UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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RESEARCH PROJECT:

‘Assessing of active substance releasing from drug nanocarriers based on zinc oxide and titanium (IV) oxide’.

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1. INTRODUCTION

Metal oxide nanoparticles are currently being studied and used for biomedical applications and they are becoming more and more important for the treatment of illnesses such as cancer thanks to their special properties. Zinc and titanium dioxide are both metal oxides which will have several uses regarding to this fields and they are the compounds that are going to be studied.

Zinc oxide and titanium oxide nanoparticles have overall similar properties. ZnO is an n-type, II-VI semiconductor oxide that is found naturally in the form of zincite. It has a 3.37eV direct band gap and an exciton binding energy of 60me. ZnO is characterized by unique physical and chemical properties such as a variety of structures and less-complicated fabrication techniques, eco-friendliness and high electron mobility among others. As mentioned before ZnO has a wide band gap permits luminescent and high optical transparency properties in the visible and near ultraviolet regions. ZnO has excellent disinfecting and antibacterial properties hence it is widely applied in the medical field it can be used in dental pastes or alimental supplements. ZnO is also used in the textile industry for UV-blocking textile coatings that protects the body from the UV radiation.

On the other hand, TiO_2 is a semiconductor with a bandgap value of 3.2 eV. TiO_2 is the most widely used photocatalytic semiconductor oxide with its anatase phase exhibiting the highest photocatalytic activity. It is applied in photocatalytic processes such as self-cleaning, air-purification and waste-water treatment. TiO_2 owes its application to its high oxidizing effect which makes it suitable for decomposition of organic and inorganic compounds. TiO2 is also non-toxic and highly abundant and has a high chemical and optical stability.

They both have unique chemical and physical properties and significant advantages in biomedical applications. Their wind gap allows them to have many applications in optical devices, sensors or solar cells due to their long-term stability, good photocatalytic activity and their ability to absorb UV radiation. That is why they are also employed in sunscreens as inorganic physical sun blockers for the UV radiation. Here is a table to compare their most important properties. Another application that both oxides are used for is as drug carriers, this application is going to be studied more deeply after in the report.

Table 1: Properties of ZnO and TiO_2

Property	Zinc Oxide (ZnO)	Titanium Oxide (TiO_2)
Molecular weight (gm/mol)	81,4	79,84
Appearance	White powder	White powder
Natural existence	Zincite	Anatase, rutile
Odour	Odourless	Odourless
Structure	Hexagonal wurtzite	Tetragonal
Solubility	Insoluble in water but soluble in acid/base	Insoluble in water
Energy Gap (eV)	3,37	3,2
Boiling Point (ºC)	2360	2972
Melting point (ºC)	1975	1843

It is important to know that when a material is reduced to the nanoscale some size dependent properties are manifested. Some of the properties that have a high importance in the biomedical applications are the increase in the relative surface area, positioning the majority of the nanostructure atoms in the surface, which can produce an increase in the chemical reactivity ,the capacity of the particles of interacting and forming agglomerates and with that the capacity of being loaded with drugs for illness treatment. Another example is the nanoparticles size is comparable to the biological molecules making it easier for them to interact.

2. Toxicity of ZnO and TiO_2:

The new properties that emerge when reducing a material to the nano scale can also be harmful for some organism and that is why not all the nanoparticles can be used for biomedical applications only those with good compatibility with the living cells. In this case ZnO and TiO_2 are both considered 'GRAS' (generally recognised as safe) by the U.S. food and drug administration which means that the can contain up to 1% without having to add it on the ingredient label, but many studies have noticed an increase of the toxicity can happen when this compounds are reduced to the nanometric size. The mains ways nanoparticles can enter the organism are by inhalation, ingestion, or dermal contact. The inhalation of metal oxides can cause an illness related to the lungs known as 'metal fume fever' caused by the irritation of the respiratory track. The dermal contact of the nanoparticles usually happens through the application of cosmetic products on the skin, being the most common the sunscreen because the ZnO and TiO_2 nanoparticles both are normally used as a protector against the UV rays because they can reflect them, it is believed that the nanoparticles can pass through the skin and reach the bloodstream but this is currently in high doubt.

The toxicity of ZnO and TiO_2 is believed to be caused mainly due to the release of metallic ions, which are toxic in excess for the human body, they generate reactive oxygen species (ROS). The main problem with the metallic ions is that they are strongly bind with the thiol groups of the cysteine amino acids and when they bound cysteine is oxidized and the metal is reduced. This reduced metal can react forming hydroxyl radicals (Fenton reaction) that are highly reactive and can cause an oxidative stress

condition, this condition takes place when an organism is unable to cope with an imbalance between free radicals and antioxidants in his body and can cause the cell death. The cell has some defence mechanism to minimize the damage, but it may not be enough to reduce the oxidative stress.

$$2\,O_2^{\bullet-} + 2\,H^+ \longrightarrow H_2O_2 + O_2$$

$$\mathbf{M^{(n)}} + O_2^{\bullet-} \longrightarrow \mathbf{M^{(n-1)}} + O_2$$

Fenton $\quad \mathbf{M^{(n-1)}} + H_2O_2 \longrightarrow \mathbf{M^{(n)}} + HO^{\bullet} + OH^-$

Haber Weiss $\quad O_2^{\bullet-} + H_2O_2 \longrightarrow O_2 + HO^{\bullet} + OH^-$

The toxicity of TiO2 and ZnO can be reduced, the main strategy used is the addition of protective coats to modify their properties, mainly improving the stability of the carrier and preventing the release of metallic ions. Examples of protective coats that are currently being tested are the glutathione (GSH) and sugars.

a. Protective coats: Glutathione.

The glutathione (GSH) is a molecule composed by 3 peptides glutamate, cysteine and glycine. GSH can prevent the damage caused by oxidative species (such as Zinc and Titanium ions) due to the thiol group presented in his structure. The GSH is an important antioxidant mainly in plants.

By adding glutathione to the metal oxide carrier, the realising of metallic ions is reduced and with that the toxicity and side effects that they produce. As we said before the metallic ions are strongly bind to the thiol groups and the glutathione also has a thiol group in his structure.

$$RSH + M^{n+} \leftrightarrow RS\text{-}M^{(n-1)+} + H^+ \qquad (1)$$

$$RS\text{-}M^{(n-1)+} + RSH \leftrightarrow (RS)_2\text{-}M^{(n-2)+} + H^+ \qquad (2)$$

$$n\,RSH + M^{n+} \leftrightarrow (RS)_n\text{-}M + n\,H^+ \qquad (3)\ \text{global}$$

The structure form for a metal with 2 valence electron such as Zinc is shown in figure X, it needs 2 electrons to be neutral and each electron is coming from 1 molecule of glutathione:

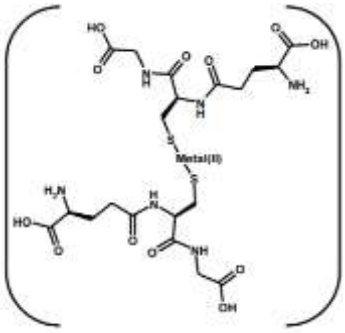


Figure 1: Molecule of deprotonated metal (II) glutathione

As it is shown in the image the metal (in this case zinc) is bonded with the glutathione molecules so it can not react with the thiol group of the cysteine and form the hydroxyl radicals, the formed complex is more stable now than without the glutathione.

It has been demonstrated that the decomposition of the complex is as shown on the figure 2:

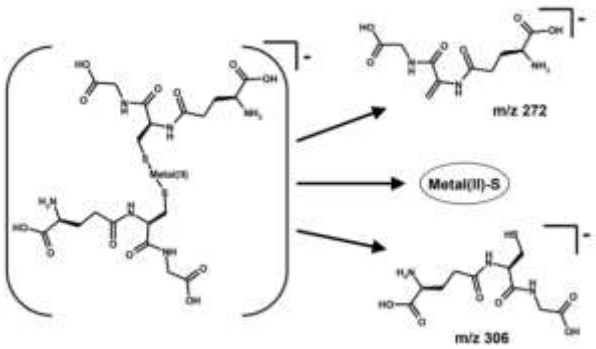


Figure 2: Decomposition of metal (II) glutathione

This information is useful because in case this happens the Zinc sulphide is less harmful than the Zinc ions for the human body. The zinc sulphide can form toxic gases but only in acids environments but the ingest of trace amounts is not dangerous.

b. Protective coats: Sugars.

In the same way glutathione is used, other type of coating are the sugars. Sugars are technically called the carbohydrates that generally have a sweet taste, for instance the different monosaccharides, disaccharides and polysaccharides. They form low stability complexes with metal ions because of the poor donor ability of hydroxylic oxygen atoms. This can be change easily introducing an anchoring group, increasing the ability to form complexes. It is easy to obtain this anchoring groups by oxidizing the

sugar, one useful example is the addition of nitric acid to galactose forming galactaric acid.

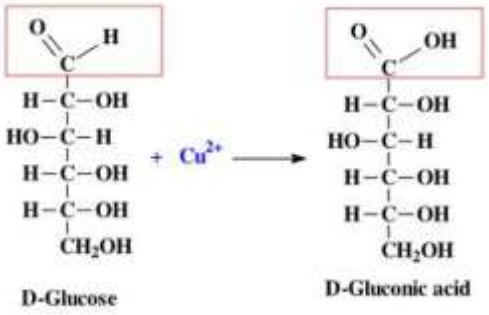


Figure 3: Oxidation of glucose.

Many structural studies have shown that the interaction of sugar acids with metal ions involves both, the carboxylic oxygen, and the alcohol group next to him. The different complexes that can be formed are:

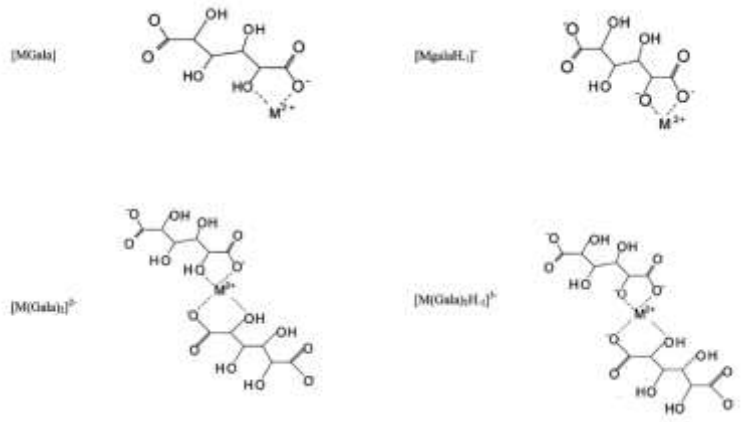


Figure 4: Different complexes formed with metal and galactaric acid.

Depending on the conditions it is more likely to form one or another. When the pH is low, the complexes on the left are the most common, pHs below 8 and when the pH is over 8 the complexes on the right are the more stable. The explanation for that may be for deprotonation of the coordinated α-alcohol group.

c. Sugar coating and cancer:

The use of sugar coating can also have a special importance for the treatment of a specific illness, cancer. It can be a stabilizer and a form of increasing the specificity of the drug release.

The cancer cells have an unusually high demand for glucose compared to normal cells, to understand why we first need to know how their metabolism works. All type of cells gets their energy from transforming the glucose into adenosine triphosphate (ATP), and there are 2 main ways of obtaining ATPs, through Oxidative phosphorylation or glycolysis. The first one occurs under aerobic conditions (presence of oxygen) and the production of ATPs is really high, 36 moles of ATPs per mole of glucose, the second one

doesn't need the presence of oxygen to take place but the efficiency of the process is lower, obtaining only 2 moles of ATPs per mole of glucose. Taking this into account the logic would make us believe that every cell would prefer the oxidative phosphorylation when there were aerobic and that is what happens normally, but the cancer cells work in a different way.

The cancer cells mainly obtain their energy through glycolysis even when there are aerobic conditions, this is called the Warburg effect. The reason why this happens is because the main objective of the cancer cells is the proliferation and glycolysis provides the building blocks for this goal, other reason can be that the glycolysis is a quicker reaction and can have a quicker response to fluctuations, and tumour cells may have more frequent perturbations than the normal cells.

This way of obtaining the energy may have some advantages for the tumour cells but the main disadvantage is clear, they need a lot more of glucose than a normal cell. This problem can be used by us to elaborate cures for cancer.

In the first impression it may seem a bad idea to use sugars to reduce de release of metallic ions because as it is said before cancer cells need a lot of glucose and by taking drugs that contain sugar the only thing you are doing is feeding the cells and help them with the proliferation. But if you think about it you can use this high glucose demand of the cancer cell to our advantage.

The nanoparticles of sugar-coated and metal oxides are attractive to the cancer cells they can be used to easily target them and deliver the drug with more efficiency. This strategy is known as glyconjugation. By doing this it is possible to reduce the side effects, because the doses taken are lower and the particles target toxicity locally (toward the cancer cells that have ingested them). There are also other advantages such as no immunogenicity, good biocompatibility, and biodegradation.

3. Drug delivery systems:

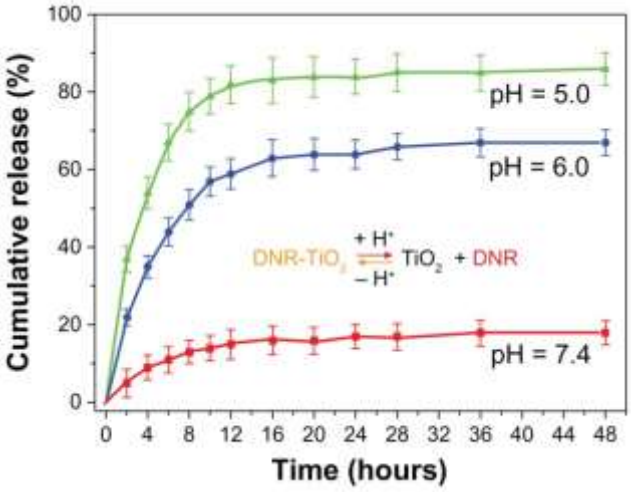
Controlled drug delivery has gained increasing interest in recent decades due to improved drug efficacy. Controlled release drug delivery systems are advantageous over the classic dosage forms in many ways, such as maintaining the optimal therapeutic concentration of the drug in blood with predictable release rates over a long period of time, minimizing harmful side effects, improvement of activity, protection of sensitive drugs against enzymatic or acid degradation in the gastrointestinal tract, reducing the number of administration or cost, due to reduced medication waste and improved patient compliance. Nowadays the metallic oxides nanoparticles such as ZnO and TiO_2 are compounds that have and can gain importance in this area due to their properties.

The release of the drug can respond to different stimuli or changes such as changes in redox state, enzymatic activity, photoirradiation, temperature, magnetic actuation, electric field or pH. When certain changes happen in these parameters the drug is released, so for designing a correct drug delivery system it is important to understand how the drug carriers react to these changes to maximize the benefits.

The most important parameter to regulate the drug release is the pH. The main reason of this importance is due to their application in the treatment of the illness that causes the most deaths every year, the cancer. It is known that the extracellular environment of the cancerous cells is more acid than the normal cells, being a normal pH around 7,4 and in the tumour cells between 5 or 6. So the perfect drug delivery system should retain the drug at a normal value of pH and quickly release it when there is a change to a lower value.

To understand the efficiency of each of the 2 metal oxides studied, the results of 2 studies will be observed, one studies the TiO_2 response and the other the ZnO response to changes in pH. In both studies the metal oxide nanoparticles are attached to different drugs. The TiO2 is attached to daunorubicin, a chemotherapy medication to treat cancer and the ZnO nanoparticles are integrated covering the pores in a system composed by mesoporous silica nanoparticles (MSNs) that contain doxorubicin (DOX).

For the first case of TiO_2 and daunorubicin, there are results from the study of the release of the drug in different pH conditions shown in the graph 1.



Graph 1: Drug release of DNR-TiO_2 complex

At biological ph (7,4) the drug release is close to 20% after 48 hours. When the pH gets lower there is a high change in the cumulative release, being almost 70 % at ph 6 and more than 85% at ph 5, a very significant change of drug release taking to account that the ph change is not that much. The change of accumulative release can be easily explained, in presence of an acidic medium, the TiO_2 nanoparticles turned positive, that changed the electrostatic relation between the daunorubicin and the TiO_2, facilitating the drug release process. The low release at the biological pH also means that the drug will remain attached to the carrier while circulating through the blood which means that less side effects would be presented and the release will mainly occur when the drug complex approaches the low pH mediums, which means that a fast release will happen in the surroundings of the tumour cells.

Regarding the ZnO nanoparticles, there are some studies of the viability and efficiency of the complex mentioned earlier. In figure 1 it is shown how the system is formed the MSNs containing DOX inside the pores and ZnO covering the pores.

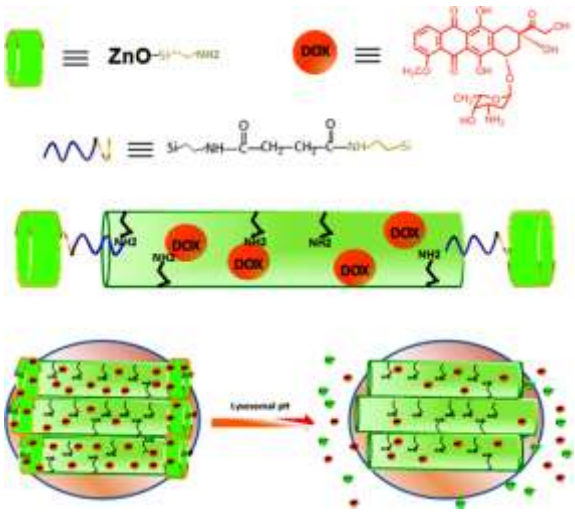
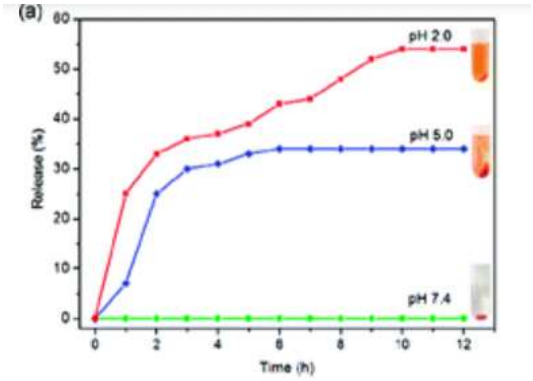


Figure 5: Scheme of DOX-MSN-ZnO

The system works similarly to the one explained previously of TiO_2, the complex keeps stable at biological pH but rapidly dissociates at lower pH. The results of the drug release are shown in graph 2, and they are now nearly as good as the one in graph 1.

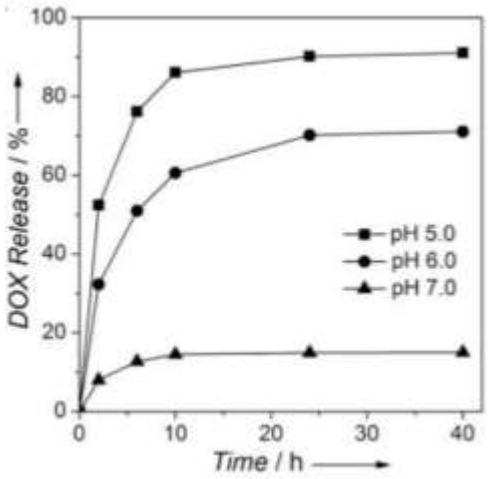


Graph 2: Drug release of DOX-MSN-ZnO complex

The drug release is only close to 30% at pH 5, a low value compared to the other results. At pH 2 the value increases to 55% but this result is not significant, because it is not possible to have a pH this low in the human body, not even in the acid medium of the cancerous tissues. Furthermore, the MSNs degradation in the human body is in discuss and the ZnO can also be toxic when transformed to Zn^{2+}, and it is important for the drug delivery systems carriers to be biodegradable and excretable. In conclusion this complex may not be the best due to the low efficiency in drug release and the doubts in his toxicity.

Considering all the problems of the previous complex and noticing the high potential of the ZnO nanoparticles as a drug delivery system another research group

develop a new one changing the MSN for a biodegradable polymer. The results shown in graph 3 are noticeably better.



Graph 3: Drug release of DOX-polymer-ZnO complex

The drug release is close to 70% in pH 6 and 90% in pH 5, a high improvement in this field compared with the previous MSN-ZnO complex. As mention before, the polymer that forms the complex is biodegradable, so it has a high biocompatibility with the cells, this means that the substance is less toxic. The only negative part of the ZnO-polymer-DOX complex is that at biological levels of pH certain percentage of the drug is realise as shown in the graph 3, in the case of the ZnO-MSN-DOX complex for this pH was 0, this means that the polymer complex can have more side effects due to the release of drugs in undesirable areas.

As mentioned before there are other forms of designing drug delivery systems that can be effective. It is known that the ZnO and TiO_2 are a wind gap semiconductor with many applications due to their ability to absorb UV radiation. The photocatalytic degradation capacity of this organic compounds when expose to UV light can also be interesting for delivery drug to certain areas.

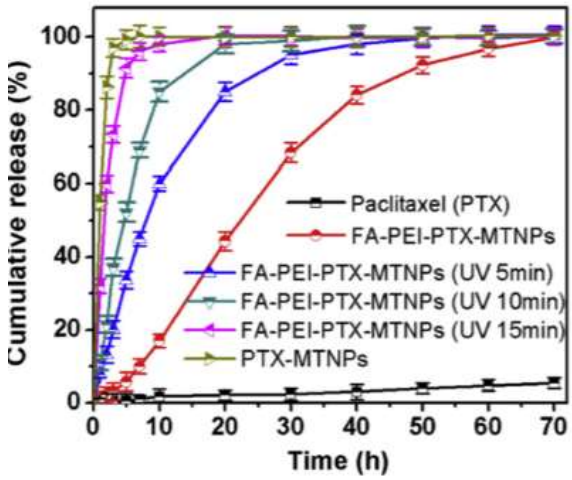
ZnO and TiO_2 nanoparticles can induce free radical formation under light exposure. When they are exposed to UV light the energy absorbed can be higher than their band gap energy, in that case the valence electrons get excited creating and electron and a H^+ and also generating free radicals that are the reason why the organic compounds are decomposed and if they are carrying a drug the drug is released. It is also worth to mentions that when the free radicals are released, they can also induce the cells into oxidative stress by the formation of ROS which that can cause the cells death. This situation may seem bad but that the main goal is to target some specific areas, for example in the case of cancer, the death of the tumour cells is good for curing the disease.

There is a study for to see the results of this type of drug delivery system, in this case the carriers are composed by polyethylenimine (PEI) and porous TiO_2 nanoparticles and the drug contained is also an anticancer drug named paclitaxel (PTX). The complex

is as shown in figure 2, the surface TiO_2 was covered by hydrophilic PEI that allows the entry of the PTX in an inorganic solvent but that quickly forms a layer when it dissolves in water, preventing the exit of the drug. The complex formed can be destroyed by the application of UV light because the free radicals can react with the PEI allowing the drug release.

Figure 6: Scheme of PEI-PTX-TiO_2.

The results of this study are shown in graph 4:

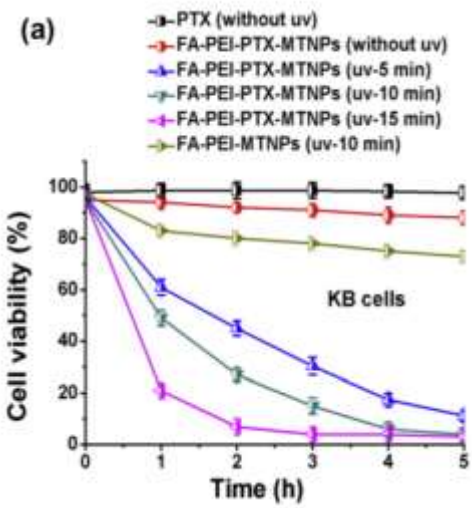


Graph 4: Drug release of PEI-PTX-TiO_2.

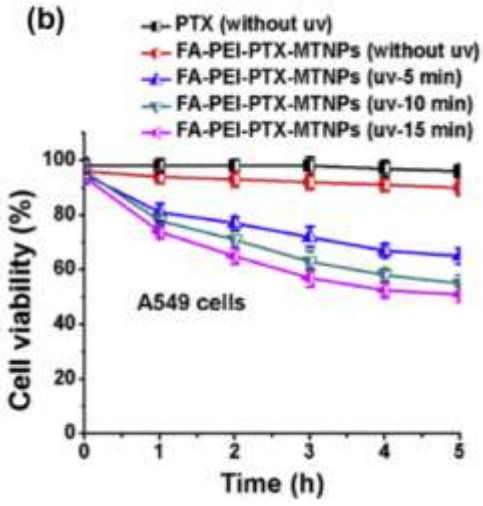
The first thing to notice is that in the experiment with only TiO_2 as carrier of the drug (PTX-MTNPs complex) the drug is released after less than 5 hours; this suggest that the PEI is necessary to control the release.

Looking at the results of the full FA-PEI-PTX-MTNPs complex a high influence of the exposure to UV radiation can be appreciated. Without the UV light the release is slow, 40% at 20 hours and with 15 minutes of UV exposure almost the 100% of the drug is released at 10 hours, a huge improvement.

To get a full understanding in whether the drug complex is toxic to every cell or only the cancerous cells there are studies comparing the toxicity.



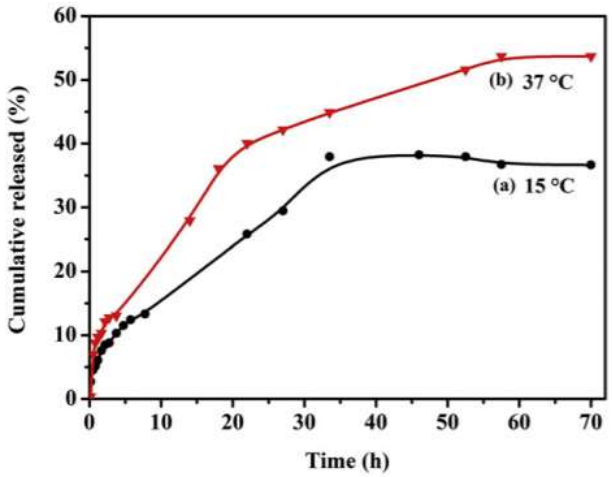
Graph 5a: Release on KB cells



Graph 5b: Release on A549 cells.

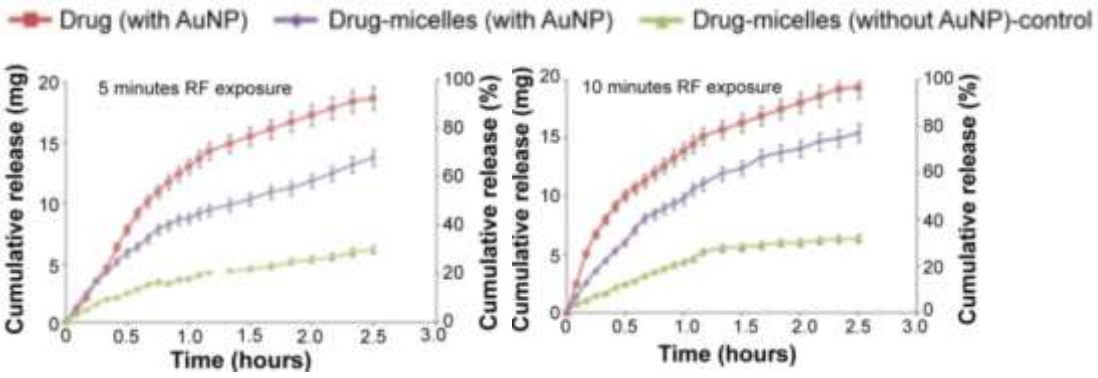
The results in the graph 5a reaffirm the importance of the UV application in order to maximize the release of PTX for the death of the KB cells, a subline of the ubiquitous KERATIN-forming tumour cell line HeLa, and also that the PEI-MTNPs system has some lethality for the cells but the PTX is the main form of destroying the tumour. In graph 5b the results are similar regarding the importance of UV application, but the cell viability is much higher for A549 cells (human lung epithelial cells), this is a good result because it reflects that the PTX mainly attacks the cancer cells.

Furthermore, there are also evidences that the temperature can have influence in the release of drugs, but as can be seen in figure the influence is not very noticeable and it is hard to think of an application for biomedicine since there cannot be very sharp changes in temperature in living beings. The results are for a complex form by poly(N-isopropylacrylamide) (PNIPAM) on zinc oxide (ZnO) nanoparticle.



Graph 6: Influence of temperature in drug release.

Another viable strategy that is a perfect example if you are searching for a non-invasive external stimulus is the radiofrequency. The drug delivery system is based mainly in TiO_2 nanotubes, radiofrequency was used as an external stimuli to trigger the release of polymeric micelles and drugs from TNT deposited gold nanoparticles that are used as thermal transducers, they transfer the radiofrequency energy to induce the drug release from the TiO_2 nanotubes. The results for 2 different times of exposure are shown in figures 5a and b.



Graph 7a: 5 min exposure ***Graph 7b****:* 10 min exposure

The first this noticeable from the results obtained is that extending the exposure for 5 minutes does not make much of a difference, the release is faster, but it may be not worth it. The presence of AuNp as thermal transducers has a lot of importance without them the results are worse and with them the 100% release is achieved at 2.5 hours.

Finally, there are other drug delivery systems that are currently being developed on magnetic and ultrasound stimuli. The first on is based on drug encapsulated in nanomagnetic structures that possess excellent possibilities for magnetic field-triggered drug release. This strategy is effective, it can release the 100% of the drug in 1 or 2 hours, the main disadvantage is the uncontrolled release by existing magnetic fields in the environment. The main application can be for drug-releasing implants in orthopaedics and bone surgery where on-demand release is needed under emergency. The ultrasound-sensitive delivery it is believed to be more reliable than the magnetic sensitive due to the uncontrollable release mentioned earlier, ultrasonic waves can be used as the trigger for stimulus-responsive local drug delivery system an be applied for bone therapies, local drug delivery, and implantable drug delivery systems including stents and brain drug delivery. Of course, more studies of ex vivo or in vivo models using different drug-releasing implants and drugs are needed to achieve significant understandings based on both concepts.

In conclusion drug delivery systems can be a huge improvement in the biomedical field improving the efficiency of the drugs and reducing their side effects. There should be a lot of studies regarding this because it can have great importance in the treatment of many diseases including cancer.

4. EXPERIMENTAL METHODS

Obtaining of ZnO and TiO_2 nanoparticles:

For the obtention of ZnO and TiO_2 nanoparticles modified with sugars the process followed is the same, only changing the reagents used.

In the first place, the obtention of ZnO follows the next reactions:

$$ZnSO_4 + 2\ KOH \longrightarrow Zn(OH)_2 + K_2SO_4$$

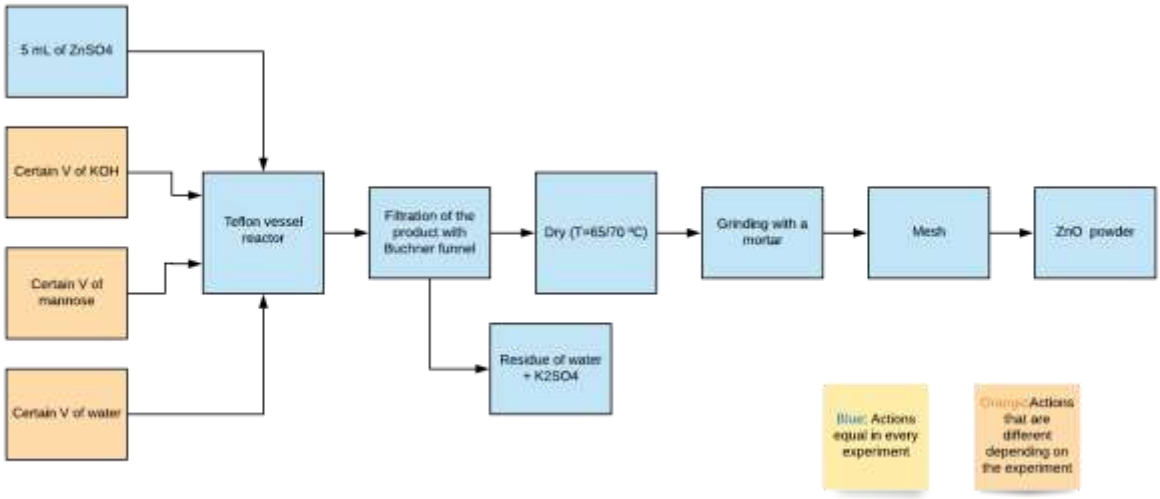
$$Zn(OH)_2 \longrightarrow ZnO + H_2O$$

Firstly, 5 ml of ZnSO4 were taken of an aqueous solution at a concentration of 2,5mol/L then a certain volume of a KOH solution with a concentration of 5 mol/L was added (the volume variates depending on the experiment) and a certain volume of Mannose if needed from a solution with a concentration of 0,2 mol/L. The final volume should be 35 mL so if the solution does not have this volume it is important to add water till the volume is reached. The volume of each reagent needed for each experiment is shown in table 2:

Table 2: Distribution of reagents in the different experiments for ZnO.

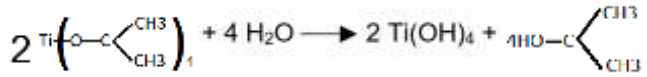
sample number	molar ratio n MAN : n ZnO	fold of stoichiometric n KOH : n ZnSO4	Moles of MAN	V Man (mL)	Moles KOH	V KOH (mL)	V water (mL)		temp, °C	time, min
E1	0	1	0	0	0,025	5	25	Microwave process	150	5
E2	0	2	0	0	0,05	10	20		150	5
E3	0	3	0	0	0,075	15	15		150	5
E4	0,1	1	0,00125	6,25	0,025	5	18,75		150	5
E5	0,1	2	0,00125	6,25	0,05	10	13,75		150	5
E6	0,1	3	0,00125	6,25	0,075	15	8,75		150	5
E7	0,2	1	0,0025	12,5	0,025	5	12,5		150	5
E8	0,2	2	0,0025	12,5	0,05	10	7,5		150	5
E9	0,2	3	0,0025	12,5	0,075	15	2,5		150	5
E B	0	1	0	0	0,025	5	25		150	5

All the reagents are added into a Tefflon vessel reactor, were the reactions take place. Once the reaction time has passed the product is filtrated with a Buchner funnel obtaining a residue of Water and K2SO4. The desired ZnO product is wet, it dries to remove water at about 70 degrees and then grinded with a mortar and meshed, then the final product is obtained. In scheme 1 the full process can be seen.



Scheme 1: Obtention of ZnO np

In the case of TiO2 the reactions that take place are:



$$2\ Ti(OH)_4 \longrightarrow Ti(OH)_3 - O - Ti(OH)_3 + H_2O$$

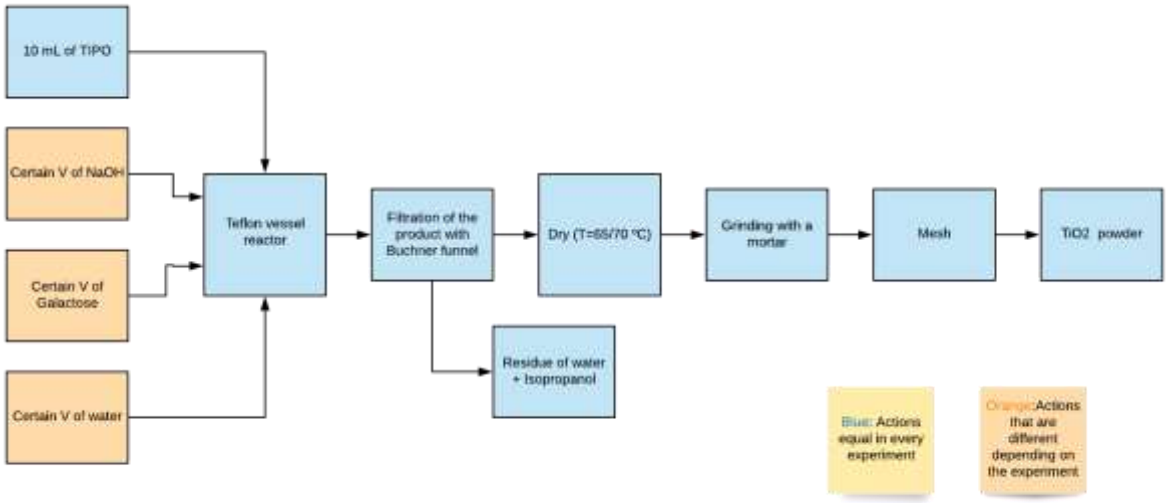
$$Ti(OH)_3 - O - Ti(OH)_3 \longrightarrow 2\ TiO_2 + 3H_2O$$

Firstly 10 ml of TIPO solution (100% of isopropoxide) were taken, a certain volume of a NaOH solution of 0,5 mol/l and a certain volume of a 0,5 mol/L solution of Galactose also depending on the experiment. Lastly add water until a volume of 40 mL is reached. The volume of each reagent needed for each experiment is shown in table 2:

Table 3: Distribution of reagents in the different experiments for TiO_2.

sample number	molar ratio n GAL : n TiO2	fold of stoichiometric n NaOH : n TIPO	Time, min	Moles of GAL	V Gal (mL)	Moles NaOH	V NaOH(mL)	V water (mL)		temp, °C
C1	0,02	0,059	2	0,0006754	1,3508	0,0028	4	24,65	Microwave process	150
C2	0,02	0,1184	20	0,0006754	1,3508	0,004	8	20,65		150
C3	0,02	0,1776	11	0,0006754	1,3508	0,006	12	16,65		150
C4	0,11	0,059	20	0,0037147	7,4294	0,002	4	18,57		150
C5	0,11	0,1184	11	0,0037147	7,4294	0,004	8	14,57		150
C6	0,11	0,1776	2	0,0037147	7,4294	0,006	12	10,57		150
C7	0,2	0,059	11	0,006754	13,508	0,002	4	12,49		150
C8	0,2	0,1184	2	0,006754	13,508	0,004	8	8,49		150
C9	0,2	0,1776	20	0,006754	13,508	0,006	12	4,49		150
C B	0	0,059	11	0	0	0,002	0,004	30		150

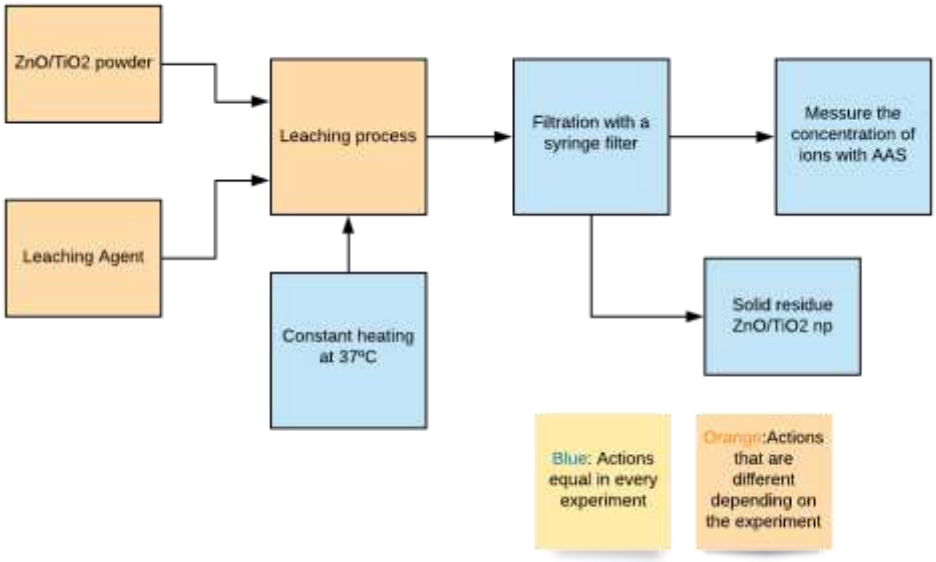
The process followed to obtain TiO_2 nanoparticles is the same:



Scheme 2: Obtention of TiO_2 np

Leaching process:

Once the nanoparticles are obtained the goal is to study the release of metallic ions under specific conditions to see the influence of different factors in the stability of the product. A leaching agent is added to de metal oxide powder obtained previously, this leaching agent can be water, stimulated body fluid or ringer solution. The mass ratio ZnO/TiO_2:leaching agent was 1:20. The process is constantly heated at 37 degrees to simulate the temperature of the human body and once a certain time has passed the solution is filtrated with a syringe filter and sampled to measure the ion concentration with the atomic absorption spectrometry (AAS), a technique in which free gaseous atoms absorb electromagnetic radiation at a specific wavelength to produce a corresponding measurable signal. Scheme 3 illustrates then process:

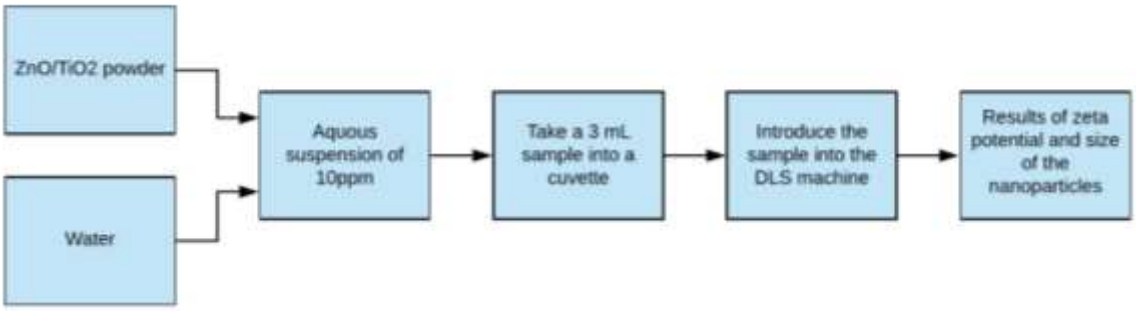


Scheme 3: Leaching process.

Analysis of size and stability:

The dynamic light scattering (DLS) is a technique that can be used to determine the size distribution of small particles in suspension and their stability. The method is based on the reflection of the light when it hits the particles.

The process followed in the DLS technique consist in forming an aqueous suspension of the ZnO or TiO2 powder with a concentration of 10 ppm and take a 10 mL sample in a cuvette. The sample is introduced in the DLS machine and wait for the results.



Scheme 4: DLS technique

5. RESULTS:

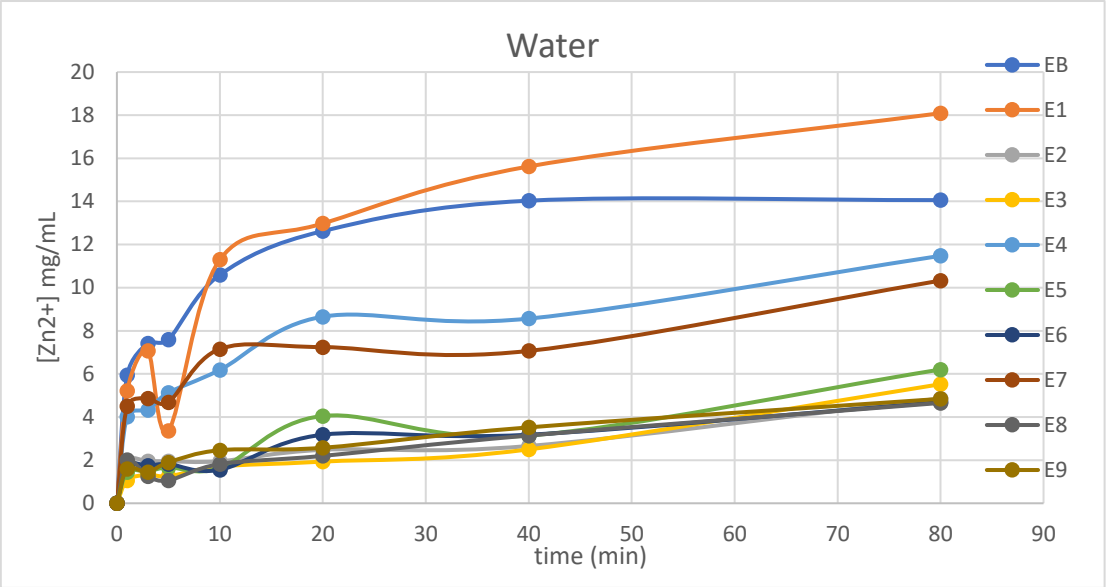
a. Analysis of releasing of ions from the products.

The results obtained of Zinc ions release in water are shown in table 4.

Table 4: Concentration of Zn ions released in water.

c	C Zn [mg/ml]									
	EB	E1	E2	E3	E4	E5	E6	E7	E8	E9
0	0	0	0	0	0	0	0	0	0	0
1	5.9391	5.2209	1.9509	1.0629	4.0113	1.4409	1.7433	4.5027	2.0079	1.5921
3	7.4193	7.0665	1.9509	1.3275	4.3323	1.4787	1.7433	4.8615	1.2519	1.4409
5	7.5957	3.3621	1.9509	1.2519	5.1261	1.6677	1.8189	4.6851	1.0629	1.8945
10	10.5945	11.3001	1.9509	1.7055	6.1845	1.6299	1.5543	7.1547	1.8189	2.4615
20	12.6231	12.9759	2.4801	1.9323	8.6541	4.0491	3.1797	7.2429	2.1969	2.5749
40	14.0343	15.6219	2.6565	2.4993	8.5659	3.1419	3.1797	7.0665	3.1419	3.5199
80	14.06	18.0915	4.8615	5.5233	11.4765	6.2037	4.6917	10.3299	4.6539	4.8429

To understand further the results, it is important to do a representation:



Graph 8: Concentration of Zn ions release in water.

The first analysis of Zinc ions release is done with water as the leaching agent, we can see 4 curves clearly above the rest, EB, E1, E4 and E7. What EB, E1, E4 and E7 have in common is that all of them have 1 as the fold of stoichiometric n KOH : n ZnSO4, this value is the lowest value of all the experiments this means that when the number of moles of KOH is low the release of Zinc ions is the highest. To understand what happens we need to take into account that KOH is a base in solution so when the fold of stoichiometric is 1 the moles of KOH are the lowest observed in the experiments, and that means that the pH is also the lowest observed. It can be concluded that the pH of the solution where the ZnO nanoparticles are obtained has a high influence in the Zn ions release, and the higher the pH is, the stability of the ZnO nanoparticles is also higher. The reactions that can take place of ZnO in aqueous solutions are:

$$ZnO_{(s)} + 2H^{+}_{(aq)} \leftrightarrow Zn^{2+}_{(aq)} + H_2O_{(l)}$$

$$ZnO_{(s)} + H^{+}_{(aq)} \leftrightarrow Zn(OH)^{+}_{(aq)}$$

$$ZnO_{(s)} + OH^{-}_{(aq)} + H_2O_{(l)} \leftrightarrow Zn(OH)_3{}^{-}_{(aq)}$$

$$ZnO_{(s)} + 2OH^{-}_{(aq)} + H_2O_{(l)} \leftrightarrow Zn(OH)_4{}^{2-}_{(aq)}$$

Obviously the last 2 reactions take place mainly at high pH, this can result in a hydroxide layer with low solubility and this low solubility means that ZnO can be more stable at this high pHs.

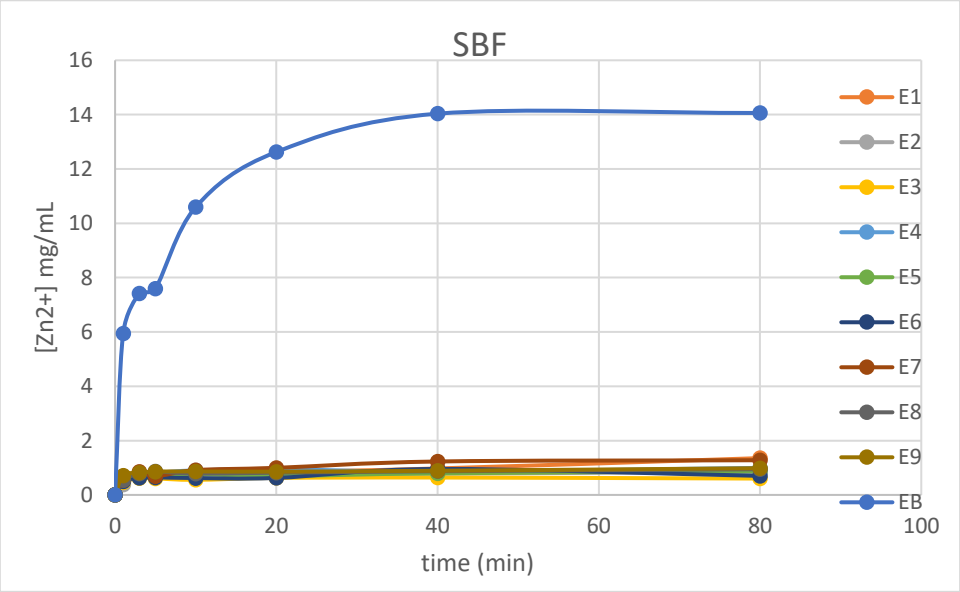
Regarding the presence of mannose, a certain influence can be appreciated in EB, E1, E4 and E7. In EB and E1 molar ratio of mannose is 0 for both and their curves are both higher that E4 and E7. This means that mannose also has an influence in the Zn ions release, maybe not as high as the pH because the effect of the presence of mannose cannot be appreciated clearly in the rest of the experiments (E2, E3, E5, E6, E8 and E9). The influence of mannose in release of ions makes sense because as explained earlier

the sugars can form a complex with the Zinc with high stability and this results in less ions release.

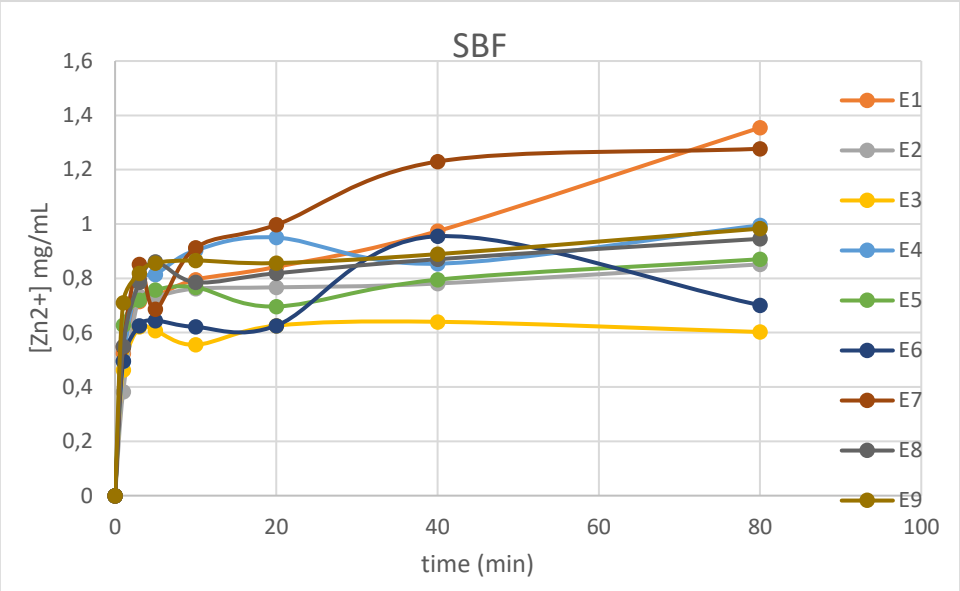
As mention before, the curves E2, E3, E5, E6, E8 and E9 are really similar and almost overlapping, even though there are difference in both of the factors mentioned earlier that can have an influence in the ion release, pH and mannose. This can mean that when the fold of stoichiometric n KOH: n ZnSO4 has value of 2 or 3 the changes in the pH are not as significant for ion release.

Table 5: Concentration of Zn ions released in SBF.

C	SBF									
	EB	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**	**E9**
0	0	0	0	0	0	0	0	0	0	0
1	5,9391	0,5235	0,3821	0,4625	0,4941	0,6282	0,4958	0,5475	0,5496	0,7102
3	7,4193	0,7152	0,734	0,6212	0,7951	0,7199	0,6259	0,8515	0,7857	0,8186
5	7,5957	0,734	0,734	0,6071	0,8139	0,7575	0,6447	0,687	0,8609	0,8562
10	10,5945	0,7951	0,7622	0,5554	0,9032	0,7669	0,6212	0,9126	0,7857	0,8656
20	12,6231	0,8421	0,7669	0,6259	0,9502	0,6964	0,6259	0,9972	0,8186	0,8562
40	14,0343	0,9737	0,781	0,64	0,854	0,7951	0,9549	1,23	0,8703	0,8891
80	14,06	1,3547	0,8515	0,6024	0,995	0,8703	0,7011	1,277	0,9455	0,9831



Graph 9: Zn ions release in SBF.

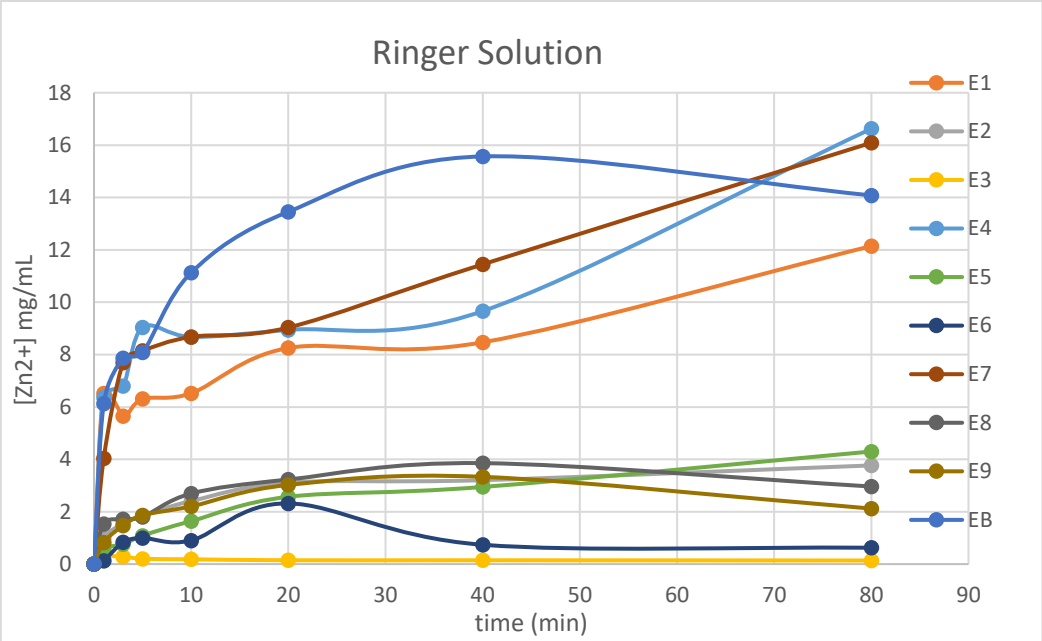


Graph 10: Zn ions release in SBF without EB.

The second analysis of Zinc ions release uses simulated body fluid as leaching agent a fluid that with ion concentrations approximately equal to those of human blood plasma, and the first thing to notice is that the concentration if Zn ions is lower in general than in water being 1,357 mg/mL the highest value compared to 18,09 mg/mL in water, this observation is positive, because as mentioned earlier the SBF is more similar to the conditions in the human body than water is, which means that the toxicity of the ZnO nanoparticles would be less than expected in the body if we only take into account the water as leaching agent. This also means that the influence of the factors mentioned earlier is less clear. Like in the previous case the curves that are above the rest are E1,E4 and E7 for the reason mentioned earlier but in this case we do not see a clear impact of the presence of mannose because the curve of E1 is not above the rest of curves.
As mentioned before the difference in the curves is unclear so maybe for this low release of Zinc ions the pH and the mannose does not have that big of an impact.

Table 6: Concentration of Zn ions released in SBF.

C	RINGER SOLUTION									
	EB	E1	E2	E3	E4	E5	E6	E7	E8	E9
0	0	0	0	0	0	0	0	0	0	0
1	6,1245	6,5228	0,9972	0,2875	6,3555	0,5883	0,1324	4,0337	1,5333	0,828
3	7,8657	5,658	1,559	0,2828	6,802	0,7575	0,8327	7,695	1,7119	1,4712
5	8,0789	6,3066	1,841	0,2029	9,0345	1,0865	0,9972	8,1415	1,8012	1,866
10	11,1278	6,5228	2,405	0,1841	8,6773	1,6404	0,9032	8,6773	2,6942	2,2044
20	13,4532	8,2524	3,11	0,1465	8,9452	2,5696	2,3172	9,0345	3,23	3,0222
40	15,5673	8,4686	3,204	0,1465	9,6596	2,9456	0,738	11,4456	3,8551	3,3324
80	14,078	12,144	3,768	0,1371	16,625	4,2992	0,6252	16,0892	2,9621	2,1198



Graph 11: Zn ions release in ringer solution.

The last analysis of ions release is done in Ringer solution, a solution of several salts dissolved in water for the purpose of creating an isotonic solution relative to the body fluids of an animal. It is worth to mention that the results obtained in this case in difference to the ones obtained for SBF are more like the ones with water about the concentration maximums.
Like in the other 2 analysis the curves of E1, E4 and E7 are clearly on top of the rest and the explanation is the same, the pH, but in this case we do not see any influence of the presence of mannose either, because E1 is the lowest curve at all points and is the one that doesn't have any mannose. There is a huge difference in these curves and the rest of the curves.
The regarding the curves E2, E5, E8 and E9 are more or less together on the second spot all of them with the same pH except E9 and lastly E3 and E6 that have the highest pH.

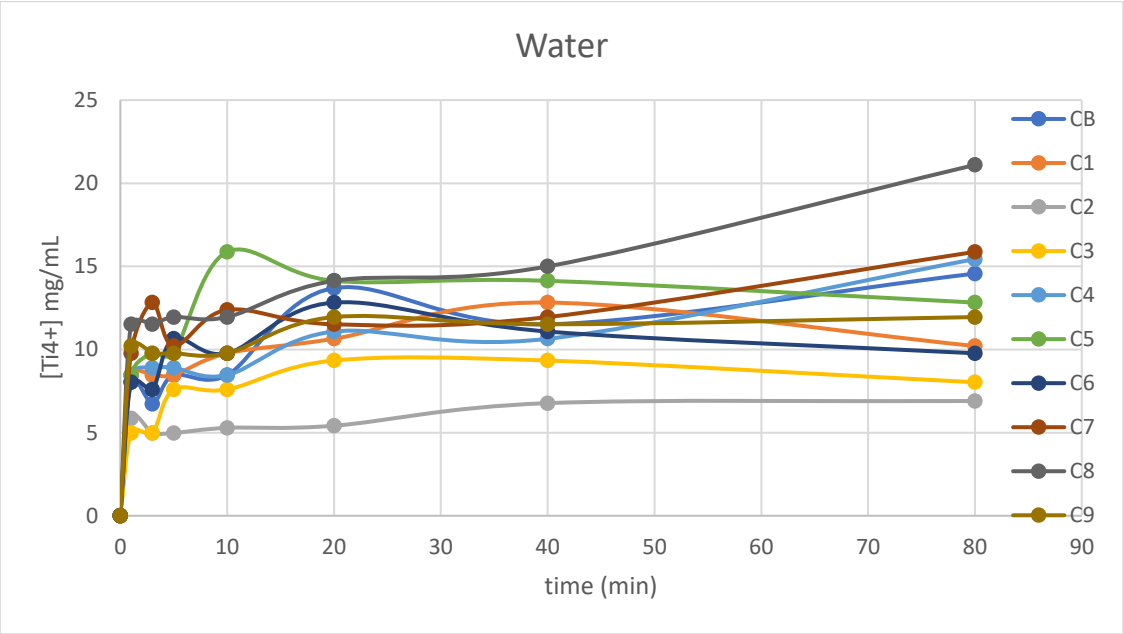
In conclusion for the 3 graphs can be appreciated that the pH in the obtention of ZnO is the factor with the highest impact in the further ion release, being the nanoparticles obtained at high pH the one that release the less ions. On the other hand, the influence of mannose is less than what could be expected theoretically and that is bad news because the goal of adding mannose is to reduce the release of ions. Another explanation for the low influence of the mannose in zinc ions release can be that quantity of moles of mannose added were too low, only 1 or 2 moles of mannose per 10 moles of ZnO, as studied in the previous essay the metal ions need 1 or 2 molecules of mannose for each ion to form a complex so adding a proportion this low could be the reason for the mannose to not influence in the ions release.

The same leaching process was studied for TiO_2 nanoparticles, obtaining the following results:

For the case of Ti ions there are only results for water as leaching agent:

Table 7: Concentration of Ti ions released in water.

Time, min	C Ti [mg/ml]									
	CB	C1	C2	C3	C4	C5	C6	C7	C8	C9
0	0	0	0	0	0	0	0	0	0	0
1	8,0296	8,4653	5,8511	4,9797	8,4653	8,4653	8,0296	9,7724	11,5152	10,2081
3	6,7225	8,4653	4,9797	4,9797	8,901	9,7724	7,5939	12,8223	11,5152	9,7724
5	8,4653	8,4653	4,9797	7,5939	8,901	9,7724	10,6438	10,2081	11,9509	9,7724
10	8,4653	9,7724	5,2868	7,5939	8,4653	15,8722	9,7724	12,3866	11,9509	23,7148
20	13,6937	10,6438	5,4154	9,3367	11,0795	14,1294	12,8223	11,5152	14,1294	11,9509
40	11,5152	12,8223	6,7724	9,3367	10,6438	1,4941	11,0795	11,9509	15,0008	11,5152
80	14,5651	10,2081	6,901	8,0296	15,4365	12,8223	9,7724	15,8722	21,1006	11,9509



Graph 12: Ti ions release in water.

First, it is important to mention that 2 values were eliminated from the graph because they were clearly anomalous data to not alternate the rest of the curve, C5 time 40 and C9 time 10.

For understanding the results, it is important to consider that the pH is not an important factor to difference the results in the different experiments. The impact of the pH is not appreciated because the moles added of base in this case are really low compared to the ones added for the obtention of ZnO np (more than 10 times less), so the variation of pH isn't as high either.

The presence of Galactose does not impact the results either, curves of C7, C8 and C9 should be the lowest because of the stability obtained by the formation of the TiO2-galactose complex but that does not happen. The explanation can be the same for this case too, the quantity of moles added of Galactose is really low and each Ti ion needs 1 or 2 molecules of sugar to form the complex, so this low influence can be solved

with the addition of more quantity of Galactose. Even though the influence of the sugars in ion release is not as high as expected the presence of sugar can also be important for the drug release in cancer cells due to their high affinity to sugar molecules because of their high demand of energy so their addition is not useless.
So, as it is explained earlier, the 2 main factors that influenced the ion release in ZnO nanoparticles does not have impact in this case, so it is important to find another explanation for the results. The zeta potential of each experiment is measured by the dynamic light scattering technique (DLS technique) and it measures how stable is a suspension, the DLS technique is going to be explained in the future. A suspension is considered stable when the zeta potential is greater than 20 and the greater the more stable. The results obtained for the TiO_2 aqueous suspension are shown in the next table:

Table 8: Zeta potential of TiO_2 nanoparticles obtained by DLS technique.

	zeta / mV
C1	24,5
C2	24,7
C3	28,2
C4	25,2
C5	23,1
C6	23,7
C7	22,3
C8	21,7
C9	26,8
C/B	24,5

Every value of zeta potential is above 20 so every suspension is stable but for example the lowest curves of the graph are E2 and E3 and the both have high values of zeta potential 24,7 and 28,2 respectively. The highest Ti ion release curve is by far the C8 curve and its value of zeta potential is the lowest. Other significant cases are C5 a curve that is high at all points except for the last one and C7 and they both have a low value of zeta potential. In conclusion there is a certain influence of the value of zeta potential in the release of Ti ions, the more stable a suspension is the less ions it releases. The DLS techniques also provides data for the nanoparticle size and particle size can also have an impact on the ion release because of the contact surface area, the ion release should increase when the particle size decreases. Looking at the results in table 10, this does not happen so it is safe to say that in this case the influence of size cannot be appreciated, there are other factors that matter more.

b. Analysis of size and stability:

The results obtained using the DLS technique for the size of ZnO and TiO_2 nanoparticles are shown in the next tables:

Table 9: Size of the ZnO particles obtained by DLS technique.

	d1 /nm	I1 /%	d2 /nm	I2 /%	Average d (nm)
E1	1573	100			1573
E2	282	100			282
E3	1744	100			1744
E4	1090	100			1090
E5	303	100			303
E6	256	98	5259	1,2	313,988
E7	1231	100			1231
E8	649	100			649
E9	268	100			268
E/B	22	100			22

Table 10: Size of the TiO_2 particles obtained by DLS technique.

	d1/nm	I1 /%	d2 /nm	I2 /%	d3 /nm	I3 /%	Average d (nm)
C1	311	98,2	5408	1,8			402,746
C2	453	100					453
C3	217	100					217
C4	300	100					300
C5	327	98,9	5560	1,1			384,563
C6	724	59,1	214	37	5386	3,9	717,118
C7	764	72,6	181	27,2			603,896
C8	399	93,1	5227	6,9			732,132
C9	596	100					596
C/B	310	100					310

The first thing to notice is that there are some particles that are above the 1000 nm (in the case of ZnO) and it can be too big to be considered nanoparticles. The size of the particles is important because there is a space between the cells called the intracellular space and that space has a different size depending on if the cell is healthy or not. As it has been mentioned earlier cancer treatment is the main application of the ZnO and TiO_2 for drug delivery systems, and the intracellular space in tumour cells goes from 50-800 nm a lot bigger than in a normal healthy cell that is around 2-10 nm. Considering this information the ideal size of the ZnO and TiO_2 should be around 50-800 nm because it would help the delivery of the drugs, the nanoparticles can pass through the intracellular space of cancer cells but they can not pass through the healthy cells, so they will act in the ill tissue and not in the healthy one and this is traduced in a reduction of the side effects.

Looking at the results in the case ZnO nanoparticles (table 8) the premise of being between 50-800 nm is fulfilled by almost every experiment except E1, E3, E4 and E7. E1, E3 and E7 all have in common that they all use in their preparation the lowest amount of KOH so the pH during the preparation is higher than in the rest of the experiments

during the preparation, so there is a influence in the case of ZnO that with a lower pH in the preparation the size of the particles obtained are bigger and this can mean that the ideal range of size is not achieved. For E6 there is 1,2% of the particles that are 5259 nm but the percentage is really low and does not really have an impact in the final result.

On the other hand, the TiO_2 nanoparticles are all in the desirable range of particle size, that means that is easier to obtain the ideal size in the preparation of TiO_2 nanoparticles than in the ZnO nanoparticles.

As explained before the DLS technique is also applied for the determination of the stability of suspension, the results obtained are in the tables bellow:

Table 11: Zeta potential of ZnO nanoparticles obtained by DLS technique

	zeta / mV
E1	13,3
E2	23,6
E3	21
E4	18,9
E5	22,8
E6	26,7
E7	15,6
E8	21,2
E9	23,5
E/B	11,3

Table 12: Zeta potential of TiO_2 nanoparticles obtained by DLS technique

	zeta / mV
C1	24,5
C2	24,7
C3	28,2
C4	25,2
C5	23,1
C6	23,7
C7	22,3
C8	21,7
C9	26,8
C/B	24,5

For a suspension to be considered stable the value of zeta potential should be of 20 mV or higher. For the ZnO nanoparticles experiments E1, E3 and E7 are bellow the 20 mV value so are considered unstable solutions, the consequences of an unstable solutions is the formation of aggregations and the possible sedimentation. These

experiments are also the ones with bigger nanoparticles and with low amount of KOH in the preparation, so it can be concluded that with big particles is harder to have a stable solution. The rest of the experiments for ZnO and TiO_2 are all stable.

To finish with this part, it is important to mention the correlation between all the parameters studied. E1, E4 and E7 had the highest release of Zinc ions, the undesirable particle size and the lowest stability of the suspension, this means that the pH during the preparation of the particles has a huge impact for the case of ZnO and it is not enough to use stoichiometric amount of KOH, it is better to have excess.

6. CONCLUSIONS:

To conclude, the applications of the ZnO and TiO_2 nanoparticles are extremely interesting and the only way to fully develop them is to continue with their study. Reducing its toxicity to the human body is of vital importance and can be achieved through protective coating, although the results obtained in this study with respect to this topic have not been as expected, other ways to reduce toxicity have been discovered, such as increasing the pH in obtaining nanoparticles or that their size is optimal to only act on diseased tissues.

The application of these compounds in the drug delivery systems is also very interesting, especially those based on pH to help increase efficacy and reduce side effects in the treatment of cancer.

In terms of reducing the ion release the pH in which the nanoparticles of ZnO was obtained was the factor with the highest impact, the higher the pH the lower the ion release. The presence of mannose also had some impact but not as expected since the amounts of moles of mannose added in the experiments were too low. The pH of the preparation also has an impact in the stability of the suspension and the size of the particles, a low pH negatively affected these parameters.

The pH during the obtention of TiO_2 nanoparticles was not a huge factor, mainly because the quantity of base added in this case was not as high, so there were not high fluctuations on the pH. The presence of galactose did not affect either for the same reason mentioned earlier, so the main factor affecting the ion release was the zeta potential.

Considering the results obtained, TiO_2 nanoparticles may seem a more appropriate option, all the particles obtained formed stable suspensions and their size was the desirable size to focus on cancer cells. The ion release was similar for ZnO and TiO_2 at least in water.

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