Functional polymer - clay nanotube biocomposites with sustained drug release

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Abstract. By adding 5% (w/w) of halloysite nanotubes that have been modified (loaded) with proteins or drugs it is possible to produce strong and functional biocomposites. Materials loaded with both types of materials were investigated using ultraviolet-visible spectrophotometry and thermogravimetric analysis to determine their release kinetics and overall loading efficiency. It was found that both released over a period of 5-20 hours with two distinct phases being present. An initial “burst stage” of release followed by a period of sustained release. Specifically, for proteins it has been shown that a significant amount (50-75%) remain immobilized even after being dispersed. The typical loading efficiency for both classes of molecules was 10-15%. These modified nanotubes can both strengthen a material and give it unique functionality and possible uses include more effective externally applied antibiotics and immobilized proteins with enhanced stability and reusability.

1. Introduction
Halloysite nanotubes (HNT) of 50 nm diameter and 1-1.5 µm length are a perspective dopant for stronger and functional polymer composites. We exploit the advantages of halloysite nanotubes for polymeric composites and its unique ability to act as a container for macromolecules (proteins and drugs). It was found that the addition of 3-5 % halloysite to polypropylene increases its mechanical strength by ca. 50 %. Besides, it is possible to take advantage of the unique chemical storage ability of halloysite to add needed functionality to polymers. Halloysite nanotubes are capable of storing biomolecules that are negatively charged inside of its lumen at 10 -15 wt. percent. Anti-septic chemicals[1], anti-corrosion chemicals[2], proteins, and a subclass of biocatalytic proteins called enzymes may also be stored and slowly (5-50 hrs) released from these natural biocompatible nanotubes.

By simply mixing HNTs and a protein, or drug, together it is possible to immobilize the protein or drug inside of the tubes. We found that loaded proteins and are split into two essential categories: protein molecules that are permanently attached inside of the nanotube lumen and proteins that will be released back into solution during 4-5 hours. Having two different categories and therefore two different “lifetimes” for a selected protein immobilized with halloysite allowed for the study of a burst phase of activity and a sustained phase. These phases correlate to the proteins that do not reside permanently and those that are tightly bound in the tube lumen, respectively. Drugs also show a similar phenomenon of a two-phase release pattern. Therefore, by taking halloysite, a naturally abundant nanocontainer, and utilizing the unique catalytic properties of enzymes and the potent effects of drugs it is possible to create strong and biofunctional composite materials.
2. Experimental
The first necessary step in our research was to study the loading efficiency and release kinetics of the selected proteins loaded inside of halloysite nanotubes. In Table 1 we summarize some proteins that were used in our experiments. An essential feature of these protein group is that they may be overall negative or positive depending on the pH at which we are making loading and release (e.g. above or below their isoelectric point).

There is a standard procedure that can be used to load any molecule inside of halloysite and can be found in various pieces of literature[3]. Proteins are very sensitive to their environment and in particular to pH and temperature. Temperatures above 60°C often cause irreversible thermal denaturation, effectively destroying the protein. Therefore it is suggested to use ice during sonication to neutralize heat generation from this technique. Also, water based buffers are the preferred solvent for proteins, and although it is possible to use organic solvents like acetone it’s not suggested. After performing the loading procedure the enzymes release kinetics were studied by studying the concentrations of the protein after halloysite being dispersed in water by measuring the UV absorbance of a sample at 280 nm. Thermogravimetric analysis (TGA) was employed to measure the tube loading efficiency and to elaborate on the two categories of proteins (permanently immobilized and capable of release). Some of the enzymes, glucose oxidase and horseradish peroxidase, had their enzymatic activity qualitatively tested. The results from those experiments confirm that the enzymes retain their enzymatic activity after loading and drying.

Table 1. The isoelectric point of a protein can be used to manipulate its charge. At a pH above the isoelectric point the protein will have a net negative charge and at a pH below the pI the protein will be positively charged.

| Enzyme            | EC Number | Molecular mass (kDa) | pI (Isoelectric point) |
|-------------------|-----------|----------------------|------------------------|
| Bovine Serum Albumin | N/A       | 70 (monomer)         | 5.0                    |
| Glucose Oxidase   | 1.1.3.4   | 65 (dimer) = 130     | 4.2                    |
| Lipase            | 3.1.1.3   | 50 (dimer) = 100     | 5.0                    |
| Myoglobin         | N/A       | 17 (dimer) = 34      | 7.0                    |
| Pepsin            | 3.4.23.1  | 35 (monomer)         | 1.0                    |
| Urease            | 3.5.1.5   | 90 (hexamer) = 540   | 5.0                    |

3. Results and Discussion
The first data retrieved from the experiments was release kinetics of the enzymes. A large compilation of data for a wide variety of enzymes can be seen in Figure 1. From this figure several observations can be made about how proteins interact with halloysite nanotubes. First, is that in comparison to other molecules proteins have a relatively short release time frame. Typically, within the first hour over 75% of the total amount of protein found was already in solution. This graph is somewhat misleading however in that 100% of release is shown. The reality is that it is impossible to have every molecule of protein released from the halloysite nanotubes. However, after many experiments it was found that after 6 hours there is not a significant increase release. This is exemplified in Figure 2 where at 6 hours there is 86% release and at 50 hours when the experiment stopped there was only 15% more. When converted to mass this 15% is in the microgram range.
Figure 1. In these experiments the proteins were loaded at a 1:1 ratio (w/w) of protein to halloysite in water. The release study was also done using water. Absorbance was read at 280nm.

Figure 2. The same procedures as in Figure 1 were applied to this experiment. However, in this experiment the protein was positively charged and the ratio was 2:1 protein to HNT.

Ideally we would want the release of the protein to be extended over a much larger period of time. Somewhere along the lines of days to weeks would correlate better with other results from other chemicals. Despite this seemingly short release we were able to come across an interesting...
observation while using TGA to determine the loading efficiency. Figure 3 is an example of what would be constructed for every protein that was attempted to be loaded inside of the nanotubes.

Figure 3. In this particular experiment glucose oxidase was loaded with halloysite when the protein had both a positive and negative charge. The positively charged sample shows a 7.11% decrease in mass while the negatively charged sample showed a 4.425% decrease.
There are couple meaningful observations of this TGA data. First the positively charged sample of glucose oxidase showed a significantly higher loss of mass over the same temperature range as the negatively charged sample. This can be attributed to the increase surface area on the outside of the nanotube which also happens to be negatively charged. The next is that both samples show almost the exact same maxima of degradation. This is indicative that the samples are stabilized in a similar fashion. For example, they may adsorb in the same orientation on both the outer surface and the inner surface. As mentioned earlier an interesting observation was made when a sample of protein loaded halloysite nanotubes was analyzed in this fashion after going through a release study. Figure 4 shows the result of this experiment.

**Figure 4.** This sample of lipase loaded halloysite was prepare and release in deionized water. The ratio at loading was 1:1. The weight (%) curve for lipase before release was offset +1 unit to account for a larger loss of water in the first 100°C.

It can be seen that 76 % remained after the release study had been completed. This led to the development of the idea that there may be two “categories” of proteins and essentially two phases of enzymatic activity that could be expected. The first phase is the burst phase as mentioned before. In this phase the enzyme that is release from the tube is allowed to react freely with any available substrate while at the same time the protein that is immobilized inside of the tube is also free to react albeit likely with less efficiency due to diffusion constraints or reduction in activity due to immobilization. Typically proteins and enzymes degrade over time and the free protein will eventually stop working. This is where the immobilized protein, which is likely to be much more stable will be able to continue to act as a catalyst, hence the sustained phase. There has been a lot of work done on immobilized protein catalysts and they have found important uses in industry[4] hence exploring this phenomena could find potential large scale use.

We must continue to work to establish the parameters that control the loading efficiency of proteins. As of now the mass, charge, and secondary structure of the protein seem to be the main factors that influence the loading efficiency. Big heavy proteins seem to be loaded less efficiently,
while proteins with a condensed β-sheet structure seem to load well regardless of weight as opposed to those with mostly α-helixes. Charge is not as simple as mass structure to state a correlation. In general the loading efficiency for positively charged proteins is 40 % higher than that of negative proteins. However, it is likely they these proteins are adsorbing to the surface of the tubes and not on the inside. Finally, we need to produce quantitative data on enzymatic activity after loading.

Loading and release of different drugs was also studied. In Figure 5 we show results on sustain release of three drugs (dexamethasone, furosemide and nifedipine) from halloysite nanotubes. These drugs loading rate was 6-7 wt. % and release time increase from 5-10 min in non-encapsulated state to 10-20 hours for the drugs encased in the clay nanotubes.

![Figure 5](image)

**Figure 5.** Release profiles of the drugs from halloysite nanotubules in water at pH 7.4: a-loading from 10 % alcohol; b- loading from 50 % ethanol / aqueous solvent.

Using for the loading the more concentrated drug solution in 1:1 mixture of water and ethanol we reach higher 9-10 wt. % loading efficiency and more linear though shorter time release characteristics (Figure 5 b).

**4. Conclusion**

Natural biocompatible halloysite clay nanotubes may be used for storage and sustained release of bioactive compounds, such as drugs and proteins. Typical release time is between 5 and 20 hours which is perspective for biomedical applications. However, one has to mind that these nanotubes are not biodegradable and some mechanisms of the nanotube removal from organisms have to be considered or such formulation can only be used for external (non-injective) applications.

We found that negatively charged proteins may be loaded into positively charged clay nanotube lumens at 5-8 wt. % and positively charged proteins may be loaded both inside and outside tubes at 10-15 wt. percent. Both type of immobilized proteins may be release within 4-5 hours, however in the case of negative proteins loaded into the tube lumen, approximately half of the load (3-4 %) are strongly bound and remain immobilized for much longer time.
5. References

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