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Bioremoval of Antibiotics by Using Biodegradable Hydrogel Beads from Aqueous Solutions

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Abstract

Elimination and proper disposal of pollutants of emerging concern are a major challenge in environmental remediation. On the other hand, biodegradable and naturally-occurring materials have been successfully used as adsorbents of inorganic and organic contaminants from solutions with very positive results. This research proposes the use of alginate (AB) and chitosan (CH) hydrogel beads for the bioremoval of antibiotics, which are listed as top priority pollutants in water. Batch experiments were used to determine the experimental conditions at which the uptake of Enrofloxacin (En) and Penicillin G (Pe) antibiotics is maximized. Parameters like pH, adsorbent dose, initial antibiotic concentration, salinity and presence of other interferences were investigated. Results indicate that pH plays an important role on the adsorption of both antibiotics, whereas salinity and the presence of other interferences (heavy metals and surfactants) have a negative effect on the encapsulation. En removal was maximized at pH 6 whereas Pe was readily trapped at pH 2 with AB. A better adsorption of antibiotics was observed when CH was used at high antibiotic concentrations. Time-dependent experiments indicate than less than 60 minutes are needed to reach equilibrium. Finally, morphological analyses show the high porosity and optimum properties of both beads as adsorbents. These natural beads have not only proven to be potential antibiotic adsorbent, but also have a low cost and toxicity.

Keywords: Enrofloxacin; Penicillin G; Hydrogel Beads; Alginate; Chitosan; Bioremoval; pH

Introduction

Numerous studies are being carried out to achieve the preparation and characterization of new materials for the elimination of organic pollutants from contaminated water. Naturally occurring adsorbents are potential candidates for this application as they are biodegradable and inexpensive. There are diverse types of polymeric particles that are commonly used in the removal of organic pollutants (i.e. drugs and antibiotics) from solutions. According to their size, they can be classified as micro- and nanoparticles. Microparticles are polymeric spheres whose size ranges from 1 to 250μm (ideal diameter is <125μm). Within this group, we can include microcapsules, which are vesicular systems where the pollutant is confined in a cavity and surrounded by a single polymeric membrane; and the microspheres that are matrix systems in which the pollutant is dispersed in the particle. On the other hand, nanoparticles are polymeric systems with smaller sizes (<1μm) [1].

The polymers that are used for the preparation of these particles are macromolecules formed by hundreds or thousands of functional units, named monomers. These polymers can be synthetic, artificial and/or natural. These last years, natural polymers or biopolymers have attracted the scientific interest in biotechnology [2], where biopolymers are utilized to protect cells and tissue or as encapsulating and delivery agents of several drugs and biologically active molecules.

An ideal polymer should possess these characteristics to succeed as an adsorbent: biodegradable, mechanically resistant, chemically stable and low toxicity [3].

Amongst the broad number of natural polymers that are used for pollutant adsorption, this study uses alginate and chitosan. Chitosan is a lineal biopolymer that can be produced by the partial deacetylation of chitin by alkaline hydrolysis at high temperatures. Chitosan has several applications due to its biocompatibility and low toxicity [4,5]. Perhaps one of the most important advantages of using chitosan in several applications is its high water solubility in acidic conditions. This is crucial, because pollutant adsorption can be tuned by simply adjusting the pH of the medium where the micro- or millisphere is utilized. Polyalginate are chains of alginic acid, a natural polysaccharide that is formed by lineal chains of α-L-guluronic and β-D- mannuronic acids, linked by α1→β4 glycosidic bonds. Polyalginates are the most important components of brown algae like Lessonia trabeculata and Macrocystis integrifolia [6,7]. One important characteristic of alginates is its capacity of forming hydrocolloids in hot and cold water, creating solutions with a high viscosity, dispersions and hydrogels. Alginates are ionizable polymers, where the negative charge resides on the deprotonation of carboxylic acids. Alginate has been used in the production of films, used as a thickening agent, and in the removal of pollutants from wastewaters like heavy metals and organic compounds [8-10].

The chemical structures of chitosan and alginate polymers are shown in Figure 1. As observed, both biopolymers share the same glucose-like scaffolds. This particular similarity to glucose makes them biodegradable and avoids any secondary contamination by the accumulation of these materials. Figure 1 also displays the presence of important functional groups in both polymers. Chitosan has an amino group that can become positively charged (NH3+) in response to acidic pH values. On the other hand, polyalginic acid shows carboxyl groups that are pH-dependent and can become negatively charged at relatively high pH values (pH higher than 3) [6] .

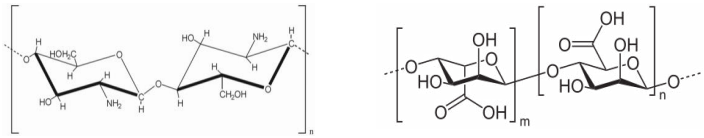

Figure 1. Chemical structure of chitosan (left) and polyalginic acid (right)

The American Environment Protection Agency (US-EPA) has listed antibiotics and other pharmaceutical products as top priority pollutants of emerging concern [11,12]. The prevalence of these substances in wastewater puts in danger not only the aquatic life, but also contributes to the development of microbial resistance [12]. Enrofloxacin (En) and Penicillin G (Pe) were chosen as our target antibiotics due to their widespread use in the population. En is prescribed for the treatment of infection in animals. Pe is typically given intravenously or intramuscularly in humans. Chemically speaking, En and Pe belong to different antibiotic categories. En is a fluoroquinolone with different functional groups like carboxyl, amine, aromatic and ketone [11]. Conversely, Pe is a β-lactam with carboxyl, thioether, amide and aromatic as the most important functional groups [12].

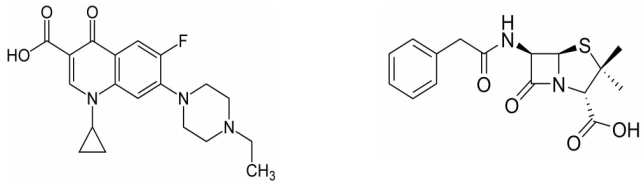

Figure 2. Chemical structure of the antibiotics Enrofloxacin (left) and Penicillin G (right)

For this study, alginate (AB) and chitosan (CH) hydrogel beads were prepared and tested as potential adsorbents for these antibiotics. The industrial application of these beads in envisioned in the large scale. Experimental conditions such as pH, mass of dry beads, antibiotic concentration, salinity, presence of ionic and molecular interferences, and time were explored to maximize the uptake of En and Pe. The relevance of this research goes beyond the encapsulating properties of AB and CH. In the short term, these hydrogels could be used for the treatment of residual waters of pharmaceutical industries. Most of the available literature focuses on the development of materials as drug delivery agents [2-4], but few studies have addressed the potential application of these materials in environmental remediation.

Materials and Methods

Reagents and solutions

Stock solutions of 1000ppm (mg of antibiotic per liter of solution) were prepared by dissolving Enrofloxacin (En) and Penicillin G (Pe) in deionized water. Both reagents were of analytical grade (Fisher Scientific). Solutions of varying concentrations were produced by dilution of the stock solutions until the desired concentration was reached. No buffer was used in the preparation of the solution to minimize salt effects in

the experiments. This protocol has been previously used with phenols [6], heavy metal ions [9], and dyes [10] with positive results. The initial solution pH of each solution was adjusted to the required values by adding aliquots of diluted HCl and NaOH prior contact with the hydrogels.

Preparation of the hydrogel beads

Alginate beads (AB) were prepared by a protocol that is available elsewhere [8]. In summary, sodium alginate (reagent grade, Fisher Scientific) was dissolved in deionized water and left under magnetic stirring overnight for its complete dissolution (a thick and dense solution was obtained). In the meantime, a calcium chloride (reagent grade, Fisher Scientific) solution (0.2M) was also prepared with deionized water under magnetic stirring. Upon complete dissolution of both reagents, the alginate solution was added drop by drop into the calcium chloride solution by using a peristaltic pump. Alginate beads (AB) were immediately observed upon contact of both solutions. Finally, AB were rinsed and suspended in deionized water and finally stored in the refrigerator at 4°C degrees. This technique allows obtaining millispheres of AB a diameter of 2mm.

Chitosan beads (CH) were prepared following a commonly used protocol [13]. It consists on the dissolution of chitosan in a mixture of 10mL of acetic acid in 240mL of deionized water. Chitosan took about 24h to totally dissolve in the solution, and generated a highly viscous solution. In a separate plastic container, a 2.5M solution of NaOH was prepared and kept under magnetic stirring. Finally the chitosan/acetic acid solution was added drop by drop into the NaOH solution using a peristaltic pump. CH beads were formed by neutralization of the acetic acid in NaOH. Chitosan beads were also rinsed, suspended and stored, using a procedure similar to AB.

Determination of the dry/wet mass ratio of the beads

AB and CH hydrogel beads have high water content. These samples are highly porous and need water to maintain their structure and porosity. Prior adsorption experiments, the dry/wet mass ration of both beads was obtained to report actual dry mass values (dry alginate or chitosan). To do so, the masses of wet beads were recorded and then placed in an oven at 60°C for drying (close to 12h). Higher temperatures were not used to avoid organic decomposition. Then, the masses of dry beads were taken and compared to those of the wet beads. A linear calibration curve was constructed to correlate the wet and dry masses.

Adsorption Experiments

The uptake of En and Pe was studied in discontinuous experiments in duplicates using capped plastic polyethylene tubes with a capacity of 50mL. Experiments were run at room temperature under orbital agitation of 250rpm in an incubator shaker (New Brunswick Scientific, Model C24). Preliminary experiments indicated that less than 24h are needed to reach the equilibrium (maximum encapsulation). For the discontinuous experiments, a given mass of AB and CH were placed in contact with solutions of variable concentrations of En and Pe. Different equilibrium parameters like initial solution pH, concentration of antibiotics, dry mass of the hydrogel, salinity, and presence of ionic and molecular interferences were optimized at the time. Tubes were sealed with parafilm to avoid leaks. Upon equilibrium, antibiotic adsorption by both beads was obtained by comparison of the initial and final antibiotic concentrations. These concentrations were determined by UV-vis spectrophotometry at wavelengths of 334 and 220nm for En and Pe, respectively [14,15]. These analyses were carried out using an automatized microplate reader (Synergy4, Biotek). To confirm that adsorption was not reported from plastic adsorption or antibiotic degradation, preliminary assays demonstrated that both antibiotics do not adsorb onto any plastic surface and showed the same UV absorbance for at least 48 hours.

Time-dependent Experiments

The actual time that is needed for the adsorption of the antibiotics was determined by time-dependent experiments. Antibiotic concentration was kept as 100ppm, and this time the reaction setup was kept under magnetic stirring at 250rpm, to resemble the same stirring as the orbital shaker. Upon contact of the beads with the solution a timer was started and aliquots of the solution were taken at different time intervals with a plastic pipette. These samples were taken to the microplate reader to determine the residual antibiotic concentrations.

Data Analysis

The amount of adsorbed antibiotics onto AB and CH was expressed as Adsorption Percentage and calculated as shown in Equation (1):

$$\%ADS = \frac{(C_i - C_{eq}) * 100}{Ci} \qquad (1)$$

where Ci and Ceq are the initial and final concentrations of En and Pe in mg/L, respectively.

Characterization of the Encapsulating biopolymers

Surface texture and morphological properties of the beads were explored before and after the adsorption (encapsulation) of the antibiotics by scanning electron microscopy (SEM) using a Tabletop microscope (TM3000, HITACHI) with low vacuum. No gold coating was needed for these tests.

Results and Discussion

pH effect

Residual waters are subject to different composition, depending on their origin and the industry or environment at which it was exposed. These characteristics can be attained under different conditions like acidity (pH), presence of solvents, changes in pressure, alkalinity, and other parameters. pH is one of the most important parameters to evaluate during the adsorption of a molecule. Results of the effect of pH are shown in Figure 3. According to the data, the adsorption of En and Pe is highly dependent on the pH. The data indicates that En is better adsorbed by AB, whereas CH shows almost no response to En. The adsorption of En onto AB is maximized at a pH value of 6 and almost zero at low pH. These results totally agree with the chemistry of the functional groups that are present in En and AB.

Previous reports demonstrate that the pKa value of polymeric alginic acid is 3.0 [6]. This means that at pH values higher than 3, the surface of the alginate beads is negatively charged and therefore should have a higher affinity towards neutral or positively charged compounds. As observed in Figure 2, En also has a carboxyl group which should also get deprotonated at pH 3-5 [16]. However, En has three basic nitrogen atoms (as tertiary amines) that are highly basic and most likely protonated at pH values lower than 7 (NR3+). Based on these evidences and on the experimental pH results, we can conclude at pH 5, the surface of AB is negatively charged and En is negatively charged on the carboxylate side, but positively charged in the amino groups. This hypothesis is corroborated by the fact that the adsorption of En slowly decreases as the pH increases. At higher pH values the protonated amines of En lose their hydrogen atoms and become neutral, and decrease their affinity towards the negatively charged alginate of AB. At very low pH, AB is fully neutral and cannot interact with the positively charged En.

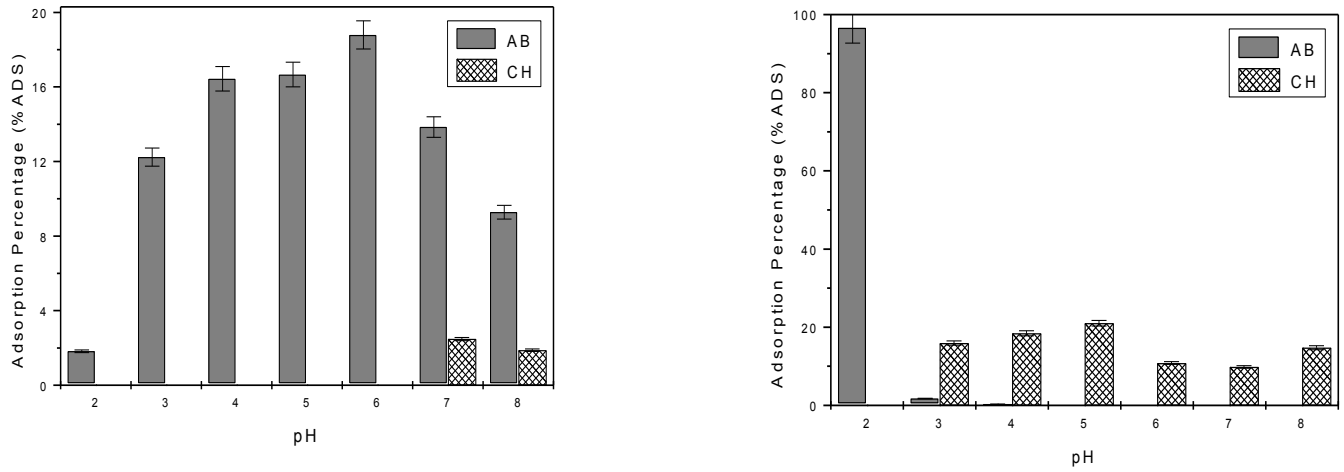

Figure 3. Effect of the initial solution pH on the bioremoval of En (left) and Pe (right) onto AB and CH.

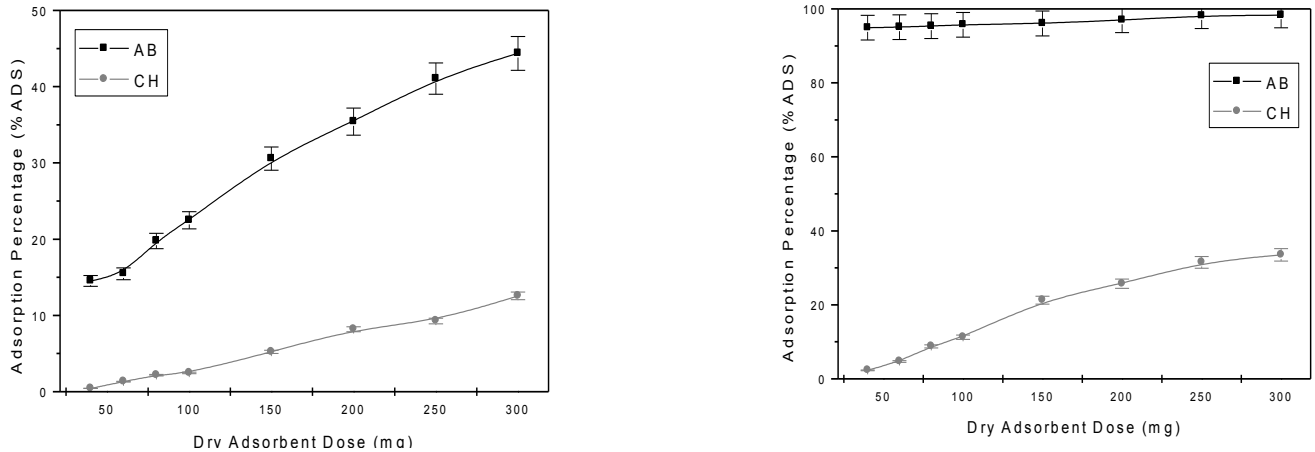

Figure 4. Effect of the mass of beads on the adsorption of En (left) and Pe (right) onto AB and CH.

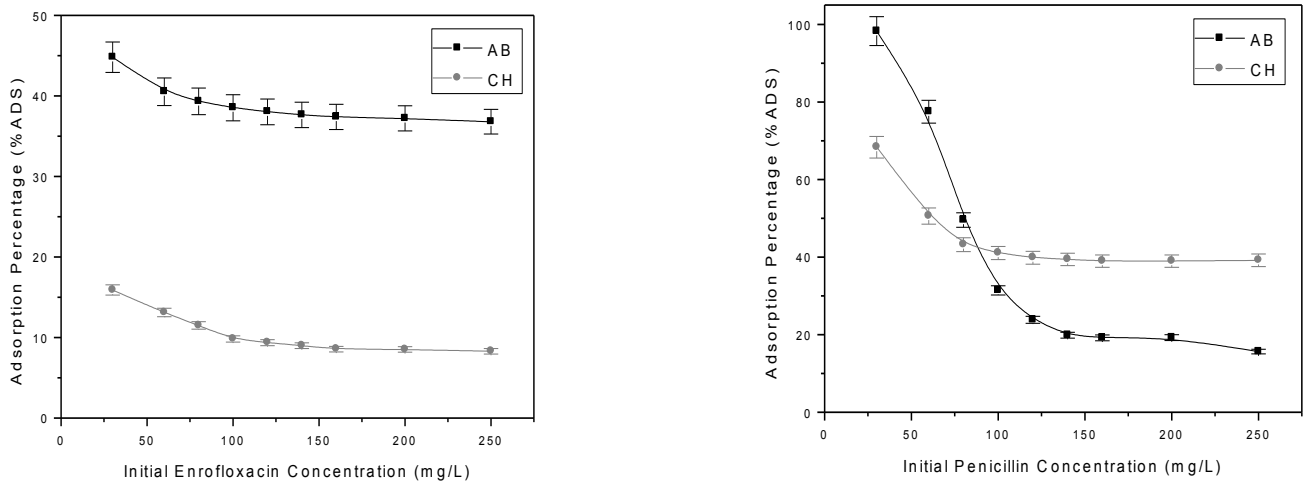

Figure 5. Effect of antibiotic concentration on the adsorption of En (left) and Pe (right) onto AB and CH.

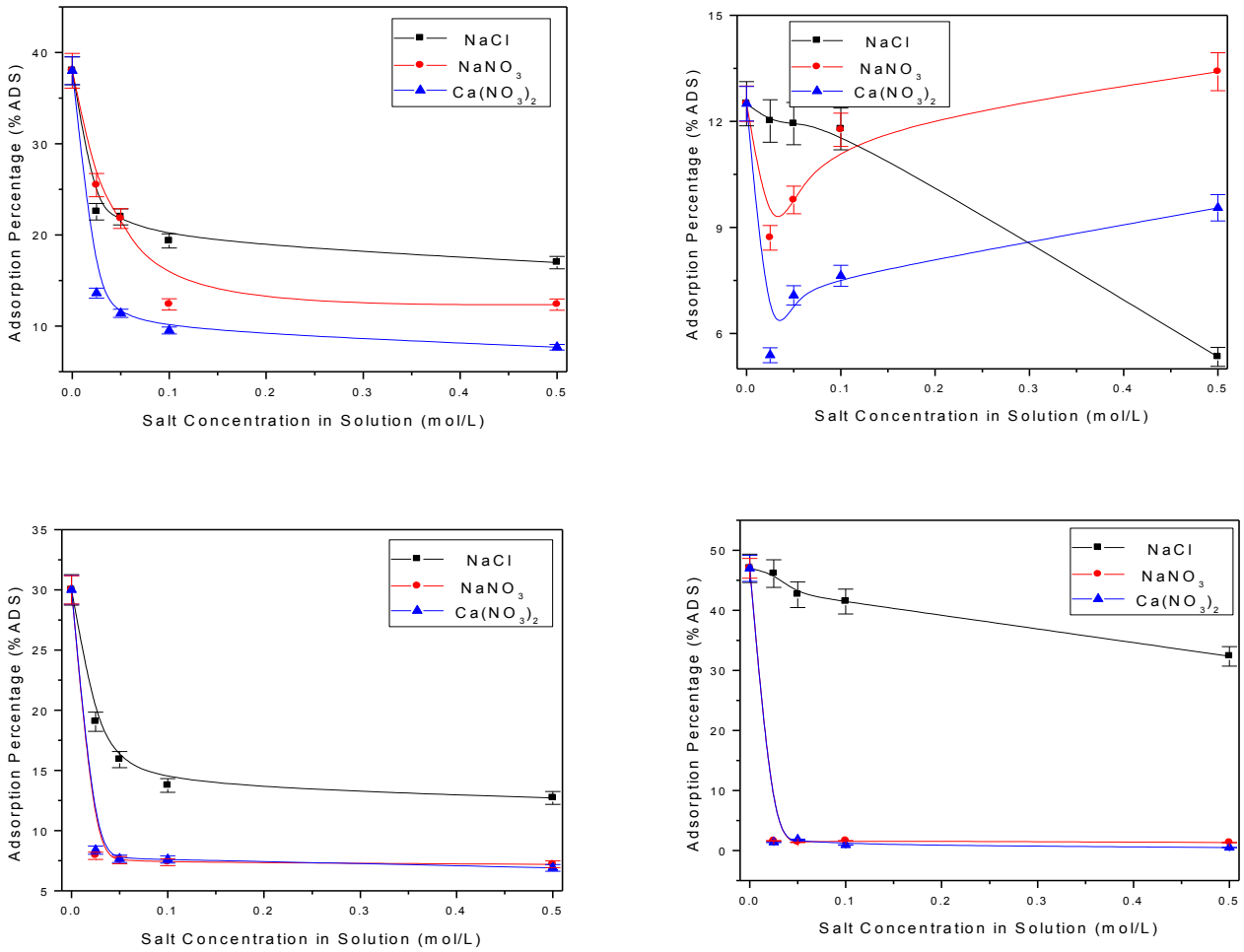

Figure 6. Salinity Effect on the adsorption of En with AB (top left), En with CH (top right), PE with AB (bottom left) and Pe with CH (bottom right).

Therefore, the adsorption of En onto AB could be mostly driven by electrostatic interactions between the carboxylate groups of AB and the protonated amines of En. In addition to these results, CH shows a very low adsorption due to the same reason. At low pH values, the surface of CH is positively charged (on the amino groups) and En is also positively charged, inhibiting their attraction.

In the case of Pe a similar behavior is observed. AB shows a high adsorption at pH 2 (close to 100%). Surprisingly, higher pH values show zero adsorption. This could also be explained by electrostatic interactions. At pH 2, the surface of AB is neutral and according to the structure of Pe (Figure 2) at that pH, Pe is also neutral. However, the presence of several polar groups in Pe (amides and carboxyl) can potentially form dipole-dipole interactions and hydrogen bonds with the hydroxyl and carboxyl groups of AB. At pH values higher than 2, both species become negatively charged, increasing the repulsion. As for CH, it shows a decent adsorption of Pe at pH 5 (slightly acidic). At this pH, a high encapsulation is expected because the surface of CH is positively charged and the carboxyl group of Pe is negatively charged. However, the carboxylate ion in Pe is surrounded by very hydrophobic groups, like two methyl groups and a thioether on one side and the nitrogen of a tertiary amide on the other side. These groups greatly increase the hydrophobicity and could prevent the interaction with the protonated amino group of CH. Likewise, the β-lactam ring, where the carboxyl group is attached to, is known to be highly hydrophobic [3].

Mass effect

A cost-effective mindset not only involves the elimination of antibiotics, but also the minimization of the amount of biopolymers that are used. For this purpose, different masses of AB and CH were put in contact with En and Pe solution to determine the minimum mass of the beads to remove the maximum or a reasonably high adsorption percentage. According to the results (Figure 4), all the adsorbents reach a steady adsorption percentage at high dry bead masses. Therefore, a higher mass of beads do not improve the adsorption, it actually creates bead aggregates.

The concentration of beads increases the traffic in the solution and reduces the active surface of the beads, preventing the diffusion of the antibiotics inside of AB and CH. The optimum masses for AB and CH are 250mg for both antibiotics. A steady adsorption of a very small increase was observed with 300mg. Mass effect indicated that the adsorption percentage for En was 43% and 8% with AB and CH, respectively. For Pe, adsorption percentages of 98% and 33% were observed with AB and CH, respectively.

Effect of the initial antibiotic concentration

A given mass of biopolymer will adsorb a certain number of antibiotic molecules (surface saturation). The concentration effect determines the concentration of antibiotic at which a given mass of hydrogel beads cannot adsorb any more En or Pe. As observed in Figure 5, all the adsorbents show a plateau, indicating that the saturation has been achieved and no more antibiotics can be taken after that point. In the case of En, 125ppm and 140ppm are enough to saturate 250mg of AB and CH, respectively. Penicillin G shows a similar behavior, showing that 140ppm and 100ppm are needed to saturate 250mg of dry AB and CH, respectively. It is important to highlight the difference in the curves for both antibiotics, Pe displays a more abrupt fall in the adsorption with concentration. This supports our previous hypothesis with the pH effect, when Pe is more hydrophobic and higher Pe concentrations are not friendly with highly polar environments like the watery inside of hydrogel beads.

Salinity effect

Pharmaceutical industries, normally, use additives in the formulation of medications. Moreover, the industrial process also involves the use of other inorganic ingredients that are needed to the synthesis and manufacture of pills and capsules. The effect of salinity explores the role of different salts in the retention of the adsorbed antibiotics. Since the adsorption of these antibiotics is mainly driven by polar and electrostatic forces, the presence of salts should be important. Figure 6 displays the effect of three salts on the adsorption of En and Pe. On average, salinity has a negative effect on the encapsulation of antibiotics in AB and CH. This effect is stronger at high salt concentration and with Ca(NO3)2, where the adsorption is almost inhibited. Since the affinity between the biopolymers and the antibiotics is mediated by electrostatic and polar forces, the salt ions act as shielding species and prevent the attraction of our target antibiotics by competing for the active sites. Na and Ca ions will always be more hydrophilic than the antibiotics that are organic compounds. The graphs also demonstrate that NaCl has the weakest effect on the adsorption. This is important in terms of recovery of these antibiotics from seawater.

Ca ions shows the strongest effect, most likely due to its higher charge, that allows it to have a higher affinity towards the carboxylate ions (in AB) or amino groups (in CH). Moreover, the adsorption of En on CH shows an initial decreased followed by a slow increase in the uptake with NaNO3 and Ca(NO3)2. NaCl shows similar effect when compared to the other cases. We can conclude that nitrate ions are responsible for this behavior. This phenomenon can be attributed to an initial adsorption enhancement at low nitrate concentrations, but a negative effect at high nitrate doses by repulsion of the negatively charged En and nitrate ions that are now in higher proportion.

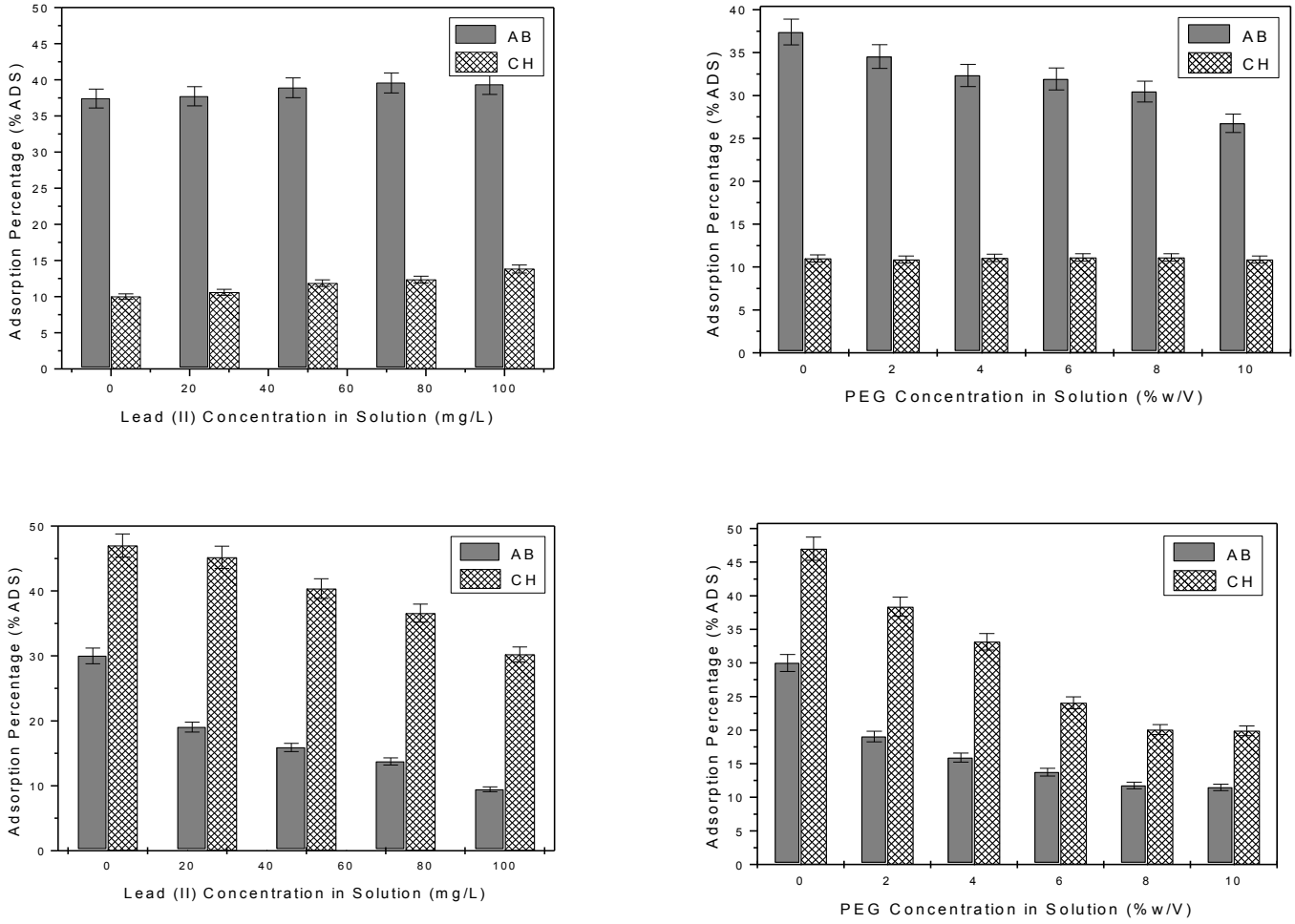

Figure 7. Effect on metal ions on the adsorption of En (top left) and PE (top right) and PEG on the adsorption of En (bottom left) and Pe (bottom right).

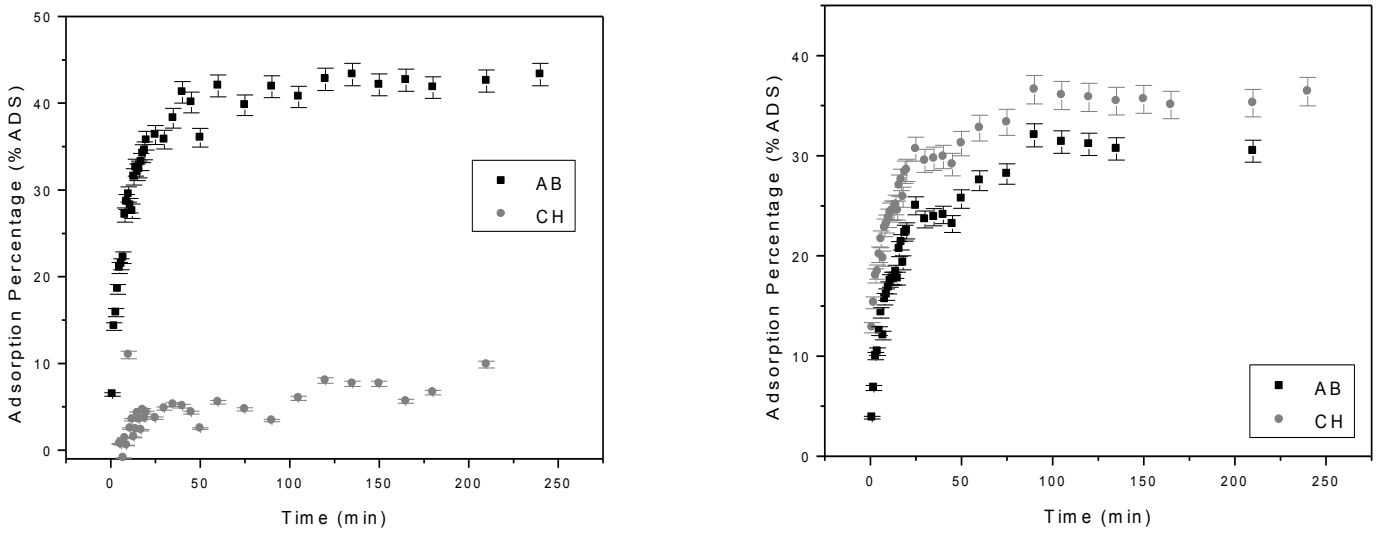

Figure 8: Time-dependence of the adsorption of En (left) and Pe (right) onto AB and CH.

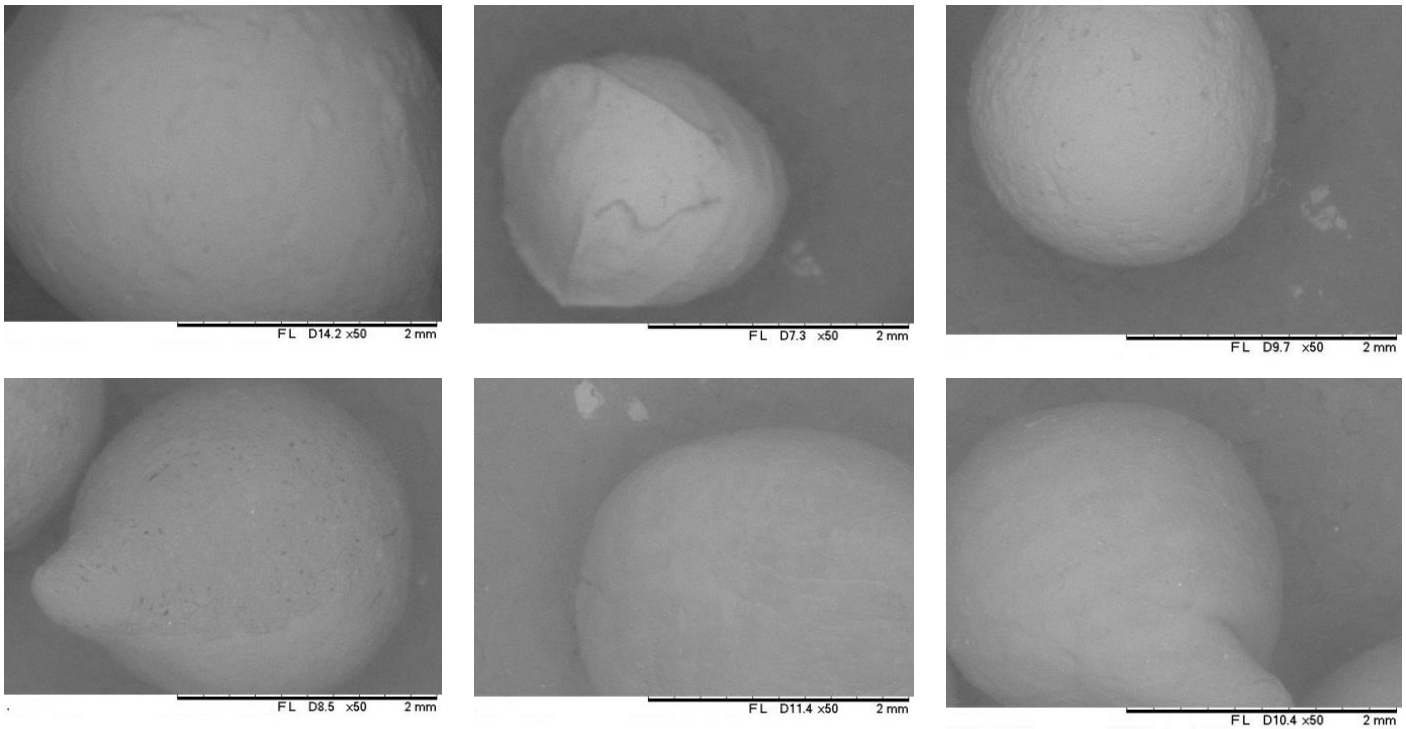

Figure 9. SEM analyses of AB beads (top left), AB+En (top center), AB+Pe (top right) and CH beads (bottom left), CH+En (bottom center), and CH+Pe (bottom right)

Effect of the presence of other ionic and covalent interferences

The bioremoval of antibiotics from solutions can also be disrupted by the presence of other solutes like other metal ions and surfactants (covalent compounds). In this test, En and Pe were mixed with a lead ions and a surfactant, polyethyleneglycol (PEG) to investigate the effect of these substances on the adsorption. Results are shown in Figure 7, indicating that lead (II) ions have a small effect on the adsorption of En for both biopolymers. Conversely, Pe shows a decrease in the adsorption at increasing doses of Pb ions. These results suggest that En targets different adsorption sites than Pb ions and Pe competes for the same adsorption sites with Pb ions for both biopolymers. The purpose of adding a crowding agent such as PEG aims to increase the molecular traffic in the solution, making the adsorption sites less accessible for the antibiotics. Due to the limited solubility and relatively larger sizes, En and Pe decrease their adsorption on AB and CH in the presence of PEG. Once again, the effect is more intense with Pe, due to its reduced water solubility.

Time-dependent experiments

Ideal industrial applications involve savings in workforce and energy. Therefore, a process that minimizes operation time and number of technicians will be preferred by industries. As shown in Figure 8, the adsorption of En and Pe was studied based on the time that is required to achieve a constant adsorption (equilibrium). According to the graphs, both adsorbents need less than 45min to reach equilibrium for En and less than 60min for Pe. The difference in time for Pe can be explained by its poor solubility and therefore slower transport to the active sites.

Adsorbent characterization by SEM

Surface area was explored by SEM to investigate the texture and morphology of the adsorbents. Figure 9 shows AB and CH before and after the adsorption of the antibiotics. As expected, both adsorbents display a very smooth surface with small hills. We suspect that those valleys are formed during the gelification and belong to the pore formation for intraparticular diffusion. The after-adsorption images show no changes on the adsorption, indicating the encapsulation of the drugs inside the hydrogel beads.

Conclusions

Development of eco-friendly adsorbents is a priority in scientific research. This research proposes biopolymers of alginate (AB) and chitosan (CH) to prepare millispheres for the uptake of the antibiotics Enrofloxacin (En) and Penicillin G (Pe). Results indicate that both adsorbents can be potentially used to

adsorb En and Pe. AB and CH report a higher affinity towards Pe with adsorption values of 98% and 70%, respectively. En is better adsorbed by AB (45%). Bioremoval of the antibiotics is greatly affected by the pH, salinity and presence of metals and surfactants. These results compare to previously reported data, for example Gomez-Gill et al. [17] used Artemia franciscana, Kim and Choi [18] used solid lipid beads with results that compare to this report. Other materials include synthetic zeolites [19] to encapsulate ketoprofen, however zeolites are not biodegradable. Nanoparticles are catching most of the attention due to their nanoscopic dimensions [3,20,21], however new controversial topics have risen due to the materials that are used in their preparation and the proper disposal of these nano-sized particles. Finally, other research groups have used activated sludge [22] and activated carbons [23] with very interesting adsorption properties. Finally, these materials also have a biomedical application as they can be potentially used as drug delivery agents for these antibiotics and other pharmaceutical products.

Acknowledgements

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