RIKILT Food Safety Institute

Assessing the transport of Iron-Zinc nanoparticles in an *in vitro* model of the Human FAE

Denisse Hernández Martínez

February 2010

WAGENINGEN UNIVERSITY
WAGENINGEN UR

Assessing the transport of Iron-Zinc nanoparticles in an *in vitro* model of the Human FAE

Denisse Hernández Martínez

Registration number: 830122329010

Supervisors:
Hans Bouwmeester
Jenneke Riethoff-Poortman

The thesis was carried out in cooperation of RIKILT Food Safety Institute and Wageningen University

February, 2010
Wageningen, The Netherlands

Thesis code number: TOX-80436
Master programme: MSc. Food Safety
Wageningen University and Research Centre

Acknowledgements

I would like to express my sincere gratitude to Hans Bouwmeester for his guidance, support and comments through all stages of this process. I will always appreciate the encouragement to try different approaches. Thank you for the lesson about making decisions at certain moments with the information available.

Special thanks and appreciation to Jenneke Riethoff-Poortman for making me feel part of the group, for her patience and support and for teaching me the techniques to carry out the experiments.

Many thanks to Ab van Polanen, Sjaak van Oostrom, and John Verbunt for the making time in their schedules to do the measurements of the samples.

Great appreciation to Bert Brouwer for the patience and help with the DLS and TEM images. I would also like to thank to Rob Bakker for the TEM images.

I would also like to express sincere thanks to TE cluster members for letting me feel part of the group. And for all the patience they have in the labs.

I am grateful to my room mates, Xiaomeng, Antonia and Andréia, it was a pleasure to share with them many experiences during these months.

Particular thanks to my brother, Hernán for his support and wise words.

Lastly, and most importantly, I wish to thank my parents Benjamin and Catalina, for their unconditional support, love and encouragement. They have been the ones that through daily life have taught me that work is a way to get to the place I want.

Abstract

There is a wide range of uses of nanoparticles within the food chain; potentially, these materials could be used as fortifiers, since there are claims about the increase in the bioavailability of food component when applied in nano form. Iron and zinc deficiencies in public nutrition have a worldwide extension, therefore with the idea of a combined fortification, at The Swiss Federal Institute of Technology Zurich and the Institute of Food Science and Nutrition nanoparticles containing Iron/Zinc at different ratios where developed. These nanocomplexes have the advantage of having less impact in the sensory characteristics of the food stuffs added with. As the potential use of the nanomaterials increases, more attention needs to be paid to their potential toxic effects. This starts with the study of the potential bioavailability of the nanoparticles; therefore, the aim of the present work was to assess the transport of Iron-Zinc nanoparticles in and *in vitro* co-culture model of the Human FAE.

Seven Iron-Zinc nanoparticle complexes were used: FeP, FeZnP, FeZnCaO, FeZnMgO and 3 different FeZnO compounds, as ion control. $FeSO_4.H_2O$ was selected. Initially, all complexes dispersed in DMEM were characterized and screened for toxicity. The complexes FeZnP, FeZnCaO and FeP were selected with basis on the stability in dispersion, which was determined by means of DLS measurements and TEM images. Furthermore, the concentrations selected to perform the transport studies were 10, 30 and 60 μg/mL, since the cell viability assay (WST-1) did not show decrease in cell viability upon exposure during 4 hrs.

The transport studies in the *in vitro model* of the Human FAE did not show a significant increase on the amount of iron present in the basolateral chamber after an exposure of 4 hrs to dispersion of FeZnP, FeZnCaO, FeP nanocomplexes in a DMEM. However, for the three mentioned complexes the amount of iron measured in the cell section was increased when concentrations of 30 and 60 μg/mL where used. This increase could be an indication of an uptake of the nanoparticles by M Cells.

Contents

1. Introduction

1.1 Nanotechnology in food

Nanotechnology is a field that in the last years has grown enormously. As published by the Scientific Committee in Emerging Newly Identified Health Risks (SCENIHR) an engineered nanomaterial is “any material that is deliberately created as such, it is composed of discrete functional and structural parts, either internally or at the surface, many of which will have one or more dimensions of the order of 100 nm or less” (SCENIHR 29 November 2007). Nanoparticles have unique properties, finding its use in a large number of different applications; therefore the products containing them have increased. Current applications are medical and cosmetic related as well as food products. Within this last mentioned field, nanoparticles can be introduced in the food chain, during primary production, processing and packaging (Bouwmeester, Dekkers et al. 2009). Among the uses that have been described are, food contact materials, additives and processing aids, biosensors for monitoring the condition of food, nanosized agro-chemicals or veterinary medicines and as food ingredients (EFSA 2009). There might be a potential use of nanoparticles either as fortifier or supplement with the claim of an increase in the bioavailability of the added compound.

The application of Nanotechnology in the food sector has raised a number of safety, environmental, ethical, policy and regulatory issues (EFSA 2009). One of the concerns about the safety in their application is that they might have toxicity profiles that might differ from their microparticulated counter forms due to properties such as: small size, surface reactivity and translocation across biological membranes.

The potential routes of exposure to nanoparticles comprise: absorption via lung, via the olfactory nervous system, dermal absorption, and gastrointestinal absorption, is the last one, the most relevant when considering the exposure to nanoparticles in food, although some of the particles found in the gastrointestinal part might have its origin in ingested products but also from other routes such as respiratory tract or dermal absorption (Oberdorster, Maynard et al. 2005).

1.2 Iron and zinc Deficiency

Iron deficiency has important consequences for human health and child development: anaemic women and their infants are at greater risk of dying during the perinatal period; children’s mental and physical development is delayed or impaired by iron deficiency, and

the physical work capacity and productivity of manual workers may be reduced (WHO/CDCP 2004). Although many efforts have been done regarding this deficiency, the conditions remain affecting up to two billion people worldwide in both, industrialized and developing countries (WHO 2001). Food fortification is one of the strategies to control iron deficiency, however the water-soluble and with an acceptable bioavailability compounds such as $FeSO_4$ cause adverse organoleptic changes in the food-stuffs, what limits its addition to a certain extent. In turn, highly bioavailable compounds such as NaFeEDTA, heme iron, ferrochel aminoacid are relatively expensive (Hurrell 2002). The downsizing of iron compounds have been shown to be a factor that might increase the iron bioavailabity causing lower sensory changes in the food (Rohner, Ernst et al. 2007).

Zinc is another micronutrient that is regarded as essential for the human health. It is well known that zinc deficiency causes numerous effects in humans, including neurosensory changes, growth retardation and delayed wound healing. It is estimated that zinc deficiency affects about one-third of the world's population. Although severe zinc deficiency is rare, mild-to-moderate zinc deficiency is quite common throughout the world (WHO 2002).

The importance of these two mentioned micronutrient deficiencies in public nutrition, and their likely coexistence in infants in developing countries support the idea, that combined supplementation with both iron and zinc would improve the cost-effectiveness (Berger, Ninh et al. 2005). At the same time, because of nanoparticles characteristics, complexes containing iron and zinc have been thought with the potential to overcome the technical difficulties posed by the macroparticulate compound and to increase the bioavailability of the referred micronutrients.

1.3 *In vitro* assessment requirements

With the increase in the use of nanosize compounds in the food field, it is of great importance to get insight on the final fate of the nanoparticles added to the food. In this sense, the oral exposure and gastrointestinal absorption are aspects to be considered. For this purpose the *in vitro* assays offer the advantage over *in vivo* in terms of cost and time. However when *in vitro* assays are to be used to assess the toxicity and bioavailabililty of nanoparticles there are aspects to be considered since nanomaterials have shown to behave in a different manner than their macroparticulate counterparts. It is necessary to have well characterized dispersions, as well as to include a series of controls when performing the *in*

vitro assays. To pay attention to both requirements will lead to have results that are meaningful and comparable.

1.3.1 Dispersions and Characterization

As a result of the increased concern about the use of nanoparticles, many *in vitro* as well as some *in vivo* studies have been performed, following the approach case by case in the assessment of their toxicological potential, however, in certain cases, the results obtained from studies of the same kind of nanoparticles are different. Thus to ensure that the results are reproducible, meaningful, an accurate characterization of the nanoparticles has to be done at different stages: after synthesis, as supplied, as administered and even after administration (Oberdorster, Maynard et al. 2005).

In experimental nanotoxicological studies, measuring the state of the dispersed nanoparticles in a biological relevant media is of great importance for assessing the nanoparticle toxicity (Powers, Palazuelos et al. 2007). Therefore, parameters such as size, agglomeration, aggregation could be relevant and/or influence the outcome of the studies (Jiang, Oberdörster et al. 2009). Among the tools frequently used for this purpose are: DLS and TEM, the former has been a commonly used approach to get an insight of the stability of the nanoparticles dispersion, as well as the size of the particle in the media, being this the advantage that the technique offers over others. TEM in turn, has been also used as an analytical tool to assess the size and state of aggregation of the particle, however in both cases, there are some limitations; DLS is a weight-averaged measurement biased towards larger particles (Park, Annema et al. 2009), in case of TEM, it has been claimed that the process for the preparation of the sample might influence the structure of the sample, another of the drawbacks is the field of vision, that could lead to do not have a representative sample, next to that is the labour intensive of the sample's preparation (Luykx, Peters et al. 2008). For the aforementioned, it is relevant to characterize the particles as fully as possible and try to crosslink the information gathered by different analytical techniques.

1.3.2 *In vitro* toxicity

In vitro assays are means for a fast approach to distinguish between low and high cytotoxicity materials, however the intrinsic characteristics of the materials screened such as size, shape, surface area, surface reactivity, solubility influence their cellular up take. In this respect, different studies differ in their findings (Park, Annema et al. 2009), for this

reason it is important to do an appropriate characterization of the nanoparticles assessed. In order to have a fair comparison of the results of different test, it has been revealed of great importance the protocol followed for screening the cytotoxicity. Due to the aforementioned it is necessary to include different controls: a positive control to ensure the ability of the cell type to produce specific markers of cytotoxicity, another control to assess a possible interaction of the nanoparticles with the WST-1 reaction product (formazan), and an extra one to assess whether the nanoparticles dispersion scatter or absorb light in the read-out system (Park M, Daniëlle PK Lankveld et al. 2009). By including the two latest controls, the possibility of underestimation or overestimation of the induced cytotoxicity can be determined.

1.3.3 Human Follicle Associated Epithelium

As it has been mentioned previously, the effects that nanoparticles might have on human body, depend on the route of exposure, thus when considering as a source of exposure the food itself, it would be of great importance to know what is the fate of the nanoparticles upon passage through the gastrointestinal (GI) tract. It is possible that due transformations such as solubilisation, agglomeration, aggregation, absorption or binding to other components of food or the reaction with GI components, the nanoparticles will not remain as such in the lumen (EFSA 2009). However considering the scenario where the nanoparticles remain intact at the time they reach the intestine, it is important to know whether there is a transport of the referred material. The term translocation from a kinetic point of view represents both, the absorption and distribution process (Hagens, Oomen et al. 2007). Thus in this report the term transport will be used to refer to the nanoparticles that are found in the basolateral compartment. It has been published that due the size of the pore at the tight junctions, the para-cellular transport of nanoparticles will be limited unless there is a damage of the junctions (Slütter, Plapied et al. 2009). However nanoparticles might pass through the epithelial well, by trancistocys by M-Cells in the Payer's Patches (PP) (Frey A 1997; Neutra, Mantis et al. 1999).

M-Cells in the follicle associated epithelium (FAE) of the intestinal PP and isolated lymphoid have as a function to sample the gut lumen and transport the antigens to the underlying mucosal lymphoid tissue for a processing and initiation of an immune response (Brayden, Jepson et al. 2005) . Considering, the potential of the M-Cells, *in vitro* models of human FAE have been developed in order to study the contribution of M-cells to the transport of nanoparticles (Gullberg, Leonard et al. 2000), (des Rieux, Fievez et al. 2007A).

Since the models developed are based in the suggestion that B-lymphocytes are the most important immune cells for the induction in the differentiation of M-cells, through the release of cytokines (Gullberg, Leonard et al. 2000), the first model was developed in order to avoid the use of murine B-Cells in the co-culture. However it was necessary to have a more functional and reproducible model, reason why the model was modified by inverting the trans-well inserts to make the caco-2 cell monolayer more accessible to lymphocytes encouraging a closer contact between the two cell types.

2. Aim of the study

The aim of the present work was to assess the transport of Iron-Zinc nanoparticles in and *in vitro* model of the Human FAE. The model used was developed by des Rieux, et al, 2007 and put in place at RIKILT, previously. Such model involves the co-culturing of caco-2 cells/Raji cells to induce M cell differentiation. Important factors for the interpretation of the transport experiments involve in one hand, the use of well characterized particles suspended in the relevant biological media, and in the other hand, the use of non toxic doses, to assure the integrity of the monolayer of the co-culture model.

3. Materials and Methods

3.1. Materials

Iron/Zinc Nanopowders. Seven Iron-Zinc nanoparticle complexes were used: FeP, FeZnP, FeZnCaO, FeZnMgO and 3 FeZnO compounds with varying Fe/Zn ratios content. all the before mentioned nanocomplexes as well as ion control selected $FeSO_4.H_2O$ were a kind gift from prof. Michel B. Zimmerman. (The Swiss Federal Institute of Technology Zurich and the Institute of Food Science and Nutrition). The compound characteristics are shown in Table 1.

Human Intestinal cell line Caco-2 and the Human Burtkett's lymphoma cell line Raji were obtained from The American Type Culture Collection (ATCC), Dulbecos's Modified Eagle's Medium (DMEM) containing and phenol red free, were purchased from Lonza, Non-essential amino-acids, (NEAA) were purchased from MP-Biomed, Penicillin Streptomycin Solution (PEST) was obtained from Sigma-Aldrich Inc., Heat Inactivated Fetal Bovine Serum (FBS) and RPMI 1640 medium were purchased from GIBCO ™ Invitrogen. Trypsin/EDTA (0.25/0.05%) and Hank's balanced salt solution buffer (HBSS) were prepared according to RIKILT protocols. Cell Proliferation Reagent WST-1, was obtained from ROCHE. Ultra pure water was obtained from PureLab Ultra ® purified water systems, HNO_3 65% was obtained from Merck and used as a solution 13% in ultra pure water.

Table 1. Compound characteristics reported by ETH Zürich PTL and HNL

Material	Specific surface Area (m^2/g)	Amorphous a/crystalline c	Fe content %w (AAS)	Zn content%w (AAS)	Ca content%w (AAS)	Mg content%w (AAS)	P content%w (AAS)
FeZnO ($ZnFe_2O_4$)	157.89	c	34	24.4			
FeP ($FePO_4$)	197.3	a	40		6	1	12
FeZnP ($FeZnPO_4$)	190.76	a	21	11	7	3	15
FeZnCaO ($ZnFe_2O_4$ + CaO)	105.16	c	20	29	29	0	0
FeZnMgO ($ZnFe_2O_4$ + MgO)	191.96	c	23	13	5	15	0
FeZnO ($ZnFe_2O_4$)	173.93	c	35	25	5	0	0
FeZnO ($ZnFe_2O_4$)	172.04	c	32.2	24			

3.2. Preparation of nanoparticles dispersions.

The working dispersion were prepared following the approach described by Bihari (Bihari, Vippola et al. 2008) as a optimal sequence to prepare dispersion of nanoparticles in physiological solutions without creating coarse agglomerates.

A stock solution of 6000 μg/mL in ultra pure water was prepared; this solution was sonicated with Sonicator XL-2000 series for 1 min at 4W having as result a specific ultrasound energy of 240 J/m^3. An initial dispersion of 120 μg/mL was prepared by adding the stock solutions with FBS in such a way that the final concentration of FBS in DMEM is 10%. The next step was to vortex the dispersions before the addition of DMEM not supplemented with FBS, after the addition of DMEM the solutions underwent a second vortexing step where the power in all case was 4W, but the time varied depending on the volume. This difference in time was used with the purpose of having a specific energy of 400 J/m^3, every time. Finally, the working solutions were prepared by making the required dilutions in supplemented DMEM (DMEM containing 10% FBS) to get the final concentrations needed. The working dispersions were further sonicated with a power of 4 W for the time required to have a specific energy of 96 J/m^3.

As control group ionic iron sulphate was used, this compound is used either as supplement or fortifier of referred metal, for this reason the concentrations used in these experiments were based on The WHO Recommendations where the advised concentrations for Fe in flour vary between the range of 10 to 60 μg/mL (WHO 2009) . The actual concentrations for the cell viability and transport studies were between the range of 10 to 60 μg/mL.

Apart from the characterization stage all the dispersions were prepared one day before exposure, and stored and 4º C, before the used of the dispersions they were sonicated at 4W for the time required to achieve 96 J/m^3 of specific energy. In order to make the sonication process, scalable and reproducible (Hielscher 2005), the time was calculated from:

$$Ev = \frac{P}{V_c} \times t_r$$

Where Ev= is the specific ultrasound energy, P= power in watts and V_c is the volume in m^3 of the dispersion to be processed and t_r is the residence time in seconds.

3.3. Nanoparticle characterization

The size distribution of the nanoparticles dispersion was analyzed with by NANOSIGHT LM10™ Dynamic Light Scattering (DLS). This technique measures the dynamic fluctuation of light scattering intensity caused by the Brownian motion particles. The measurement gives as a result the average hydrodynamic diameter distribution. Other technique used to get some images from the nanoparticles in dispersion was the Transmission Electron Microscope JEOL JEM1011 with a SIS Keenview 1K x 1K camera (TEM). Both techniques were used as a mean to get some insight of the size, aggregation and agglomeration state of the dispersions in the biological media.

3.3.1 Dynamic Light Scattering Analysis.

For the characterization of the Iron/Zinc complexes in DMEM, solutions of 2 μg/mL were prepared, since higher concentrations provoked a crossover of the particles, incrementing the light scattered by the dispersions, thereby interfering with the actual measurement of the size of the particles.

For the assessment of the dispersion's stability, after having sonicated the suspensions of 2 μg/mL (20 mL), the content was divided into four tubes, one tube for each of the following times: 0 hrs, 4 hrs and two different tubes of 24 hrs.

The tube for the condition 0 hrs, was immediately measured after preparation, the tubes of 4 hrs and 24 hrs were kept at 37 °C and 5% CO_2, another tube of 24 hrs was stored at 4°C. The last approach was in order to mimic the conditions that the working solutions would undergo either in the exposure time or during its storage, since for practical reasons the solutions should be prepared on the day before each experiment. For a summary of the conditions of the solutions see Table. 2

Table 2. Summary of the conditions of the solutions

Sample	Time (hrs)	Conditions of Storage
A	0	N/A
B	4	37ºC, 5% CO_2
C	24	37ºC, 5% CO_2
D	24*	4 ºC

N/A. Not storaged
*Sonicated prior measurement

After storage, all samples were measured directly by DLS. One exception was for samples stored at 4°C, they were sonicated (E_v of 96 J/m^3) before measurement. The videos were processed by Nanoparticle Tracking Analysis [NTA] software. The data presented in the next section was an average of 6 different measurements.

3.3.1 TEM Analysis.

TEM images from dispersions of 30 μg/mL in DMEM were taken. For practical reasons, the nanoparticles were deposited onto the copper grid within the next 4 hours after its preparation. The images were obtained with a magnification of 30 000x.

3.4. Cell culture

Human intestinal cell line Caco 2 were used from passages numbers 20-43. The cells were cultured and maintained in 75cm^2 flasks (Corning®) in DMEM supplemented with 10% FBS, 1% PEST and 1% NEAA. Before the experiments, cells with a confluence near to 90% were trypsinized, and resuspended to the needed concentration in DMEM.

The Human Burkett's lymphoma cell line Raji used belonged to the passage numbers 4-20, this cell- line was cultured and maintained in 75cm^2 flasks (Corning®) in RPMI 1640 medium supplemented with 10% FBS, and 1% PEST. Both cell lines were incubated at 37°C and 5% CO_2.

3.5. Determination of Cell Viability

Caco 2- cells were seeded in 96-well plate at a concentration of 40 000 cells/mL, 48 hrs before exposure. The assay used to determine cell viability was the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (ROCHE), better known as WST-1 assay, briefly, 50 μl of the culture media was removed and replaced by 50 μl of the different concentration of Nanoparticles in a way that the final exposure media had a concentration of 10, 20, 30, 40, 50, and 60 μg/mL. After incubation for 4 hrs and 24hrs at 37°C in 5% CO_2 the optical density (OD) was measured at 450 nm in BioTek Synergy™ HT Multi-Mode Microplate Reader.

The percentage cell viability was calculated using the following equation:

$$\frac{OD_{treated}}{OD_{solventcontrol}} \times 100$$

A possible interaction in the read out system between the NP dispersion and formazan (reaction product in the WST-1 assay) was tested. This assessment was done as follows: cells were seeded in a 96 wellplate following the procedure described at section 3.5, after 2 and 22 hrs, respectively 10 µl of WST-1 reagent was added and incubated for 2 hrs at 37°C and 5%CO_2 to allow the production of formazan. Afterwards, the formazan was collected and pooled to be plated again in a 96 well plate (100 µl per well). Next 10 µl of dispersion of concentrations 100, 300 and 600 µl/mL were added, in order to have final concentrations of 10, 30 and 60 µl/mL, respectively. Afterwards the absorbance was measured at 450 nm.

3.6. Transport studies

For the transport studies, the concentrations used, were based on the results from the cell viability test, being 10, 30 and 60 µg/mL.

A co-culture of Caco-2 /Raji cells used as *in vitro* model of human associated epithelium (FAE) described by des Rieux (des Rieux, Fievez et al. 2007A), was used to assess the transport of iron /zinc nanoparticles. Briefly, at day 1, Caco 2-cells suspended in 0.5 mL of DMEM, were seeded at a density of 80 000 cells/mL on the apical side of the Transwell® inserts. At day 5 the inserts were inverted in Petri dishes filled with DMEM. The inversion was done with the aid of a piece of silicon tube, the basolateral side was filled with 0.5 mL of DMEM, this medium was renewed every other day until day 16, when Raji cells suspended in DMEM were added at a concentration of 80 000 cells/mL to the basolateral compartment of the inserts. The co-cultures were kept as described above until day 21 when the transport studies were carried out. Prior the experiments, the silicon tubes were removed and the inserts washed with HBSS and placed in its original orientation in the Transwell® plates containing 1.5 mL of phenol red free DMEM, the apical side was added with 0.5 mL of the same medium. The plates were placed at 37°C and 5% of CO_2 to allow them to equilibrate for 30 min before the measurement of the Epithelial Electric Resistance (TEER) by Millicell®-ERS .

For the transport studies the DMEM contained in the apical chamber was removed and replaced by the Nanoparticles dispersion in concentrations of 10, 30 and 60 μg/mL. The concentrations used for Iron Sulphate were 1, 1.5 and 2 μg/mL. In the transport studies the Complexes used were: FeZnP, FeZnCaO and FeP.

The TEER values were measured again after the transport studies. For the second time point of the TEER measurement, the media in the apical compartment contained the NP dispersion, thus, in order to check whether the increase in resistance could be due the presence of the nanoparticles, in a prior experiment the resistance was measured just after the addition of the nanoparticles dispersion.

The total exposure time was 4 hrs, which has been reported as an average time for the transit of the chyme in the small intestine (Versantvoort and A. G. Oomen 2005).After collecting the solutions from the apical and the basolateral compartments, the cells on the Transwell® inserts were lysed with 2 mL of HNO_3 13% for a period of 48 hours. This last treatment will be referred as cell section from now on.

The total iron transported and contained in the cell section was measured by Atomic Absorption Spectrometer graphite furnace (PerkinElmer AAnalyst™ 800), while AAS flame (Perkin Elmer AAnalyst^{TM} 400) was used to measure the final iron concentration in the apical compartment.

3.7 Statistical Analysis

All variables were tested at least in triplicate and the values analyzed by One-way analysis of variance (ANOVA) with Dunnets's and Turkey post post hoc test performed using GraphPad Prism (version 5.02 for Windows, GraphPad Software, San Diego California USA. Differences were considered significant if $p<0.05$.

4. Results

In this report, rather than give the average hydrodynamic diameter of the solutions, the size profile of the nanoparticles dispersion is presented since a big dispersion and two peaks are observed in the data. In general, all particles showed a dynamic interaction with DMEM components along the time, some sort of aggregation/ agglomeration is observed, since the distribution becomes planar, and its size range becomes broader. Despite, the procedure used to disperse the particles in DMEM, after 4 hours there is still some sediment formed in the bottom of the container, being less for particles FeP, FeZnCaO and FeZnP and more for FeZnMgO and the three different FeZnO. For most of the particles, the main changes in increase and decrease of size distribution appears to be closely related to the changes suffered by DMEM. In the cases of particles FeP and FeZnO with an iron content of 35% the sonication post- storage at 4°C is enough to bring the dispersion to a similar state of agglomeration.

Using TEM it was possible to observe how the particles are entrapped within some of the DMEM components, the size in the images was different from the values observed by DLS, however; the images can not be taken as a representative state of the nanoparticles in media.

The results from the WST-1 assay showed no decrease in cell viability for the exposure time of 4 hrs, however, at time 24 hrs, the cell viability showed a decrease of 20% compared to the control. Finally, the outcome of the transport studies does not show an increase in the iron content in the basolateral compartment after 4 hrs of exposure, however an increase in iron content in the cell section was measured, what might suggest a potential transport of the particles by M cells.

4.1 Nanoparticle characterization

The size distribution of the particles suspended in DMEM shows two peaks. The first peak is observed between 10 and 110 nm, and the second one is observed at 110-430 nm for all particles, except FeP, whose second peak is between 110 and 300 nm. Already, after 4 hrs differences can be observed between fig. 1.A and 1B, in the latest the first peak section shows a decrease in the particle count, the same observation holds for fig 1.C representing 24 hrs, where the particle count remains similar to the one of fig. 1B.

At time 4, the opposite happened to the second peak, In the range 110-430 nm with a peak around 250 nm the count of particles is increased, however, at time 24 hrs, the number of particles decreases. A different case is FeP whose distribution range as

mentioned before is smaller having its peak around 200, this last dispersion not withstanding the treatment undergone, showed a less variation even after the sonication process after 24 hrs of storage at 4°C.

When the iron/zinc dispersions are measured, although the main changes are related to the changes undergone by the media, for some particles there are differences in the count of particles, this might suggest and aggregation-agglomeration process.

Dispersions of particles, FeP, FeZnCaO and FeZnP showed minor changes in distribution profile within the 4 hrs after their preparation. For particle FeP, the stability of the size distribution remained constant for all treatments.

For most of the complexes, the step of sonication post– storage at 4°C, does not allow to deagglomerate or disaggregate the particles in dispersion. In the cases of particles FeP and FeZnO with an iron content of 35% the sonication post- storage at 4°C was enough to bring the dispersion to a similar state of agglomeration.

As a mean to get a better insight in the aggregation/agglomeration state of the nanocomplexes, TEM pictures from concentrations of 30 μg/mL in DMEM were taken. The dispersions were fixed in the cooper grid within a period time between 1 and 4 hrs after sonication.

Fig. 2. A) shows that already in DMEM structures smaller than 20 nm can be found, a layer of DMEM components is observable as well. In Fig. 2. C and D, from nanocomplexes FeZnP, FeZnCaO, structures of 15 nm and 50 nm, respectively appear to be entrapped by a layer that could be the so called “protein-corona” . The same element that is visible at the image of DMEM alone appears to be agglutinated and surrounding the nanoparticles. When comparing the size of these structures and the size distribution got by the DLS measurements, there is a observable difference in values. However, in the case of nanocomplexe FeP (Fig. B), a polydisperse solution can be observed, the sizes of the elements, are between 10 and 300 nm, therefore the sizes of the structures observed by TEM, seems to be in agreement with the two peaks present in the size distributions obtained by DLS measurements. These results should be taken with reserves since the concentrations measured with DLS and TEM differ for practical reasons, as it has been mentioned before.

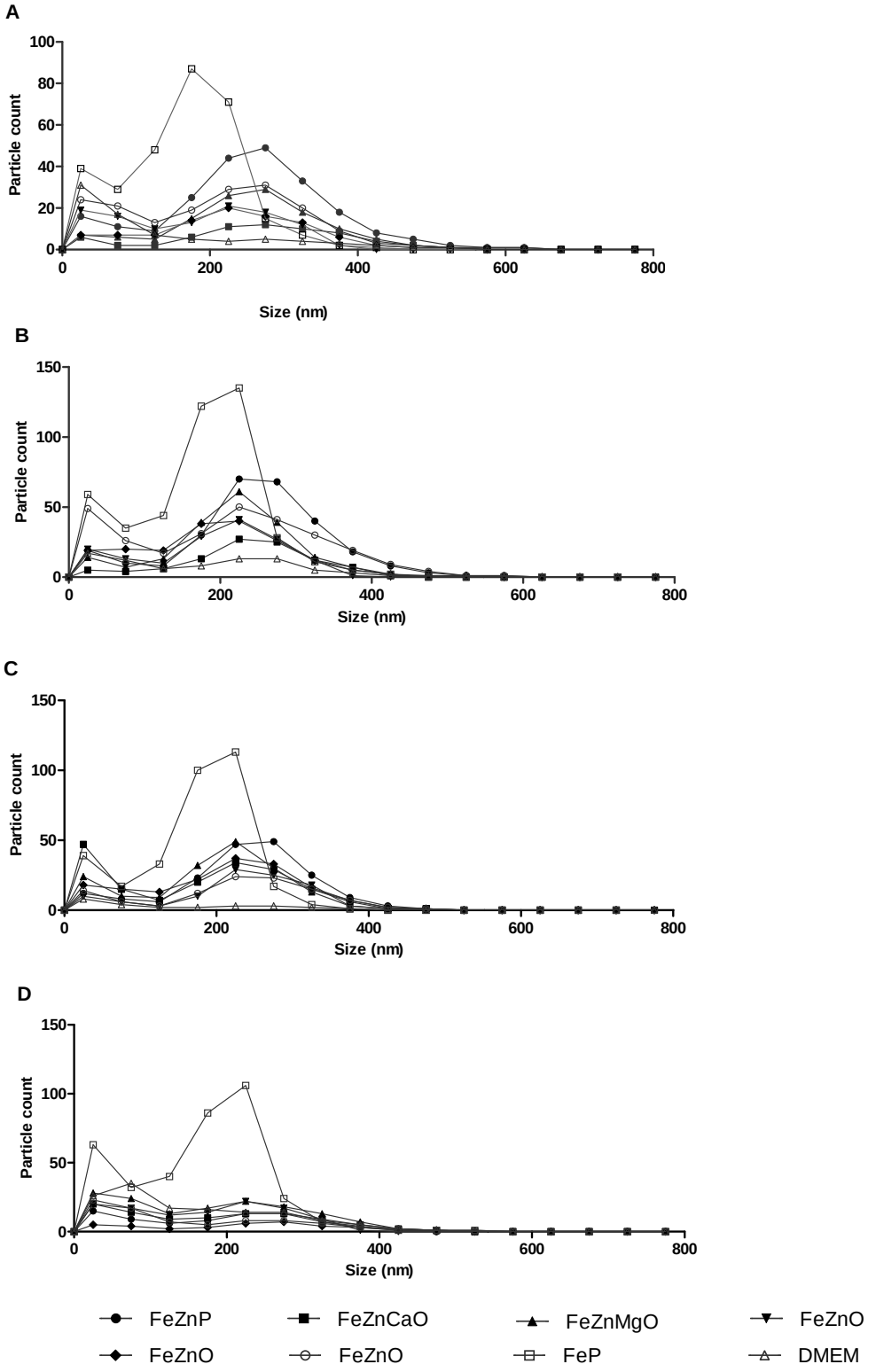


Fig. 1. Dynamic Light Scattering of Fe/Zn complexes. A) Measured immediately after dispersion, B). After an incubation time of 4 hrs under at 37ºC and 5% CO_2. C). After an incubation time of 24 hrs under at 37ºC and 5% CO_2., D). After and incubation time of 24 hrs at 4ºC. DMEM is shown as a control .

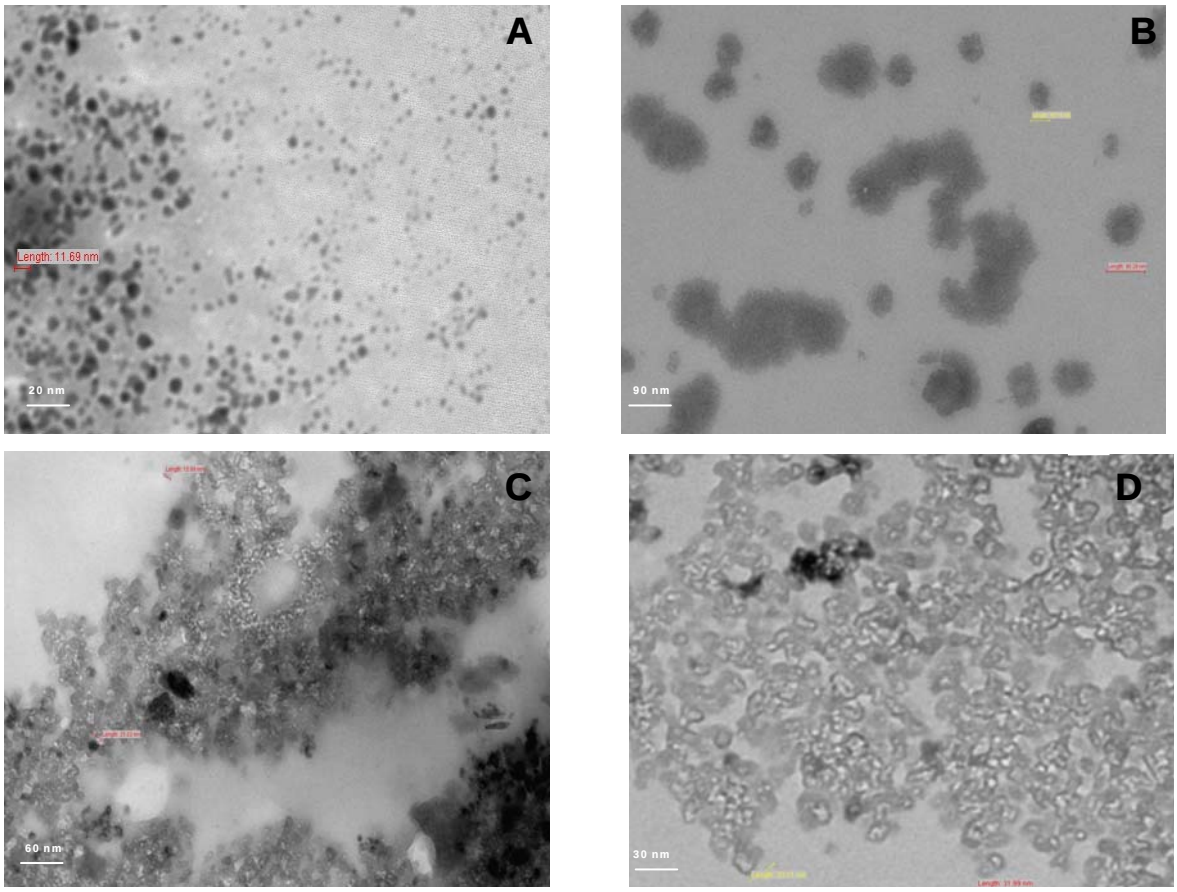
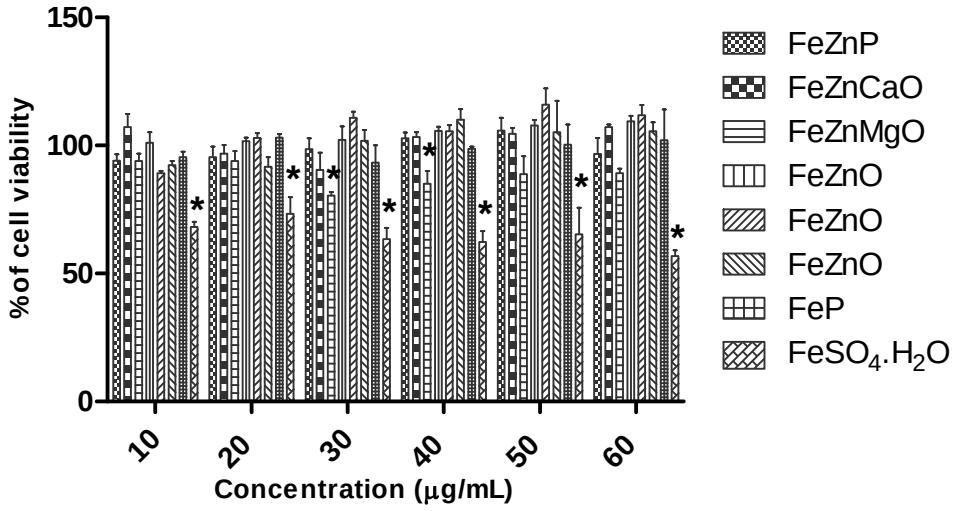


Fig. 2. Transmission electronic microscopy images of nanoparticles dispersions A). DMEM, B). FeP in DMEM, C). FeZnCaO in DMEM, D). FeZnP in DMEM

4.2. Determination of Cell Viability

In order to determine safe concentrations where the integrity of the cell monolayer on the co-culture model was not compromised due to cytotoxic effects, therefore affecting the results of the transport of iron in the FAE model, a cytotoxicity test was carried out to screen the concentrations that would be used. As described previously, the assay was performed at two different time points,. After 4 hrs of exposure, under microscopic the cells did not show any morphologic change. Fig 3.2. A. shows how the ion control $FeSO_4.H_2O$ exercises a cytotoxic effect on Caco 2-cells, decreasing down to levels of 70% the cell viability in reference to the control; this effect does not give any indication of being a dose dependant effect. In the case of the Nanoparticles complexes studied, there were not cytotoxic effects observed in any of the treatments.

A



B

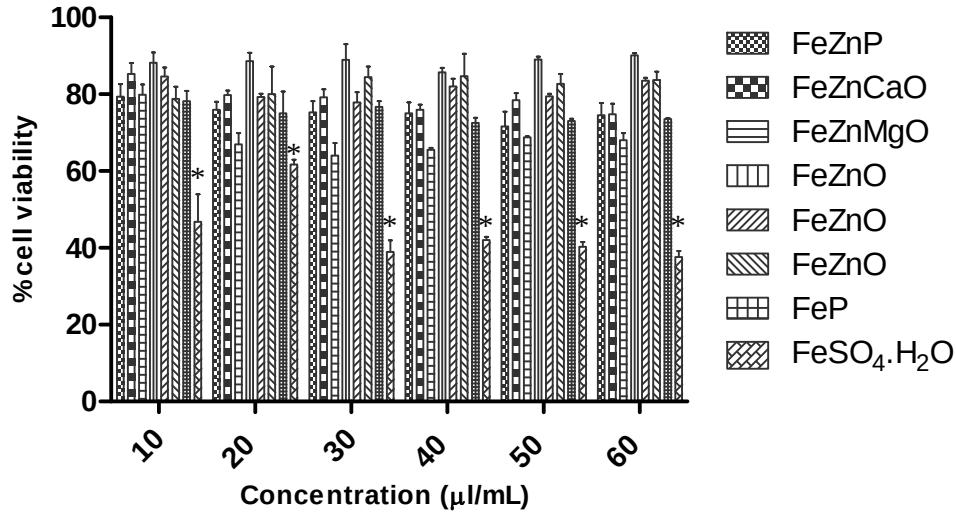


Fig. 2. A) Effect of Iron/Zinc Nanoparticles in cell viability of Caco 2-cells after 4 hrs of exposure. The values given are mean + SEM. * Indicates a statistically significant difference compared to the control by Dunnet's post-hoc analysis ($P<0.05$). B) Effect of Iron/Zinc nanoparticles in Caco 2-cells after 24 hrs of exposure. The results represent the mean of three wells compared to the control. The values given are mean + SEM. * Indicates a statistically significant difference within treatments compared by Turkey post-hoc analysis ($P<0.05$).

For the exposure time of 24 hrs, the results can be observed in fig 3.2.B. The ion control shows a decrease in cell viability from 50 to 60 %, having a trend that could be described as dose dependant. The same effect of decrease in cell viability was found in the Iron /Zinc nanoparticles, where the cell viability showed a decrease to levels of 80%, without having evidence of being a dose dependant effect.

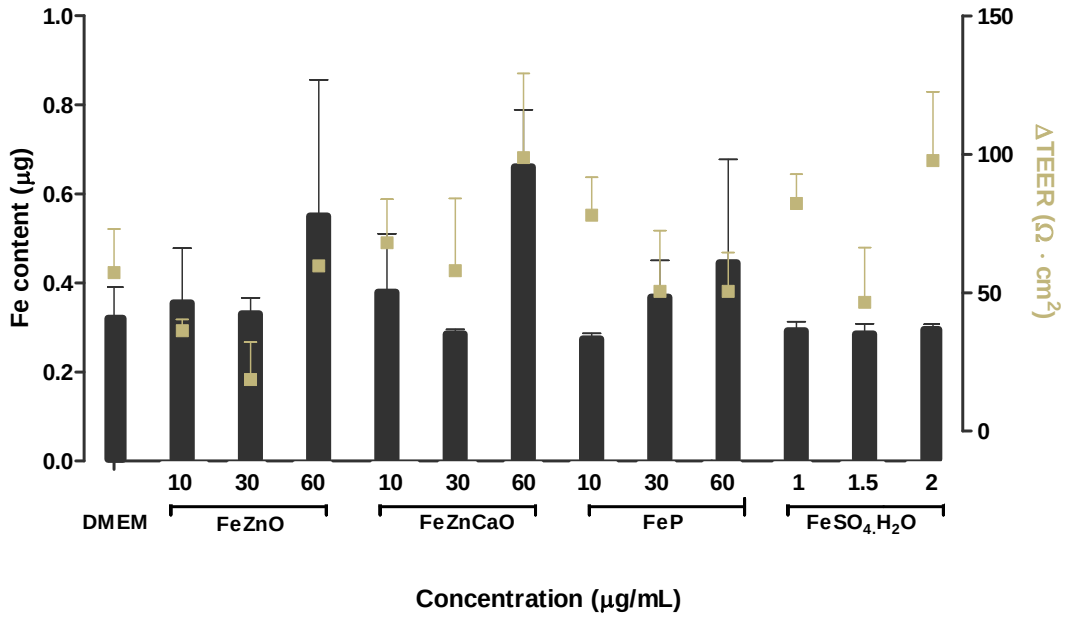
.

4.3. Transport studies

As a result of the size profiles got from the DLS it was decided to use just complexes FeZnP, FeZnCaO and FeP for the transport studies. These ere the ones that showed the most average peak size distribution, at least during the first 4 hours after sonication.

The cells used for the transport studies had a transepithelial electrical resistance of 508.6 Ω (n=49) ±41.7, which was increased to 571.6 Ω (n=49) ±54.4 during the incubation time with the Iron /Zinc nanoparticles. This increasing in the TEER measurement was measured in previous studies to check whether this increase was due to the presence of nanoparticles.

A



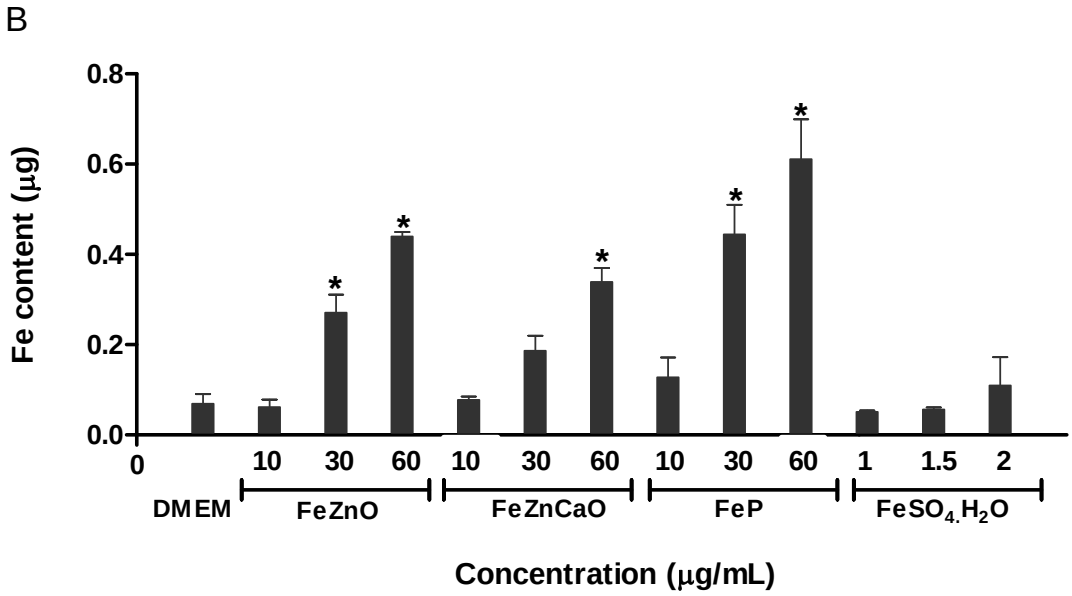


Fig. 3. A. Iron content in the basolateral compartment. B. Iron content in the cell compartment. The values given are mean +SEM. * Indicates a statistically significant difference compared to the control by Dunnet's post-hoc analysis ($P<0.05$)

Fig. 3. A. Shows the iron contained in the basolateral compartment, the statistical analysis shows that there is not a significant difference between the different treatments (tested with one-way ANOVA with Dunnets's post hoc test), thus not an increase in the amount of iron transported in reference with the treatment with DMEM. Fig. 3. B. Shows that for all particles at concentrations of 10 µg/mL, and 30 µg/mL in the case of FeZnCaO there is no increase in the iron quantified in reference with DMEM. For the remaining treatments, a significant increase compared to the initial concentration was measured.

In case of complexes FeZnP and FeP, within the samples treated with 30 and 60 µg/ml of, there is no statistical difference in the amount of iron determined in the cells. Although the ratio of iron composition of the nanocomplexes varies, there is no variation in the amount of iron quantified in the cell section.

Mass balance transport was calculated in order to check whether there is an entrapment of the Iron nanocomplexes into the cell monolayers, or they are attached to the surface of the cells, meaning in either case, that the iron has not been transported to the apical compartment Table 2.

Even after the determination iron content in the cell monolayer there is a difference from ±2% up to ±20% between the total retrieved quantity of iron and the expected one.

This percentage is lower in the case of $FeSO_4.H_2O$. From the characterization step it was observed that along the time the nanoparticles dispersion had a tendency to sediment and since the difference in the ion control is lower than in the case of nanoparticles, it can be thought that the iron remained attached to the surface of the insert.

Table 3. Mass balance after incubation time of 4 hrs (n=4). The values given are mean ± SD.

Nanoparticle	Πg Iron apical part	Πg Iron basolateral part	Πg of Iron in cells section	% of difference in mass balance
FeZnP	0.402 ± 0.008	0.355 ± 0.174	0.062 ± 0.023	18.64
FeZnP	1.417 ± 0.161	0.330 ± 0.052	0.271 ± 0.057	3.31
FeZnP	2.667 ± 0.340	0.550 ± 0.433	0.440 ± 0.014	2.87
FeZnCaO	0.688 ± 0.149	0.379 ± 0.228	0.078 ± 0.013	20.69
FeZnCaO	1.517 ± 0.076	0.285 ± 0.015	0.186 ± 0.047	2.36
FeZnCaO	2.488 ± 0.229	0.660 ± 0.223	0.339 ± 0.053	1.37
FeP	0.925 ± 0.029	0.274 ± 0.022	0.128 ± 0.076	8.16
FeP	2.463 ± 0.144	0.368 ± 0.144	0.445 ± 0.113	7.74
FeP	4.650 ± 0.436	0.445 ± 0.329	0.611 ± 0.124	16.90
$FeSO_4.H_2O$	0.273 ± 0.054	0.293 ± 0.036	0.051 ± 0.006	14.48
$FeSO_4.H_2O$	0.300 ± 0.017	0.285 ± 0.041	0.057 ± 0.008	2.12
$FeSO_4.H_2O$	0.363 ± 0.073	0.295 ± 0.017	0.110 ± 0.088	6.98

5. Discussion

The results indicate that particles FeZnP, FeZnCaO, FeP in a DMEM dispersion might be transported by M cell following an exposure of 4 hrs in a co-culture in Transwell® inserts at not toxic concentrations. In the next sections the findings regarding the requirements for the transport studies: characterization and the toxicity of cells will be reviewed. Furthermore a potential mechanism of transport of the Fe/Zb complexes by M Cells will be discussed.

5.1 Nanoparticles characterization

As it has been mentioned the characteristics of the nanomaterial, might differ once in dispersion (Schulze, Kroll et al. 2008), therefore it is important to perform a characterization of the nanomaterial in the exposure media. Metrics like size distribution, agglomeration stage, shape, chemical composition, surface area, surface charge, surface chemistry are recommended to be included, due the impact that this physico-chemical characteristics may have on the kinetic behaviour and potential responses (Jiang, Oberdörster et al. 2009).

Sizes of the NPs have been measured using two techniques, DLS and TEM. DLS results gave a size distribution of the all nanoparticles where two peaks can be clearly distinguished: one around 10-110 nm and the second one from 110 to 430 nm, the only exception was FeP, whose second peak was relatively narrower beetween 110 and 300 nm.

The measurements by DLS in case of complexes FeZnCaO and FeZnP are different from the ones imaged by TEM, but for particle FeP the values showed the presence of particles of the same sizes as the one reported by DLS. This variation has been reported (Powers, Palazuelos et al. 2007), (Bihari, Vippola et al. 2008), (Ajikumar, Wong et al. 2005), when the measurement by DLS is done in DMEM, rather than in ultrapure water.

In all cases, at DLS size profiles Fig 1. two peaks are distinguished. Regarding the first peak, there are reports in literature that alipoproteins A-1, which are DMEM components have sizes between 10 and 100 nm (Park, Annema et al. 2009), therefore it could be thought that their presence might be the reason for the constant peak measured at 10-100 nm. It has been described that alipoproteins have less rates of association and dissociation than albumin and fibrogen proteins also contained in FBS (Cedervall, Lynch et al. 2007), however it cannot be considered that the particles measured in the range of 10-110 nm correspond just to the alipoproteins. It might be the case that some of the Iron/Zinc

particles that do not have any interaction with the proteins might be free in the dispersion, thus accounting for some of the particles measured within the range of 10-100 nm.

The second peak observed in the range of 110-430 nm culture, becomes planar and wider along the time, this phenomenon could be due to the adsorption of cell culture medium components. It has been reported that bridging flocculation (when uncharged and negative areas of different particles come into contacts) is the main aggregation mechanism at both low and intermediate degree of protein coverage (Tirado-Miranda, Schmitt et al. 2003), many of the proteins contained in the media are highly surface-active and rapidly promote particle aggregation and even precipitation of colloidal materials introduced into the media (Jones and Grainger 2009). Proteins such as albumin , IgG, IgM, fibrogen, C3b, alipoprotein A-1 are binding proteins that have been reported to have been isolated from and interaction with iron oxide nanoparticles (Aggarwal, Hall et al. 2009). Although from literature those are the proteins reported to be isolated, the actual identity of the particles in interaction for these specific nanoparticles, as well as the kinetics should be determined, because protein absorption to nanoparticles may determine their interaction with cells and tissues in vivo (Deng, Mortimer et al. 2009). Ultimately understanding how and why plasma proteins are adsorbed to these particles may be important for understanding their biological responses .

TEM images from DMEM showed structures lower than 20 nm (Fig. 2A), what is closely related with the first peak of the distribution size measured with DLS. In case of FeP dispersion the image got by TEM measurement also showed the polidispersity of the dispersion (Fig 2B), where structures of different sizes were visualized, namely from 10 to 300 nm. Interestingly, the dispersion of FeP that showed an agreement between DLS measurement and TEM image appeared to be more stable in time, what could indicate that there is a stable affinity between the particles and the media components or that the interaction is low, therefore irrespective of the changes suffered by the media, there is not influence in the size (aggregation/agglomeration) of the nanocomplex. In cases of particles FeZnP, FeZnCaO, where structures seem to be entrapped by the media components, the differences in the observations derived from the two techniques used, might be due to DLS that measures the hydrodynamic diameter, therefore if the particle is entrapped, the size information will be from the aggregates/agglomerates formed with/due to the DMEM components, getting as an outcome the report of a bigger size.

Already in the initial dispersion an interaction between DMEM and the nanoparticles is observed from the size profile got by DLS (Fig 1). However these

interactions appear to be changing along time. With the information obtained it is not possible to determine if upon contact with DMEM the dispersions underwent an aggregation or agglomeration process, it is likely that the main changes observed are due to aggregation, since sonication process was able to break down the interactions as observed in Fig. 1D, but a minor aggregation process cannot be ruled out.

Because of characteristics of the DLS apparatus, it was not possible to do a follow up of dispersions in DMEM at higher concentrations, thus important is to keep in mind that the size distribution of the DLS is likely to differ from the one reported, since less amount of proteins are left for interaction as the concentration of the nanocomplex increases, and because if the available nanoparticle is in excess over the total protein concentration, lower affinity proteins tend to remain attached to the surfaces (Cedervall, Lynch et al. 2007), this might bring as a consequence a potential variation in the size profile between concentrations.

5.2. Cell viability

When using the nanoparticles FeP, FeZnP and FeZnCaO in concentrations ranging from 10 to 60 μg/mL no reduction of viability after 4 hrs of exposure in caco-2 cells was observed. When the exposure time was incremented to 24 hrs, all dispersions showed a decrease of cell viability of 80%, irrespective to the concentrations used.

There are various ways in which the nanoparticles may interfere with the WST-1 cytotoxicity assay compromising the reliability of the results. Different characteristics of nanoparticles like intrinsic photometric absorbance, or fluorescence, high surface energy and surface area may alter colorimetric or fluorimetric assay reporting (Jones and Grainger 2009). Therefore, in order to avoid an underestimation or overestimation of the cell viability the interaction between the dispersions and the end product of the WST-1 assay (formazan) was performed. Besides that, a positive control was used to ensure the ability of the cell type to produce specific markers of cytotoxicity (Park M, Daniëlle PK Lankveld et al. 2009) . The control regarding formazan, indicates a negligible interaction with the different nanoparticles tested.

With the approach followed in the determination of cell viability, the cell viability of all cells was measured, notwithstanding the possibility that some cells started to show a cytotoxic effects, thus if a washing step was included the cells that showed a detachment process, would be lose.

No reduction of cell viability was observed after 4 hrs of exposure to the different nanoparticles at different concentrations. It is known that free iron can be detrimental to cells; it is involved in forming reactive oxygen species which cause oxidative stress to the cells. The Fe (II) is a main actor in the Fenton response, producing excessive free radical to attack cell membrane and depress the stability of the membrane. The effect of this ion can be appreciated in the high reduction of cell viability in the cell treated with $FeSO_4$. H_2O.

The iron in the particles used is present in its ferric form Fe (III), and although this iron state takes part of the Fenton reactions as well, the reduction of cell viability was observed irrespective of the iron content. In the same way, Zn^{2+} release has been introduced as a possible cause in decrease of cell viability (Horie, Nishio et al. 2009), however the effect of reduction was not only observed in particles containing zinc. In literature it is found that the particles can interact in the media component thus leading to a decrease in cell viability caused by depletion of the media (Veranth, Kaser et al. 2007), (Casey, Herzog et al. 2008) , which might be the case for the decrease in the cell viability, specially for particles considered as low-cytotoxic (Horie, Nishio et al. 2009). This media depletion might be the reason why at 24 hrs there is a reduction of 20% in cell viability. As it has been mentioned, the effect observed was not concentration dependant, despite this; it is possible that at a concentration of 10 µg/mL, the particles are enough to deplete the media. Another important factor to include and that might be of relevance, is the possible existence of metal impurities of the particles that might be responsible for the cytotoxic response, but this possibility was not further investigated.

From literature, there have been reported different mechanisms through which nanoparticles might exercised their toxic effect on cells upon exposure. The one discussed here has been media depletion (Veranth, Kaser et al. 2007), (Casey, Herzog et al. 2008), and although TEM images and the size profile of the dispersions give and indication of the interaction between the nanoparticles complexes there is no a clear indication to which extent this interaction may have influenced the depletion of some nutrients that might be necessary for the viability of the cells. Another potential effect of nanoparticles on cells is the induction of reactive oxygen species (ROS) that has been reported as one of the mechanism for the toxicity of nanoparticles (Kreuter 2007). Due to restriction of time as well as the nature of the particles the performance of a screening of possible ROS production was not performed. ROS generation is linked to another potential toxic effect that is DNA damage. Inflammation, mitochondrial perturbation, uptake by reticulo-endothelial system, protein denaturation, degradation, generation of neoantigens,

breakdown in immune tolerance are other nanoparticles effects reported (Nel, Xia et al. 2006).

5.3 Transport studies

After an exposure of 4 hrs, FeZnP, FeZnCaO, FeP in DMEM did not show a significant increase on the amount of iron present in the basolateral chamber, however, the amount of iron measured in the cell section was increased at concentrations of 30 and 60 μg/mL for the three complexes.

There are not many transport studies with a model of caco-2 cells used in this project. The results of transport studies using the same co-culture model showed an enhancement on the amount of nanoparticles transported by the presence of M-cells (Slütter, Plapied et al. 2009), (des Rieux, Fievez et al. 2007A), (des Rieux, Fievez et al. 2007B) even though the time of exposure in all cases was 3 times lower than the 4 hrs of exposure to the Iron/Zinc dispersions. The nanoparticles used in such studies were carboxylated polyestirene, helodermine encapsulated in 200 nm nanoparticles containing a mix of Polyethilenglycol monomethyl ether: Poly(lactide-coglycolide: Poly(lactic-glycolic acid), and chitosan. Therefore, it is important to keep in mind that the particles assessed in such studies have characteristics that differ greatly from the iron/zinc nanoparticles. In addition, the particles used in two of those experiments underwent a coating process with Poly(lactic-co-glycolic acid), treatment that reduces the binding of proteins unto the surface the nanomaterials used (des Rieux, Fievez et al. 2007B). This treatment potentially could influence the nanoparticles transport by M-Cells.

When looking at the iron quantified in the cell compartment there is an increase of iron for treatments: at 30 and 60 μg/ml what might be an indication of the uptake of iron by the M cells. Iron absorption usually happens in the duodenum or upper jejunum where the pH is 5-6. There, the iron is in a large extent absorbed by the enterocytes as Fe (II), this process involves a membrane protein called divalent metal transporter. Therefore it is not likely an uptake by the mechanism described previously from the iron contained in the nanoparticles dispersion, since iron in the media is its ferric state.

Increased of iron measured in the cell compartment does not necessarily reflects an uptake and if this is the case, that the uptake of iron has been in its nanosize and by M cell. To be able to make such a kind of asseveration it is necessary to have more information about the state of iron quantified. For this purpose, determination of ferritin could be used, if an increase was determined, it would indicate that the iron has been absorbed as Fe (II),

other possibility would be to look for the nanoparticles within the FAE model making use of TEM, Confocal Laser Lcanning Microscopy (CLSM), or Scanning Electron microscope (SEM).

M cells have been reported to have the ability to transport nanoparticles (Neutra, Mantis et al. 1999), the mechanism as well as what are the characteristics that render nanoparticles to be likely transported for this type of cells have not been fully described yet (Vila, Sánchez et al. 2002). Thus, it is likely that the dispersion characteristics of the nanoparticles used make them not a suitable candidate to be transported by M cells, what would potentially lead to an immune reaction. A paper making a comparison of iron uptake from different iron powders, where $FeSO_4$ of 100 nm was included, demonstrated that there is an increase in iron uptake when a monoculture of caco-2 cells is used, in this model the particles were pre-treated in an *in vitro* digestion model. There, the uptake by the enterocytes might be done from the reduced iron (He, Feng et al. 2008), these findings appear to be in line with the findings in animal studies where iron nanoparticles have shown an increase in the iron bioavailability after a diet supplemented with iron in form of nanoparticles. In such studies it is reported that the decrease in particle size increased the iron absorption, without showing any indication of cytotoxicity (Rohner, Ernst et al. 2007), but also, there was not indication of an iron uptake as nanoparticle either through the tight cell junctions or the through the Peyer's Patches in the small intestine.

As it has been reported in literature, the gastric conditions degrade the nanoparticles, (Wiecinski, Metz et al. 2009), (Wang, Nagesha et al. 2008) unless they have undergone a specific treatment to increase their resistance during the gastric passage of engineered nanoparticles. Although in this study it was not possible to observe a substantial increase of the iron content in the basolateral chamber during the period of exposure, the model might differ from the *in vivo* situation since the role of digestion processes might have a big influence on the final bioavailability and or toxicity of the compounds.

6. Conclusions and Recommendations

The results of this study showed a not significant increase in the iron quantified in the basolateral chamber after 4 hrs of exposure, however it can be speculated about a possible uptake by M cells since there is an increase in iron content in cells and the iron state in the nanoparticles makes not likely that the absorption has been done through the usual mechanism, however it is necessary to get more information about the state of the iron of the nanoparticles in this section to be able to confirm the increase in bioavailability and/or the uptake by M-cells.

Nanoparticles dispersion should be prepared in a relevant media of exposure and thoroughly characterized prior its use in transport studies. In the same way it is important to get and insight of the interaction that the particles have with the media components, since this might influence the outcome of the *in vitro* toxicity studies as well as the up take by M-cells.

While preparing the nanoparticles dispersion, the approach suggested by Kato et al. 2009 could be used. In the referred approach, a pre-adsorption of the nanoparticles is done prior the preparation of the dispersions at the concentrations required. This pre-adsorption is done in two steps, one with FBS alone and the second one with DMEM supplemented with FBS (Kato, Suzuki et al. 2009). This approach could lead in one hand to have stable solutions and in the other to block a potential media depletion.

It might be interesting to look for the mechanisms by which the particles are exercising the cytotoxic effect, therefore it could be relevant to look which are the interaction that the nanoparticles have with the media components, to try to determine which are the elements of the long lasting protein corona. In the same way to look for known mechanisms of cytotoxicity as the generation of ROS, in this case different methods should be screened, HDCF-DA, Superoxide dimutase determination, etc.

The iron determination was done as elemental iron, however is of relevant importance to be able to differentiate if the changes observed in such a kind of studies are due to the transport of the compounds as nanoparticles, in both parts the basolateral compartment as well as the cell section. Tools as TEM, SEM, CLSM, might be used.

The inclusion of a positive control would be of great utility to assure the reproducibility of these transport studies. This control should be well characterized in the dispersion media as well as in its rate of transport.

Finally, it is necessary to take in consideration the conditions of exposure (matrix and gastric passage) and to mimic them in the *in vitro* assessment to determine whether there is an enhancement of Fe/Zn bioavailability.

References

Aggarwal, P., J. B. Hall, et al. (2009). "Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy." Advanced Drug Delivery Reviews **61**(6): 428-437.

Ajikumar, P. K., L. G. Wong, et al. (2005). "Synthesis and Characterization of Monodispersed Spheres of Amorphous Calcium Carbonate and Calcite Spherules." Crystal Growth & Design **5**(3): 1129-1134.

Berger, J., N. X. Ninh, et al. (2005). "Efficacy of combined iron and zinc supplementation on micronutrient status and growth in Vietnamese infants." Eur J Clin Nutr **60**(4): 443-454.

Bihari, P., M. Vippola, et al. (2008). "Optimized dispersion of nanoparticles for biological in vitro and in vivo studies." Particle and Fibre Toxicology **5**(1): 14.

Bouwmeester, H., S. Dekkers, et al. (2009). "Review of health safety aspects of nanotechnologies in food production." Regulatory Toxicology and Pharmacology **53**(1): 52-62.

Brayden, D. J., M. A. Jepson, et al. (2005). "Keynote review: Intestinal Peyer's patch M cells and oral vaccine targeting." Drug Discovery Today **10**(17): 1145-1157.

Casey, A., E. Herzog, et al. (2008). "Single walled carbon nanotubes induce indirect cytotoxicity by medium depletion in A549 lung cells." Toxicology Letters **179**(2): 78-84.

Cedervall, T., I. Lynch, et al. (2007). "Understanding the nanoparticleâ€“protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles." Proceedings of the National Academy of Sciences **104**(7): 2050-2055.

Deng, Z. J., G. Mortimer, et al. (2009). Differential plasma protein binding to metal oxide nanoparticles.

des Rieux, A., V. Fievez, et al. (2007A). "An improved in vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells." European Journal of Pharmaceutical Sciences **30**(5): 380-391.

des Rieux, A., V. Fievez, et al. (2007B). "Helodermin-loaded nanoparticles: Characterization and transport across an in vitro model of the follicle-associated epithelium." Journal of Controlled Release **118**(3): 294-302.

EFSA (2009). "The potential Risk arinsing from Nanoscience and Technologies on Food and Feed Safety." The EFSA Journal **958**: 1-39.

Frey A, N. M. (1997). "Targeting of mucosal vaccines to Peyer's patch M cells." Behring Institute Mitteilungen(98): 376-389.

Gullberg, E., M. Leonard, et al. (2000). "Expression of Specific Markers and Particle Transport in a New Human Intestinal M-Cell Model." Biochemical and Biophysical Research Communications **279**(3): 808-813.

Hagens, W. I., A. G. Oomen, et al. (2007). "What do we (need to) know about the kinetic properties of nanoparticles in the body?" Regulatory Toxicology and Pharmacology **49**(3): 217-229.

He, W.-L., Y. Feng, et al. (2008). "Comparison of Iron Uptake from Reduced Iron Powder and FeSO4 Using the Caco-2 Cell Model: Effects of Ascorbic Acid, Phytic Acid, and pH." Journal of Agricultural and Food Chemistry **56**(8): 2637-2642.

Hielscher, T. (2005). Ultrasonic production of nano-size dispersions and emulsions Germany.

Horie, M., K. Nishio, et al. (2009). "Protein Adsorption of Ultrafine Metal Oxide and Its Influence on Cytotoxicity toward Cultured Cells." Chemical Research in Toxicology **22**(3): 543-553.

Hurrell, R. F. (2002). "Fortification: Overcoming Technical and Practical Barriers." J. Nutr. **132**(4): 806S-812.

Jiang, J., G. Oberdörster, et al. (2009). "Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies." Journal of Nanoparticle Research **11**(1): 77-89.

Jones, C. F. and D. W. Grainger (2009). "In vitro assessments of nanomaterial toxicity." Advanced Drug Delivery Reviews **61**(6): 438-456.

Kato, H., M. Suzuki, et al. (2009). "Reliable size determination of nanoparticles using dynamic light scattering method for in vitro toxicology assessment." Toxicology in Vitro **23**(5): 927-934.

Kreuter, J. (2007). "Nanoparticles--a historical perspective." International Journal of Pharmaceutics **331**(1): 1-10.

Luykx, D. M. A. M., R. J. B. Peters, et al. (2008). "A Review of Analytical Methods for the Identification and Characterization of Nano Delivery Systems in Food." Journal of Agricultural and Food Chemistry **56**(18): 8231-8247.

Nel, A., T. Xia, et al. (2006). "Toxic Potential of Materials at the Nanolevel." Science **311**(5761): 622-627.

Neutra, M. R., N. J. Mantis, et al. (1999). "The composition and function of M cell apical membranes: Implications for microbial pathogenesis." Seminars in Immunology **11**(3): 171-181.

Oberdorster, G., A. Maynard, et al. (2005). "Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy." Particle and Fibre Toxicology **2**(1): 8.

Park M, Daniëlle PK Lankveld, et al. (2009). "The status of in vitro toxicity studies in the risk assessment of nanomaterials." Future Medicine **4**(6): 669-685.

Park, M. V. D. Z., W. Annema, et al. (2009). "In vitro developmental toxicity test detects inhibition of stem cell differentiation by silica nanoparticles." Toxicology and Applied Pharmacology **240**(1): 108-116.

Powers, K. W., M. Palazuelos, et al. (2007). "Characterization of the size, shape, and state of dispersion of nanoparticles for toxicological studies." Nanotoxicology **1**(1): 42 - 51.

Rohner, F., F. O. Ernst, et al. (2007). "Synthesis, Characterization, and Bioavailability in Rats of Ferric Phosphate Nanoparticles." Journal of Nutrition **137**(3): 614-619.

SCENIHR (29 November 2007). Opinion on the scientific aspects of the existing and proposed definitions relation to products of nanoscience and nanotechnologies.

Schulze, C., A. Kroll, et al. (2008). "Not ready to use: overcoming pitfalls when dispersing nanoparticles in physiological media." Nanotoxicology **2**(2): 51-61.

Slütter, B., L. Plapied, et al. (2009). "Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination." Journal of Controlled Release **138**(2): 113-121.

Tirado-Miranda, M., A. Schmitt, et al. (2003). "The aggregation behaviour of protein-coated particles: a light scattering study." European Biophysics Journal **32**(2): 128-136.

Veranth, J., E. Kaser, et al. (2007). "Cytokine responses of human lung cells (BEAS-2B) treated with micron-sized and nanoparticles of metal oxides compared to soil dusts." Particle and Fibre Toxicology **4**(1): 2.

Versantvoort, C. H. M. and e. a. A. G. Oomen (2005). ""Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food."." Food and Chemical Toxicology **43**(1): 31-40.

Vila, A., A. Sánchez, et al. (2002). "Design of biodegradable particles for protein delivery." Journal of Controlled Release **78**(1-3): 15-24.

Wang, L., D. Nagesha, et al. (2008). "Toxicity of CdSe Nanoparticles in Caco-2 Cell Cultures." Journal of Nanobiotechnology **6**(1): 11.

WHO (2002). Childhood and maternal undernutrition. Geneva.

WHO, F., UNICEF, GAIN, MI, & FFI (2009). Recommendations on wheat and maize flour fortification. Meeting Report: Interim Consensus Statement. Geneva.

WHO, W., UNICEF (2001). Preventing and controlling micronutrient deficiencies in populations affected by an emergency. Geneva.

WHO/CDCP (2004). Technical Consultation on the Assessment of Iron Status at the Population Level. Geneva.

Wiecinski, P. N., K. M. Metz, et al. (2009). "Gastrointestinal biodurability of engineered nanoparticles: Development of an *in vitro assay*." Nanotoxicology **3**(3): 202 - 214.