption, reaching values as low as 22 and 19 m²g⁻¹ for the bio-hybrids Z-SEP48 and Z-PALY28, respectively, which have the highest zein content. Given that both clays exhibit structural micropores whose dimensions are only accessible to small molecules (like the N₂ used in the specific surface area measurements), adsorption of the voluminous zein polypeptide chains inside the tunnels of these clays is not possible and so it could only take place on the external accessible surface of the clay (150 m² g⁻¹ in sepiolite and 120 m² g⁻¹ in palygorskite). Although they are not able to penetrate in the nanosized tunnels, the important decrease in specific surface area of the bio-hybrids with respect to that of the starting clay minerals confirms that the protein molecules are blocking the access of nitrogen to the nanopores during the BET measurements, but without entering them. The decrease in the specific surface area values as zein content increases points out to the agglomeration of the particles by the action of this biopolymer (Sanchez *et al.*, 2011).

High resolution solid-state NMR spectroscopy was applied in order to investigate the molecular interaction between the protein and the fibrous clays. The ¹³C-NMR spectra of zein and selected samples of zein-sepiolite are presented in Figure 4.4. In the ¹³CNMR spectra of zein-sepiolite samples, the intensity of the characteristic signals of zein increase with the coverage of the silicate surface by protein. Thus, the spectra of the hybrid compounds Z-SEP16 and Z-SEP24 (Figure 4.4 a) still show a very poor signal to noise ratio on account of their carbon content, which is in accordance to the results of elemental chemical analysis of these compounds (7.4 and 9.9 % of C, respectively). Despite this difficulty, from the spectra of the bio-hybrids deconvoluted in Figure 3.3b, it is possible to evidence a small shift of the 174 ppm signal to lower ppm, suggesting the existence of interaction of zein with the silanols groups. This effect is more accused for the bio-hybrids with lower content in zein. In the spectrum of sample Z-SEP24 a signal at 163 ppm is also observed, which can be also related

to the presence of perturbed carbonyl groups. In contrast, the Z-SEP48 bio-hybrid sample with high content of adsorbed zein (Figure 4.4 a), presents a spectrum more similar that of the starting zein, indicating that a large part of the macromolecules are not interacting with the silicate surface. The deconvolution of its spectrum shows the presence of a shoulder in the 172 ppm signal (Figure 4.4 b) as well as of a new peak at 29 ppm. This latter evidence could to be associated with the existence of interactions between the protein and the silicate. A similar effect cannot be deduced for other samples due to the low resolution of their spectra in this region (Svagan *et al.*, 2010).

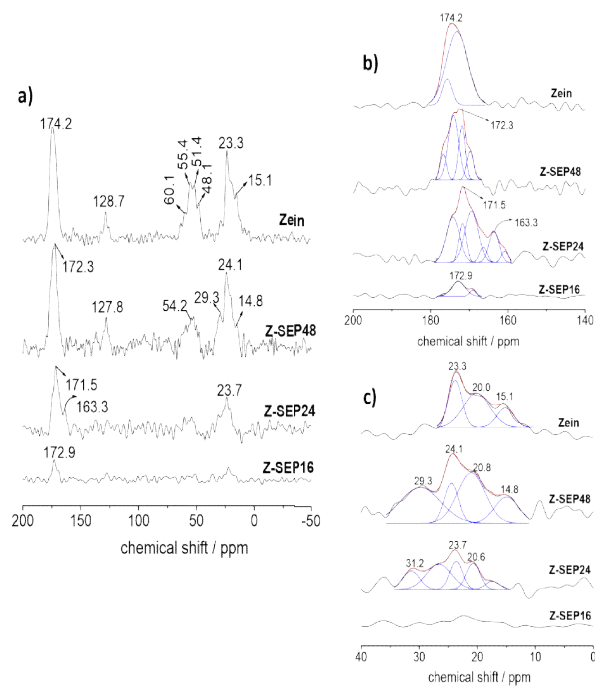


Figure 4.1 ¹³C CP/MAS NMR spectra of pure zein, Z-SEP48, Z-SEP24 and Z-SEP16. Deconvolution details of those ¹³C NMR spectra in the (b) 200-140 and (c) 40-0 ppm range

The morphology of prepared bio-hybrids was investigated by FE-SEM technique, which allowed to observe the textural differences in the bio-hybrids compared to the starting zein. This latter shows the presence of globular aggregates, which result from the strong zein-zein and zein-solvent interactions during the drying process, leading to the formation of those stable aggregates. In contrast, in the hybrid materials it is not possible to distinguish the presence of such type of protein agglomerates. In bio-hybrids based on both type of clays, the silicate fibers appear to be well integrated inside the structure of the zein, making difficult the protein-protein interaction which is the cause of formation of the aggregates.

Therefore, this observation points out to the existence of a considerable interaction between the clay fibers and the protein, as already deduced by FTIR and NMR spectroscopic results. However, a careful analysis of the FE-SEM images of ZSEP54 with the highest zein content (Figure 4.2 c and d), reveals the presence of small spheres that could be attributed to the presence of segregated particles of this protein. This observation points out to the existence of zein-zein interaction that stabilizes increasing amounts of zein on clays, as 53.59 g zein/100g sepiolite, presented in Table The TEM image of the same sample, presented as insert in the Figure 4.5 c, corroborates the presence of these small zein aggregates in the bio-hybrid that contain high amounts of the protein.

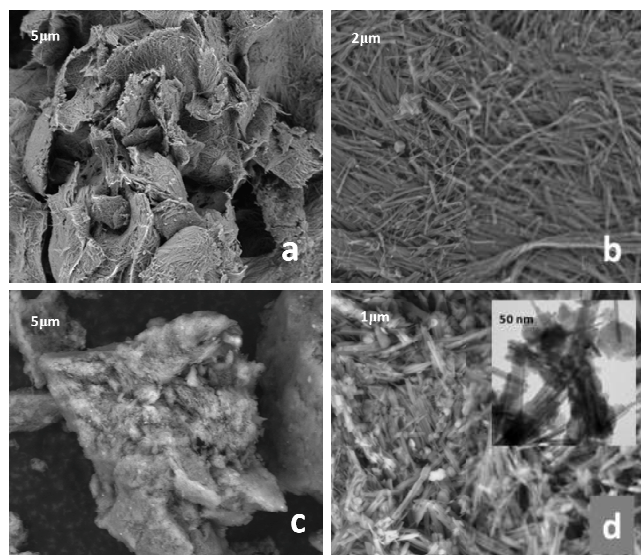


Figure 4.2 FE-SEM images of Z-PALY28 (a and b) and Z-SEP54 (c and d) bio-hybrids. Insert in the image (d) shows the TEM image of Z-SEP54

ZEIN- FIBROUS CLAYS BIO-HYBRIDS AS FILLER IN BIOPOLYMER MATRICES

Zein-fibrous clays bio-hybrids could be advantageous as an ecological alternative to alkylammonium-based organoclays for different applications, especially in the field of nanocomposites, i.e. acting as bio-organoclays. For this application, stability tests of these bio-hybrids were carried out. Washing tests in pure water of the Z-SEP48 biohybrid, chosen as example, provoked only a 2.6 % weight loss. This result indicates that zein-clay hybrids have a good stability in water, which could be also a proof of the strong interaction between both components (Svagan *et al.*, 2010; Swiegers, 2013). Taking into account that zein-fibrous clay bio-hybrids show on the one side lower hydrophilicity than the unmodified clays, and on the other side organophilic properties, together with biocompatibility and biodegradability properties, this new class of materials could be good candidates as fillers or additives of polymer matrices in the preparation of nanocomposites in general and bionanocomposites in particular. Those properties may help to improve the characteristics of polymers from biological origin such as polysaccharides or proteins, as they usually show high hydrophilicity and low stability in water.

Thus, the polysaccharide alginate was chosen as a model biopolymer matrix to test the efficiency of zein-clay bio-organoclays as nanofillers in the development of bionanocomposites. This application has been chosen because natural polymers, such as polysaccharides and proteins, are receiving considerable attention for the development of green-plastics due to their availability, low cost, high biocompatibility and biodegradability, as well as good film-forming ability and flexibility in most (Takahashi *et al.*, 1996; Bessadok *et al.*, 2009) However, much work is still needed to improve the mechanical and physical properties as well as the low water resistance of this type of biopolymer films (ZarateRamírez, 2011; Jerez, 2007, in order to allow their use in wet environmental conditions. In the present case, these novel bio-organoclays based on zein-fibrous clay systems may afford both the reinforcing role and the enhancement of water barrier properties due to the presence of zein.

In this way, bionanocomposite films were prepared by dispersion of the zein-sepiolite or zein-palygorskite bio-hybrid compounds within an alginate matrix. For the sake of comparison, alginate bionanocomposites using neat sepiolite as filler as well as alginate films without incorporation of bio-hybrid particles have been also evaluated.

Mechanical properties and transparency of alginate-based bionanocomposites films

Tensile properties (E) and the percentage of elongation at break (CB) of ALG/Z-SEP and ALG/Z-PALY bionanocomposite films were evaluated. Films of pure alginate and pure zein (2% w/v) have tensile modulus of approximately 3.9 and 0.5 GPa, respectively. The incorporation of neat zein in alginate diminishes both tensile modulus and elongation at break, presenting values of 1.12 GPa and 3.7%, respectively, in the case of an alginate film loaded with 25% of zein (w/w). Sepiolite alone acts a filler enhancing the modulus (e.g. 5.1 GPa in alginate films containing 50% sepiolite), but it diminishes the plastic behaviour of the system up to 3.3%.

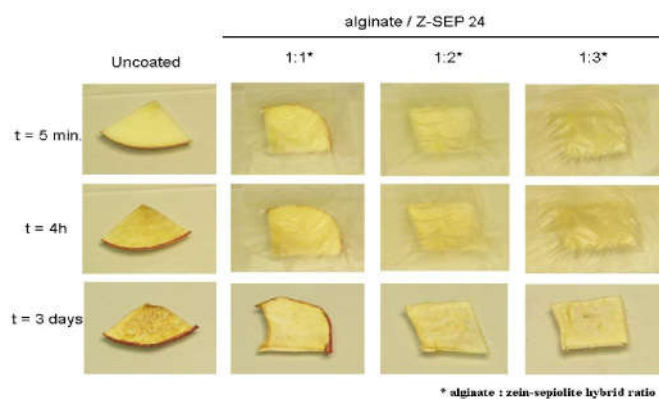
Contact angle measurements

Measurements of contact angle constitute a useful tool to determine the hydrophobic or hydrophilic characteristics of a surface. Table 4.5 shows contact angle (θ) values determined in alginate-based bionanocomposite films. A quantitative definition of the relative terms hydrophobic and hydrophilic surfaces has been done for surfaces exhibiting water contact angles higher than 65° and lower than 65° , respectively, (Vagler, 1998). Pure alginate film shows a value around 90° , which may be related to the slow diffusion of the water from the outside to inside of the film due to their crystallinity. In the case of the alginate-based bionanocomposites, all the films here analyzed showed hydrophobic surfaces with very close values. Films of alginate loaded with neat sepiolite shows a decrease in the contact angle to a value close to 67° in comparison to the film of pure alginate. The bionanocomposites incorporating ZSEP bio-hybrids show contact angles between 81° and 88° (Table 4.5). This significant increase in the contact angle in comparison to the alginate-sepiolite film may be related to the reduced hydrophilicity showed by sepiolite fibers once assembled to zein protein, as observed in moisture sorption isotherms (Figure 4.7), making these alginatebio-hybrid films have a higher hydrophobic surface than the alginate films loaded only with pristine sepiolite. Preliminary verification of the good barrier properties of the alginate/zein-clay bionanocomposites towards the passage of water vapour and oxygen, being so promising materials for packaging applications in the food area

Sample	Contact angle / degree
Alginate	90.4 ± 20.08
Alginate/Sep	67.1 ± 15.33
Alginate/Z-SEP10	88.2 ± 4.95
Alginate/Z-SEP29	81.3 ± 2.17
Alginate/Z-SEP47	84.3 ± 5.13

SYNTHESIS AND CHARACTERIZATION OF ZEIN-SEPIOLITE BIONANOCOMPOSITE FOAMS

Zein-sepiolite bionanocomposite foams were prepared by a mechanical mixing of zein with 0, 3.5 and 7% (w/w) of pure sepiolite (Sep) or sepiolite modified with magnetic



* alginate : zein-sepiolite hybrid ratio

Evaluation of the protective role of various alginate/[Z-SEP24] bionanocomposite films against oxidation of apple slices

nanoparticles (SepNp). As commented previously in the Experimental section (see Chapter 2, § 2.3.3, Figure 2.2), a mechanical mixture of both components was conformed as pellets, and then the soluble fractions of protein were extracted in absolute ethanol. The alcohol-treated pellet was then immersed in pure water to swell the material for subsequent freezing and lyophilization, giving rise to the zein bionanocomposite foam. Given that the strategy employed in the preparation of these zein foams is new, a study of each individual step of the foaming process was carried out, being presented at the beginning of this section. The study of the relationship between the structure and the properties of the resulting foams was also addressed in this section, as well as the possibility to provide the foam with superparamagnetic properties by using a magnetite-modified sepiolite instead of neat sepiolite (Yang *et al.*, 2013).

Study of the foaming process in the preparation of zein foams

Zein bionanocomposite foams were prepared considering the solubility of zein in pure ethanol and pure water. In this context, the foaming process of zein was investigated in detail. In the study of this process, two steps have been considered: i) the immersion of the zein-based pellet in absolute ethanol:[35] ii) the posterior immersion of this alcohol treated pellet in pure water, followed by the consolidation of the obtained structure by means of freeze-drying technique. In order to have a comprehensive understanding of the phenomena that take place during the foam formation, a careful study of the process was carried out in pellets made of neat zein.

Immersion of the pellet of absolute ethanol

A weighted amount of zein was conformed as a pellet and immersed in absolute ethanol (Figure 5.1 a), where the alcohol provokes the extraction of soluble components in a process described in Chapter 3, 3.2.2. Figure 5.1 b shows the cross-section of the zein pellet, in which it is clearly evidenced the aspect change to a rubbery and sticky material, like a resin, whose handling became extremely complicated. Actually, it seems that the zein particles are agglutinated by the alcohol penetration that induces partial solubilization of zein and agglomeration of non-soluble fractions, being an important step for the stabilization of the pellet conformation. The morphology of this material observed by FE-SEM microscopy once frozen in liquid N₂, and dried by lyophilization is shown in Figure 5.1 c.

This image reveals a homogeneous and compact structure in resulting material, far away from the searched porous structure.

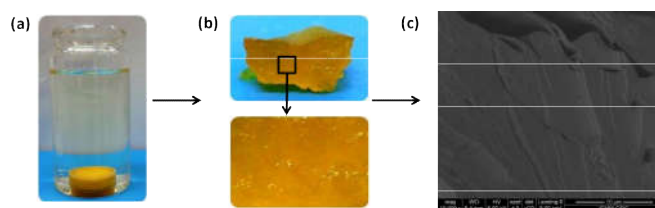
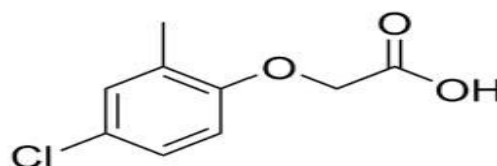


Figure 5.1 Photographs of (a) pure zein pellet when immersed in absolute ethanol and (b) cross section of the resulting alcohol-treated pellet. (c) FE-SEM image of the material once frozen in liquid N₂ and dried By lyophilization

ZEIN-SEPIOLITE BIONANOCOMPOSITE FOAMS AS ADSORBENTS FOR HERBICIDE REMOVAL

The widespread use of pesticides in agriculture and other activities increases the presence of these chemical substances in soils and ground water, becoming a serious environmental problem. Pesticides are generally applied in higher doses than those required for the control of pests and the excess reaches soils and groundwater due to transport processes such as leaching, runoff, etc. (De Wilde *et al.*, 2007). Several adverse effects of pesticides on human health/animal were reported in the literature, which include teratogenesis, oncogenesis, mutagenesis, hemotoxic and neurotoxic effects, endocrine disorders, among other unwanted reactions (Maroni, M and Fait, A. 1993; Cardoso *et al.*, 2006; Undabeytia *et al.*, 2008). In this sense, adsorption processes are generally known to be one of the most effective techniques for removal of these environmentally hazardous pollutants. On the other hand, biosorption processes using cheap and biodegradable materials, are currently considered as a desirable technological and ecological alternative in water treatments. In this area, bionanocomposite foams become an attractive material for the removal of pollutants or harmful compounds. A recent example of this use of macroporous bionanocomposite foams based on locust bean gum (LBG) and a layered silicate in the removal of dyes from water (Instituto de Ciencia de Materiales de Madrid (ICMM). This herbicide was chosen as model due to its organophilic character and its widespread use (Wickein *et al.*, 2013).



Chemical structure of the MCPBA herbicide

Conclusion

Zein protein from corn used in this study was furnished by Sigma-Aldrich. This protein is presented as a yellow powder and was reported to be approximately 35% *azein*, which includes two sub-units of average molecular weight of 22 and 24 kDa. The novel porous materials can be provided with magnetic properties by using a sepiolite modified with magnetic nanoparticles instead of the neat sepiolite or by incorporating magnetite nanoparticles into the zein matrix. The systematic study carried out shows the possibility to prepare

zein-based bionanocomposite foams, reinforced with natural sepiolite fibrous clay, by a new, easy and ecofriendly foaming method. The existence of strong affinity between zein and the sepiolite-based fillers results in materials with good mechanical properties and improved water resistance, and in certain cases, the use of magnetite-based fillers introduces interesting superparamagnetic properties in the bionanocomposite foams. These materials offer interesting results for the retention of MCPA, tested as a model herbicide, which supports the potential use of these biocompatible and biodegradable functional bionanocomposites in environmental remediation. The zein bionanocomposite foams were prepared by a new, easy and ecofriendly foaming method. The resulting porous materials can be provided with magnetic properties by assembling a sepiolite modified with magnetic nanoparticles to the zein matrix. The structure and properties of the resulting bionanocomposite foams were studied in relation between the kinds of filler. Zein-based bionanocomposite foams were designed in order to show good water stability as well as magnetic and mechanical properties, which could be profited for applications in environmental remediation, such pollutants retention in aqueous media.

A new type of bio-organoclay material based on the combination of sepiolite or palygorskite fibrous clays with zein. The assembling of these components reduces the hydrophilic character of the pristine clays, conferring new properties to the resulting bio-hybrids, which can be applied as fillers in the preparation of bionanocomposites. A first approach of this application is the preparation of alginate-based bionanocomposites for potential use in food packaging. These alginate/zein-clay systems are able to form self-supporting films that show a marked resistance to the passage of water molecules in comparison to pristine alginate films. Mechanical properties, water uptake, contact angle measurements, water vapor transmission rate, and gas permeation properties were evaluated in the alginate-based bionanocomposite films with zein-clay bio-hybrid as filler. The results reveal that the proportion of each of the bio-hybrid employed in the formulation of alginate bionanocomposite films has a crucial role in the water vapour barrier and gas permeation properties. It is observed a significant decrease in the water uptake with the increase of the zein content adsorbed on the clays, as well as with the proportion of zein-clay bio-hybrid incorporated in the alginate matrix.

This behaviour was extremely important in the permeability of gases through the bionanocomposite films in high humidity conditions, which showed a prominent permeability toward CO₂, while the barrier properties toward O₂ were not strongly affected by the degree of plasticization occasioned by the water molecules in the bionanocomposite membranes. Indeed, the bionanocomposite films prepared here, present homogeneity, transparency, as well as improved barrier properties, being therefore promising as new ecofriendly materials with potential applications in food packaging, which supports the potential use of these biocompatible and biodegradable functional bionanocomposites in environmental remediation. Additionally, these porous materials were easily removed from the aqueous medium with the help of an external magnetic field, thanks to their superparamagnetic properties, being very attractive as bio-magnetsorbent materials. Commercial Cloisite®Na and Cloisite®30B containing a type of exchangeable cation, Na⁺ and a quaternary alkylammonium ion, respectively, were used. By a

systematic control of the synthesis conditions and the use of several spectroscopic methods, it was possible to investigate the zein intercalation mechanism into both types of homoionic clays, as well as to elucidate the structure and properties of the resulting zein–montmorillonite biohybrids. It was deduced that zein adsorption processes are strongly influenced by the kind of interlayer cation, the solvent used for the dispersion of the protein, and the order of mixing of both components. The obtained bio-hybrids were evaluated as bio-organoclays for incorporation in biopolymer films.

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